

Nutrition Society and BAPEN Medical Symposium on ‘Nutrition support in cancer therapy’

The Second World Cancer Research Fund/American Institute for Cancer Research Expert Report. *Food, Nutrition, Physical Activity, and the Prevention of Cancer: A Global Perspective*

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It has been estimated by various authorities that about one-third of cancers in Western high-income societies are attributable to factors relating to food, nutrition and physical activity. Identifying with confidence specific associations between dietary patterns, foods, body composition or individual nutrients is not simple because of the long latent period for cancer development, its complex pathogenesis and the challenge of characterising the multi-dimensional aspects of diet and activity over a lifetime. Reliable conclusions must therefore be drawn not only from randomised controlled trials but from a variety of methodological approaches, judged within a classic framework for inferring causality. Using a newly-developed method with a protocol for standardising the literature search and for analysis and display of the evidence, nine independent academic centres have conducted systematic reviews addressing the causal associations between food, nutrition and physical activity and risk of development of seventeen cancers, as well as of weight gain and obesity. A review has also examined the efficacy of such interventions in subjects with cancer. The reviews have been assessed by an independent Panel of twenty-one international experts who drew conclusions with grades of confidence in the causality of associations and made recommendations. Recommendations are given as public health goals as well as for individuals.

Cancer prevention: Systematic literature reviews: Expert recommendations

In the UK cancer accounts for about one-quarter of all deaths, second only to CVD⁽¹⁾. Although survival is increasing with improved management, many cancers still carry a poor prognosis as they are less susceptible to surgical, radiotherapeutic or chemotherapeutic intervention, either because of their inherent characteristics or because they may be detected at a late stage of development.

The most common cancers are diseases of adulthood, becoming especially more common in older age. As the population ages cancer prevalence is therefore predicted to increase. Consequently, a policy of managing the burden of cancer in the population based entirely on screening for early detection and management of detected cancers is unlikely to make a marked impact at a population level

except for a few types of cancer, and is also likely to be increasingly, and unsustainably, expensive.

Thus, it is important to consider the role for primary prevention in helping to reduce the clinical, personal, economic and social burden of cancer. Approximately one-third of all cancers in high-income countries such as the UK are attributed to smoking⁽²⁾. Authoritative reviews have further estimated that about one-third of all cancers in such countries might be attributable to factors relating to food, nutrition and physical activity, including body fatness.

In 1997 the World Cancer Research Fund (WCRF), together with its sister organisation the American Institute for Cancer Research, published a landmark report *Food,*

Abbreviations: SLR, systematic literature reviews; WCRF, World Cancer Research Fund.

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Nutrition and the Prevention of Cancer: a Global Perspective⁽³⁾. This report has become the most authoritative statement on the topic and a basis for academics, researchers and policy-makers worldwide, and has been followed by a surge in research on the nutritional links with cancer.

In view of the burgeoning body of evidence, in 2001 WCRF and American Institute for Cancer Research embarked on a new report to ensure that its recommendations remained a sound basis for its own and others' activities. This report was published in November 2007⁽⁴⁾.

Nutrition and the cancer process

Cancer develops from a clone of cells that have escaped normal regulation of growth, proliferation, differentiation and intercellular relationships. These abnormalities of function derive from disordered expression of key genes, resulting in altered cellular phenotype. The disordered gene expression may result from genetic mutation or from epigenetic factors that may silence genes that should be active or switch on genes that should be silent. Factors relating to food and nutrition may directly influence both processes.

Food components may act directly as mutagens, or conversely may interfere with the action of external mutagens either directly or by facilitating their elimination. Nutritional factors may also alter the cellular milieu by modulating hormonal axes, and so influencing growth and proliferation amongst specific cell populations. Finally, regulation of processes related to specific nutrients may directly influence the expression of key genes, for instance through abnormalities of methylation of the promoter regions of genes or of histone acetylation, which can influence DNA structure and accessibility of genes to mRNA for transcription.

While it is clear that at root cancer is a disease of gene expression, only a minority of cancers appear to be related to inherited single high-penetrance gene mutations. Consequently, a major determinant of cancer risk appears to be the interaction between genotype and environment. Amongst the earliest identified indicators of the importance of this interaction are the different patterns of cancer around the world. Low-income countries have relatively high rates of cancers of the cervix, stomach, liver and oro-pharynx (all of which are at least partly related to infections), while high-income countries are characterised by higher rates of cancers of the breast, prostate and colon-rectum (related to alterations in hormonal axes)⁽⁴⁾. Most striking is the rapid change in cancer patterns, within one or two generations, when populations migrate. Together with the secular changes in the incidence of some cancers over only decades, this rapid change effectively excludes inherited factors as a key determinant of these patterns at a population level, although clearly they may be important in determining individual risk within populations.

The rationale for considering food, nutrition and physical activity as key environmental factors in determining cancer risk derives from a mass of epidemiological and mechanistic data, and more recently with some clinical trials as well. The second WCRF/American Institute for

Cancer Research report was commissioned with an expert Panel to review this evidence, to draw conclusions and to make recommendations.

The process for the report

Cancer is the result of a complex process of accumulation of abnormal phenotypic characteristics among a clone of cells, usually over decades. This complexity and timescale present obstacles to the study of its pathogenesis and natural history, especially during the preclinical stages. Equally, it is challenging to characterise subjects' exposure to food and nutrients, and their trajectory of growth and body composition, over the life course. In these circumstances, clinical trials cannot realistically address more than a short period of the whole pathogenetic process and are usually conducted in high-risk groups who may not be representative of the general population. Often dietary intervention is in the form of isolated supplements rather than real foods, as it is difficult to achieve or maintain differences between intervention and control groups for real dietary or physical activity interventions.

Consequently, it is difficult to identify single methodological approaches that can be seen as inherently superior. With a body of evidence comprising very different approaches, from observational epidemiology to basic science, and where the generalisability of clinical trials is limited, robust conclusions can only be drawn from a review of the totality of the relevant evidence, allowing for the advantages and disadvantages of different methods. There are accepted approaches to synthesising such evidence and inferring the likely causality of observed associations, such as that proposed in 1963 by Bradford Hill⁽⁵⁾.

With a view to exploring the literature so that Bradford Hill's framework could be applied, WCRF International convened an expert Task Force to develop a standardised method for searching the literature and for analysing and displaying the evidence. This methodology Task Force met over 2 years to develop such a specification.

The feasibility, utility and reproducibility of the specification manual was tested and compared between two independent academic institutes, using endometrial cancer as a test case. As a result amendments were made to the protocol, and the final version⁽⁶⁾ was used to conduct the series of systematic literature reviews (SLR) that formed the basis of the Panel's deliberations.

It was decided to keep the process of data collection, analysis and display separate from that of judging it. Thus, nine independent SLR centres, three in the USA, four in the UK and two in continental Europe, were commissioned to conduct SLR on the links between food, nutrition and physical activity and seventeen cancer sites. In addition, SLR were conducted on the links with weight gain and body fatness and the efficacy of nutrition and activity interventions in individuals who had already received a diagnosis of cancer. Finally, a review was conducted on authoritative national and international reports that had made recommendations for the prevention of other diseases, so that any recommendations from this report would be set in a broad public health context.

Each SLR team had to include a range of expertise, in nutrition, epidemiology, statistics, cancer biology, the specific cancer site and systematic review. Each SLR was peer reviewed at the protocol and draft final report stage. WCRF International provided a coordinator whose role was to facilitate the process and ensure consistency in application of the specification.

An expert Panel was convened, comprising twenty-one internationally-renowned scientists, with a range of expertise including nutrition, epidemiology, cancer mechanisms, clinical medicine and public health. Chaired by Professor Sir Michael Marmot, the Panel met over 5 years to discuss and judge the evidence from the SLR, to draw conclusions and to make recommendations.

Judging the evidence

Given the complex nature both of the disease process of cancer and of the characterisation of diet and physical activity over a lifetime, a simple hierarchical approach to evidence on causal links cannot rely on randomised controlled trials. Apart from the obvious inability to mask differences in dietary interventions based on real foods, in practical terms it is equally impossible to secure sufficiently large or sustained differences in lifestyle including diet between intervention groups. Where such studies are done they often use synthetic supplements of isolated nutrients, at high dose, in high-risk groups, with intermediate outcomes. Extrapolation from such studies to the general population is not therefore straightforward. However, long-term observational studies of cohorts of healthy individuals can identify hard outcomes (cancer incidence or mortality) in typical populations consuming real diets over decades. Observational studies, however, unlike randomised trials, are subject to confounding, making causal inferences less robust.

The issue of identifying important causal associations from predominantly observational data where trials are uninformative is not new. Bradford Hill in the 1960s elucidated a series of characteristics of the data that could provide a framework for consideration to help infer which associations are likely to be causal⁽⁷⁾. The SLR specification required the data to be displayed in a way that facilitated the application of Bradford Hill's criteria. The evidence displayed was then judged within that framework, and the likelihood of any association being causal was graded according to how well the criteria were fulfilled.

Before any evidence was presented, the Panel agreed a set of minimum criteria for each grade. The grades were: convincing; probable; limited; substantial effect on risk unlikely. The limited category was further divided into where it was suggestive of a causal relation and where no conclusion could be drawn. In addition, to provide flexibility, certain characteristics of the evidence could be used to upgrade or downgrade conclusions.

Once conclusions were reached, they were presented graphically in the form of a matrix. Matrices were presented for each cancer, with all identified exposures categorised according to the grade of conclusion as to the likely causality of the association. In addition, matrices were presented for each main group of exposures, with

Table 1. Headline recommendations of the expert Panel convened to discuss and judge the evidence from the systematic literature reviews, to draw conclusions and to make recommendations

Category	Recommendation
Body fatness	Be as lean as possible within the normal range of body weight
Physical activity	Be physically active as part of everyday life
Foods and drinks that promote weight gain	Limit consumption of energy-dense foods Avoid sugary drinks
Plant foods	Eat mostly foods of plant origin
Animal foods	Limit intake of red meat and avoid processed meat
Alcoholic drinks	Limit alcoholic drinks
Preservation, processing, preparation	Limit consumption of salt Avoid mouldy cereals or pulses
Dietary supplements	Aim to meet nutritional needs through diet alone
Breast-feeding	Mothers to breast-feed; children to be breast-fed
Cancer survivors	Follow the recommendations for cancer prevention

the specific exposure–cancer links categorised according to their likely causality (although excluding the large category of limited–no conclusion).

Once agreed, these conclusions provided the basis for making recommendations. Recommendations were based only on conclusions for which the likely causality was judged probable or convincing.

Making recommendations

Recommendations were developed by the Panel with the aim of being as far as possible applicable throughout the world. Where evidence was specific to particular local circumstances this fact was stated. In addition, all recommendations were considered within the context of the review of authoritative expert reports on other diseases, to ensure that they were conducive generally to public health and not restricted to cancer prevention.

The Panel made ten recommendations. Each recommendation has a title to identify the broad category of food, nutrition or physical activity addressed. This title is followed by a brief headline to provide a sense of the essence of the recommendation, but without detail or quantification. Each recommendation then has a public health goal or goals, directed to policy-makers or health professionals with the aim of facilitating policies or programmes and quantified to aid target setting and monitoring of progress. This public health goal is followed by personal recommendations as a basis for the population (as communities, families or individuals) to make decisions about their own diet and lifestyle, also quantified where possible.

Each of these recommendations is accompanied by explanatory footnotes, which should be considered an integral part of the recommendation.

The headline recommendations are shown in Table 1.

Looking to the future

The conclusions and recommendations of the Panel set out in the report form a robust basis for further research and for public and professional education programmes. WCRF is preparing materials and other resources that begin this process.

However, scientific evidence is continually accumulating, and each new study needs to be considered in the context of the existing evidence. Thus, WCRF has commissioned a group from Imperial College, London, UK to combine the existing databases produced by the cancer-site SLR, and to update them as new information accrues. This process is overseen by a panel of independent experts, and will be accompanied by periodic reports of updated conclusions and of the implications for the recommendations.

Furthermore, while food, nutrition and physical activity are proximate causes of patterns of cancer, and of other chronic diseases, it is clear that patterns of food consumption and of activity are themselves determined by powerful social, cultural, political and economic factors. A further report to be published in November 2008 will address the evidence for determinants of such patterns and for what might be effective in influencing them. This report, *Policy and Action for Cancer Prevention: Food, Nutrition, and Physical Activity – a Global Perspective*, will explore the evidence in a similarly rigorous way and make further recommendations for policies and actions to politicians, policy-makers, health professionals and others.

Conclusions

The 2007 WCRF/American Institute for Cancer Research Report *Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective* was produced by

an independent Panel, based on systematic reviews conducted by independent academic institutions and conducted transparently according to the most rigorous procedures. It represents the most authoritative review of the topic ever produced and is the most robust current basis for research, education and policy.

Acknowledgements

I have no conflict of interest. I act as a paid consultant to WCRF International, and was project director for the WCRF/ACIR 2007 Report.

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Short Communication

Low-Fat, Low-Glycemic Load Diet and Gene Expression in Human Prostate Epithelium: A Feasibility Study of Using cDNA Microarrays to Assess the Response to Dietary Intervention in Target Tissues

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Abstract

Purpose: We examined the feasibility of using gene expression changes in human prostate epithelium as a measure of response to a dietary intervention.

Materials and Methods: Eight men with newly diagnosed prostate cancer were randomized to a low-fat/low-glycemic load intervention arm (<20% energy from fat and total daily glycemic load <100) or a "standard American" control arm (\approx 35% energy from fat and total daily glycemic load >200). Prostate tissue was collected before randomization and ~6 weeks later, at the time of radical prostatectomy. Epithelium was acquired by laser capture microdissection, and transcript abundance levels were measured by cDNA microarray hybridization and confirmed by quantitative reverse transcription-PCR.

Results: Men in the intervention arm consumed 39% less total energy ($P = 0.004$) and the difference in

weight change between intervention and control arms was -6.1 kg ($P = 0.02$). In the intervention arm, 23 (0.46%) of 5,711 cDNAs with measurable expression were significantly altered ($P < 0.05$; false discovery rate, $\leq 10\%$). In the control arm, there were no significant changes in transcript expression, even when using a false discovery rate as high as 50%.

Conclusions: A 6-week, low-fat/low-glycemic load diet was associated with significant gene expression changes in human prostate epithelium. These results show the feasibility of using prostate tissues collected at diagnosis and at surgery to study the effects of dietary manipulation on prostate tissue, which may give insight into the molecular mechanisms underlying the associations of diet and obesity with the development or progression of prostate cancer. (Cancer Epidemiol Biomarkers Prev 2007;16(10):2150–4)

Introduction

There is good evidence that obesity and perhaps, a high fat intake, due to its strong correlation with obesity, increase the risk of high-grade or aggressive prostate cancer, prostate cancer mortality, and the risk of poor outcome following diagnosis and treatment (1–3). However, the molecular mechanisms underlying these associations remains largely unknown. Experimental animal and *in vitro* studies have shown that dietary patterns and

food constituents can affect gene expression, protein synthesis, cell signaling, and other important events related to carcinogenesis (4–6). In humans, however, it is difficult to test hypotheses related to the mechanisms and pathways that underlie diet and cancer associations because appropriate tissues are rarely available. Human studies are generally limited to measuring circulating biomarkers, such as serum steroid hormones, which are influenced by diet and are associated with carcinogenesis.

The objective of this pilot study was to evaluate the feasibility of a randomized clinical trial to test whether short-term modifications in macronutrient intake could modify gene expression in prostate epithelium. We examined the effects of a low-fat/low-glycemic load diet in men with clinically localized prostate cancer who elected prostatectomy as their primary treatment choice. These results will help in the design of studies using gene expression or gene expression signatures as measures of the effect of dietary change on prostate cancer biology.

Received 2/18/07; revised 7/19/07; accepted 8/6/07.

Grant support: DK65083 (D.W. Lin), the Pacific Northwest Prostate Cancer Specialized Programs of Research Excellence CA97186 (D.W. Lin and P.S. Nelson), and the Fred Hutchinson Cancer Research Center P30 CA015704.

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doi:10.1158/1055-9965.EPI-07-0154

Materials and Methods

Participants were men with clinically localized prostate cancer, who elected to undergo radical prostatectomy and received no neoadjuvant therapy. Study activities began within 2 weeks of diagnosis.

Eligibility criteria included the ability to implement dietary change, no concurrent disease requiring dietary modification, no current use of hormonal treatments, and body mass index $>20 \text{ kg/m}^2$ and $<35 \text{ kg/m}^2$. In addition, men had to have participated in an independent protocol that collected and stored four prostate tissue cores at the time of diagnostic prostate biopsy. Both the dietary intervention and biopsy tissue collection protocols were approved by the Institutional Review Boards of the Veterans Administration, Puget Sound Health Care System and the Fred Hutchinson Cancer Research Center, and all patients signed written informed consents. Study was completed between August 2003 and November 2004.

Randomization and Dietary Intervention. Participants were randomly assigned to one of two groups: group I received instructions to follow a low-fat/low-glycemic load diet ($<20\%$ energy from fat and total daily glycemic load <100) and group II was instructed to follow a "standard American" diet (35% energy from fat and total daily glycemic load >200). A detailed description of the intervention is available from the authors upon request. In brief, participants were provided a dietary intervention manual that focused on meal planning. For individuals randomized to the low-fat/low-glycemic load diet, the study nutritionist provided detailed instructions and worked with the participant to plan at least three meals. Participants randomized to the standard American group were asked to continue their usual dietary habits, using lists of prohibited and permitted foods as a guideline for food choices. During the first week, the nutritionist made up to five follow-up telephone calls to review the intervention materials and provide additional meal planning support; calls were made at least once per week thereafter.

Dietary and Anthropometric Assessment. Unscheduled, telephone-administered 24-h dietary recalls were completed each week to assess adherence to the study diet, using the University of Minnesota's Nutrition Data Systems for Research software (version 37, 2006). Interviewers were blinded to the randomization assignment. On the days of randomization and prostatectomy, participants were weighed by study staff on a beam balance scale. Height was measured using a standing stadiometer. Body mass index was calculated as weight (kg) / height (m)².

Clinical Specimens. At the time of biopsy and after acquiring 12 diagnostic prostate cores, we obtained four additional tissue cores from the peripheral zone. At the time of radical prostatectomy, the prostate was exposed via a midline incision, and we obtained four *in situ* prostate biopsy cores using an 18-gauge prostate needle biopsy gun (Bard Inc.). All biopsies were collected from the peripheral zone and immediately embedded and frozen as described below. Because our study was limited to the effects of diet on normal epithelium, we attempted to intraoperatively biopsy sites that did not

contain malignant epithelium, corresponding to sites from diagnostic biopsy specimens. The *in situ* samples were collected before removing the prostate because we previously found that surgical manipulation caused artifactual changes in gene expression (7).

Specimen Handling and Laser Capture Microdissection. Biopsy cores were embedded individually in polyethylene glycol freezing media (Tissue-Tek OCT Compound, Sakura Finetek), placed in isopentane that was precooled in liquid nitrogen, and stored at -80°C (8). We used laser capture microdissection to collect 5,000 epithelial cells from histologically benign epithelial glands and extracted RNA using a standardized protocol (9). The RNA was subjected to linear amplification and converted to cDNA for microarray hybridization as previously described (7).

Gene Expression Analysis by Microarray Hybridization. We prepared and hybridized spotted cDNA microarrays as previously described (10), using RNA from a single batch of reference standards for each hybridization. Fluorescent array images were collected for both Cy3 and Cy5 using a GenePix 4000B fluorescent scanner (Axon Instruments), and GenePix Pro 4.1 software was used to grid and extract image intensity data. Spots of poor quality, as determined by visual inspection, were removed from further analysis. To normalize log ratio data, a print tip-specific Lowess curve was fit to the log-intensity versus log-ratio plot, using 20.0% of the data to calculate the fit at each point. This curve was used to center the log-ratio for each spot. Data were filtered to exclude poorly hybridized cDNAs by removing values with average foreground minus background intensity levels less than 300. We used the average of the two duplicate cDNA spots on each microarray chip in subsequent analyses. Data were filtered to include clones returning data for at least 75% of the samples in both preintervention and postintervention groups, which reduced the initial list of 6,751 clones to 5,711 clones and 5,643 clones in the low-fat/low-glycemic load and the standard American diet groups, respectively.

Statistical and Pathway Analyses. We used the significant analysis of microarrays algorithm⁵ to analyze differences in transcript levels between preintervention and postintervention specimens (11). Paired, two-sample *t* tests were calculated for each transcript, and genes differentially expressed were identified using false discovery rates (FDR) ranging from 5% to 50%.

Quantitative Reverse Transcription-PCR. We used quantitative reverse transcription-PCR to validate microarray results for selected genes. Primers specific for the genes of interest were designed using the web-based primer design service Primer3 provided by the Whitehead Institute for Biomedical Research.⁶ We determined acceptable performance characteristics of the PCR primers using normal human prostate cDNA, Biolase Taq polymerase (Bioline Inc.), and the GeneAmp PCR system 9700 (Applied Biosystems) as previously described (8).

⁵ <http://www-stat.stanford.edu/~tibs/SAM/>

⁶ http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

Table 1. Postintervention dietary characteristics and intervention differences, by study arm

	Group I: low-fat/low-glycemic diet (n = 4)	Group II: standard American diet (n = 4)	P
	Mean (SD)	Mean (SD)	
Glycemic load	135 (6)	266 (37)	0.0004
Calories	1,466 (367)	2,394 (215)	0.004
Fat (g)	51 (36)	94 (8)	0.06
Carbohydrates (g)	178 (12)	309 (47)	0.002
Protein (g)	82 (11)	83 (13)	0.9
% Calories from protein	23 (3)	14 (3)	0.007
Fiber (g)	21 (4)	13 (4)	0.02
Preintervention weight (kg)	91.4 (20.1)	90.9 (12.8)	0.97
Postintervention weight change (kg)	-5.3 (1.7)	0.8 (4.5)	0.02*

*Test for difference in weight change between study groups, adjusted for baseline weight.

Total RNA from participant samples were reverse transcribed, and cDNA was purified as previously described. Relative quantification of gene expression by quantitative PCR (40 cycles of 60°C annealing, 72°C extension, and 95°C melting) was done on a 7700 Sequence Detector using SYBR Green Master mix and gene-specific primers following the manufacturer's recommendations.

Results

Participant Demographic and Clinical Characteristics. Median participant age was 64 years (range, 59-69), median body mass index was 29 (range, 23-35), median preoperative serum prostate-specific antigen was 5.2 ng/mL (range, 2.5-16.0), median prostate volume was 50 mL (range, 35-149), and all but one participant was Caucasian.

Intervention Effects on Diet and Weight. The intervention resulted in profoundly different dietary

patterns in the two study arms (Table 1). Compared with men in the standard American arm, participants in the low-fat/low-glycemic load arm consumed 39% less total energy, 46% less fat, 42% less carbohydrate, and 62% more fiber, and had a 51% lower glycemic load. The intervention effect for weight, defined as the difference in weight change between study arms adjusted for baseline weight, was 6.1 kg (95% confidence interval, 1.6-10.5 kg; P = 0.02).

Gene Expression Changes Associated with Dietary Intervention. Table 2 gives the distributions of gene expression differences in tissues obtained before and after dietary intervention. Differences in these tables are categorized symmetrically above and below zero in units of log₂. In both study arms, the relative difference in expression of ~95% of transcripts ranged between 0.67 and 1.49. In the standard American arm, expression of 20 transcripts decreased by 50% or greater, and 32 transcripts increased by 100% or greater. In the low-fat/low-glycemic load arm, expression of 9 transcripts decreased by 50% or greater, and expression of 43 transcripts

Table 2. Distribution of preintervention and postintervention differences in transcript expression, and number of significant differences at FDRs of 15%, 10%, and 5%, by treatment arm

	Preintervention vs. postintervention difference			Significant differences		
	Absolute difference (post/pre- log ₂)	Relative difference (post/pre- ratio)	Total, n (%)	FDR < 15% n (%)	FDR < 10% n (%)	FDR < 5% n (%)
Standard American arm (5,643 transcripts)	>+1.58	>3.0	3 (0.05)	0 (0)	0 (0)	0 (0)
	+ (1.32-1.57)	2.50-2.99	5 (0.09)	0 (0)	0 (0)	0 (0)
	+ (1.00-1.31)	2.00-2.49	24 (0.43)	0 (0)	0 (0)	0 (0)
	+ (0.58-0.99)	1.50-1.99	180 (3.19)	0 (0)	0 (0)	0 (0)
	+ (0.00-0.57)	1.00-1.49	2,549 (45.17)	0 (0)	0 (0)	0 (0)
	- (0.01-0.57)	0.67-0.99	2,658 (47.10)	0 (0)	0 (0)	0 (0)
	- (0.58-0.99)	0.50-0.66	204 (3.62)	0 (0)	0 (0)	0 (0)
	<-1.0	<0.50	20 (0.35)	0 (0)	0 (0)	0 (0)
Total			5,643 (100)	0 (0)	0 (0)	0 (0)
Low-fat/low-glycemic load arm (5,711 transcripts)	>+1.58	>3.0	11 (0.19)	11 (0.19)	10 (0.18)	6 (0.11)
	+ (1.32-1.57)	2.50-2.99	8 (0.14)	5 (0.09)	4 (0.07)	0 (0)
	+ (1.00-1.31)	2.00-2.49	24 (0.42)	3 (0.05)	2 (0.04)	0 (0)
	+ (0.58-0.99)	1.50-1.99	118 (2.07)	1 (0.02)	0 (0)	0 (0)
	+ (0.00-0.57)	1.00-1.49	2,595 (45.4)	0 (0)	0 (0)	0 (0)
	- (0.01-0.57)	0.67-0.99	2,831 (49.6)	0 (0)	0 (0)	0 (0)
	- (0.58-0.99)	0.50-0.66	115 (2.01)	3 (0.05)	3 (0.05)	3 (0.05)
	<-1.0	<0.50	9 (0.16)	7 (0.12)	7 (0.12)	7 (0.12)
Total			5,711 (100)	30 (0.53)	26 (0.46)	16 (0.28)

HUGO	NAME	PRE-DIET				POST-DIET				AVERAGE RELATIVE EXPRESSION
		1	2	3	4	1	2	3	4	
MMP7	Matrix metalloproteinase 7	13.1								
OLFM4	Olfactomedin 4	6.9								
BF	B-factor properdin	6.4								
IGF-2R	Insulin-like growth factor-2 receptor	3.5								
VMP1	Likely ortholog of rat vacuole membrane protein 1	3.3								
TGFB1I4	Transforming growth factor beta 1 induced transcript 4	3.2								
IER3	Immediate early response 3	3.2								
CCT2	Chaperonin containing TCP1, subunit 2 (beta)	3.1								
HLA-DRB5	Major histocompatibility complex class II DR beta 4	3.1								
CXCR4	Chemokine (C-X-C motif) receptor 4	2.8								
LUM	Lumican	2.8								
CCL2	Chemokine (C-C motif) ligand 2	2.7								
DUSP1	Dual specificity phosphatase 1	2.6								
IER2	Immediate early response 2	2.5								
SPARCL1	SPARC-like 1	2.3								
TBX3	T-box 3	2.1								
ETS1	V-ets erythroblastosis virus E26 oncogene homolog 1	2.0								
SLC25A3	Solute carrier family 25 member 3	0.6								
PRDX1	Peroxiredoxin 1	0.5								
ABAT	4-aminobutyrate aminotransferase	0.4								
FOLH1	Folate hydrolase (prostate-specific membrane antigen)	0.4								
BPGM	23-bisphosphoglycerate mutase	0.4								
MGC15937	Similar to RIKEN cDNA 0610008P16 gene	0.3								

Figure 1. Differential gene expression of predietary versus postdietary intervention (low-fat/low-glycemic load, group I) specimens. Paired two-sample *t* test results for presurgery versus postsurgery comparison. Twenty-six unique up-regulated genes with FDR \leq 10% are listed. Average relative expression, change from preintervention to postintervention.

increased by 100% or greater. None of the differences in transcript expression in the standard American arm were statistically significant, even using a FDR as high as 50%. In contrast, in the low-fat/low-glycemic load arm, 30 (0.53%) of the cDNAs using a criterion of a 15% FDR and 16 (0.28%) of the cDNAs using a more conservative criterion of a 5% FDR were statistically different.

Figure 1 shows the list of 23 unique genes that showed significantly changed expression in the low-fat/low-glycemic load arm. Several genes are related to cell migration and tissue remodeling, including MMP7 (also called matrilysin), CXCR4, CXCL2, lumican, and SPARC-like 1. Others are involved in intracellular signal transduction, such as the immediate early response genes 2 and 3, the dual specificity phosphatase 1, and the v-ets oncogene homologue. Expression of insulin-like growth factor-II receptor transcripts increased, perhaps due to a positive feedback of the low-glycemic load diet. Genes that were down-regulated include prostate-specific membrane antigen and peroxiredoxin 1, which may play an antioxidant protective role in cells. We confirmed these microarray results by quantitative reverse transcription-PCR and found a correlation of 0.97.

Discussion

In this small randomized clinical trial, we showed the feasibility of studying dietary effects on gene expression using the preprostatectomy model. We collected both pretreatment biopsies and posttreatment *in situ* biopsies,

and delivered an effective dietary intervention within the weeks between diagnostic biopsy and surgery. We also showed that tissue from a single prostate biopsy core, weighing \sim 3 to 5 mg, yielded adequate RNA, albeit after a single round of amplification, for multiple analyses, including cDNA microarrays and quantitative reverse transcription-PCR. Lastly, we found that the intraindividual variability in gene expression in tissues collected at two time points is sufficiently small to allow the detection of intervention effects. Much larger sample sizes are needed to rigorously measure the reliability of transcript expression from prostate tissue collected at different times, but these results are encouraging for future research examining the effects of short-term interventions on prostate tissue.

The low-fat/low-glycemic load diet, and its associated weight loss, was associated with multiple gene expression changes. Many of these changes could conceivably alter the proliferation, metabolism, and redox potential of prostate epithelial cells. For example, insulin-like growth factor-I receptor binds both insulin-like growth factor-I and -II, and the role of insulin-like growth factor-I receptor in tumorigenesis and proliferation is well established (8). We emphasize, however, that much larger samples would be needed to make strong biological inferences based on gene expression arrays.

The principal limitation of this study is its small sample size. We had little power to detect modest changes in transcript expression. We also sampled only \sim 6,000 genes of the prostate tissue transcriptome, and many additional genes that were not evaluated could be

affected to an equal or greater extent. We also have not determined whether the diet-associated changes in transcript levels result in corresponding alterations in the cognate proteins. Lastly, we limited our analyses to the epithelial compartment, as prostate cancer arises from the epithelium; however, there are many interactions between the prostate stroma and epithelium (12–14).

In summary, we conclude that the effect of complex dietary changes on gene expression can be evaluated within the preprostatectomy model. These results provide important information for future studies that aim to examine the role of diet, obesity, and prostate carcinogenesis and/or progression.

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Low-Fat, Low-Glycemic Load Diet and Gene Expression in Human Prostate Epithelium: A Feasibility Study of Using cDNA Microarrays to Assess the Response to Dietary Intervention in Target Tissues

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Cancer Epidemiol Biomarkers Prev 2007;16:2150-2154.

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Determining the efficacy of dietary phytochemicals in cancer prevention

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Keywords: chemoprevention, biomarkers, indoles, polyphenols, tumour subtypes, plasma profiling

Abbreviations: DIM – di-indolylmethane; EGCG – epigallocatechin-3-gallate; EGF – epidermal growth factor; EGFR – epidermal growth factor receptor; EMT - epithelial to mesenchymal transition; ER – oestrogen receptor; ESA – epithelial specific antigen; Her2 – EGFR family member ErbB2; I3C – indole-3-carbinol; MS/MS - tandem mass spectrometry; SVM – support vector machine; uPA – urokinase plasminogen activator; VEGF – vascular endothelial growth factor

Abstract

Accumulating data suggest that dietary phytochemicals have the potential to moderate deregulated signalling or reinstate checkpoint pathways and apoptosis in damaged cells, while having minimal impact on healthy cells. These are ideal characteristics for chemopreventive and combination anti-cancer strategies, warranting substantial research effort into harnessing the biological activities of these agents in disease prevention and treatment. However, this requires further investigation into their mode of action and novel approaches to the development of reliable biomarkers.

Introduction

Epidemiology indicates that the incidence of many common cancers is geographically and environmentally determined and that diets rich in fresh fruit and vegetables confer a lower risk of developing tumours in many target tissues. Around one third of all cancers are thought to be related to unhealthy diet and therefore, in theory, preventable [1]. However, linking particular dietary components to prevention of specific cancers has proved to be a major challenge.

Many phytochemicals of differing chemical structure have anticarcinogenic properties. They activate cytoprotective enzymes and inhibit DNA damage to block initiation in healthy cells, or modulate cell signalling to eliminate unhealthy cells at later stages in the carcinogenic process. *In vitro* data for several well-studied compounds indicate that each can affect many aspects of cell biochemistry, but different agents have many similar activities (Table 1). Nevertheless effects can be cell-type specific, with transformed cells being particularly sensitive. But despite the exponential increase in knowledge regarding mechanisms of action of these molecules, their success in clinical trials has been limited. In addition to the difficulties of long-term prevention trials in healthy populations, there are other reasons for this. In cancer therapeutics, emphasis is increasingly placed on targeting tumour subtypes within a particular tissue, but this has not generally been considered for chemopreventive trials. In many instances the crucial *in vivo* targets for particular dietary molecules are unknown, making it difficult to predict which cancer phenotypes are most likely to be affected. Many phytochemicals are poorly bioavailable and evidence suggests that combinations may be more effective than single agents. There may also be advantages in combining them with chemo- or radio-therapies. Probably the greatest hindrance to more successful trials is the lack of validated biomarkers of efficacy. To enable many more agents to be tested much more quickly, validated surrogate endpoint biomarkers are

required, which will accurately determine outcome at a much earlier time in the process of tumour development.

Identifying at risk individuals

In order to optimise the chances of success in cancer chemoprevention trials, the ability to identify those individuals most likely to benefit is clearly important [2]. In the case of primary prevention to inhibit the earliest stages of tumour development, selection has traditionally been based on known environmental and lifestyle risk factors, genetic predisposition and family history. But with such cohorts, obtaining definitive evidence to directly link exposure to a particular dietary agent with prevention of cancer in any target tissue is at best extremely challenging, at worst impossible. In future it is possible that individuals suitable for primary prevention will be identified through screening for more common multiple susceptibility loci, such as those recently reported for breast cancer [3]. Such loci together could inform on a substantial fraction of the genetic variance in some cancers.

Patients can be recruited at later stages of the carcinogenic process. Secondary prevention is appropriate for those who have already developed premalignant lesions, such as intraepithelial neoplasia or intestinal polyps, the progress of which can be monitored in response to chemopreventive treatments. Several dietary compounds, including indoles and polyphenols have shown promise in this respect, with regression of respiratory papillomatosis, cervical, vulvar and prostate intraepithelial neoplasia and oral leukoplakia [4-7]. However, the relationship of some of these early lesions to tumour outcome is uncertain. Clearly a strategy involving susceptibility loci would also be relevant at this stage, adding a degree of certainty to the prognosis.

A third strategy is tertiary prevention, which focuses on patients who have been successfully treated for a primary tumour, in order to inhibit development of second primary tumours. Greatest success to date in this respect has resulted from the use of drugs such as tamoxifen and its analogues for breast cancer, and retinoids for skin, head and neck and liver cancer [8]. If phytochemicals have a role at this stage, it is most likely to be as part of a combined therapy.

Arguably a fourth category of patients who could benefit would be those with a superficial or primary tumour, where intervention is designed to prevent invasion and metastases. While the cellular process of epithelial to mesenchymal transition (EMT) is fundamental to morphogenesis, when reactivated in cancer, it facilitates invasion and metastasis[9]. Reversing this process could limit metastatic spread, achieving late-stage prevention with

enhanced survival. Ability of chemopreventive agents to inhibit EMT or angiogenesis has been demonstrated in a number of animal models. Use of phytochemicals for this purpose would be facilitated by knowledge of the characteristics within the primary tumour which predict invasive potential. We previously showed that the polyphenol, curcumin, modifies the invasive potential of breast cancer cells[10]. Another polyphenol, epigallocatechin-3-gallate (EGCG), was found to inhibit neovascularisation in the chick chorioallantoic membrane assay and when given in drinking water could significantly suppress vascular endothelial growth factor (VEGF)-induced corneal neovascularisation [11]. Such results suggest that EGCG may be a useful inhibitor of angiogenesis *in vivo*. A number of phytochemicals also affect expression of cadherins, catenins and matrix metalloproteinases (Table 1), and prognostic metastatic biomarkers for breast cancer, such as urokinase plasminogen activator, uPA/PAI1 and Her2 [12] all of which can modulate invasive capacity.

Tumour subtypes

A key aspect of targeted therapies, which has so far received much less attention in chemoprevention trials, is the concept of tumour subtypes. Tumour development involves the accumulation of multiple mutations which differ from one tumour to the next in the same target tissue. Such changes can be assessed using gene expression profiles to identify patterns of pathway deregulation which inform on disease prognosis and indicate treatment options [13]. Subtypes are perhaps best defined for the leukaemias [reviewed in 14] and in breast cancer, where at least 5 subtypes including luminal A (oestrogen receptor α (ER α) $^{+}$ positive), luminal B (ER α $^{+}$ positive), basal-like (ER α $^{-}$ negative, Her2 $^{-}$ negative, cytokeratin 5/6 $^{+}$ positive, and/or epidermal growth factor receptor (EGFR)/Her1 $^{+}$ positive), Her2 (Her2 amplicon $^{+}$ positive, ER $^{-}$ negative) and normal breast-like have been identified [15,16]. Such patterns in cancer cell lines have also been shown to be useful in predicting response to therapeutic agents [13, 17,18]. However, subtypes identified on the basis of gene expression profiles do not correspond exactly to those identified by immunohistochemistry [19,20] and used in clinical practice [21].

Additionally, gene signatures which predict the response to individual agents can be used in combination to predict the efficacy of multidrug regimes [22]. Dietary chemopreventive agents certainly exhibit cell-type specificity, but because of their broad ranging activity, they may be effective against different tumour subtypes. Recent studies show that indole-3-carbinol (I3C) induces apoptosis by decreasing expression of

genes essential for tumour cell viability, such as ER α and EGFR in breast cells of luminal A and basal-like subtypes [23,24]

Molecular biomarkers

There is no doubt that identification of reliable molecular markers, such as those in subtype molecular signatures, would be extremely advantageous to accurately predict efficacy of any intervention. Ideal markers should be indicative of early changes, relate directly to the carcinogenic process and where possible allow less invasive assessment of chemopreventive efficacy [25]. For some approaches, this requires a detailed knowledge of not only the stages of carcinogenesis for a particular tumour subtype, but also the mechanisms of action of the preventive agent. Molecular biomarkers would be valuable as targets to identify new agents or to optimise lead compounds; as risk biomarkers for selecting suitable cohorts for chemopreventive trials; or as indicators of efficacy for determining response to mechanism-based interventions or identifying potential toxicity.

There is no shortage of candidate proteins related to oncogenic processes (drug metabolising enzymes, growth factors, transcription factors, cell cycle and apoptosis related proteins) known to be aberrantly expressed in various tumours and modulated by phytochemicals *in vitro* (Table 1). The philosophy of chemoprevention suggests long-term or life-time exposure to low doses. Therefore, when selecting potential biomarkers of efficacy, it is essential to ensure that they are modulated under appropriate conditions. However, many of the *in vitro* mechanistic studies have been carried out with single high doses of chemopreventive agent that are not achievable *in vivo*. Thus some of the reported effects may not be physiologically relevant. In order to better predict how phytochemicals may act in humans, several approaches are required. Pharmacokinetic and pharmacodynamic studies allow estimates of plasma and, in some cases tissue, concentrations to be made. Where these already exist, they indicate that achievable concentrations are in the nanomolar, or at best, low micromolar range. In humans curcumin levels are typically in the low nanomolar range, although ~10 μ M can be achieved in colorectal tissue. Resveratrol plasma concentrations of ~2 μ M appear typical [reviewed in 26]. These low doses then need to be applied in cell culture studies over extended periods to better mimic *in vivo* exposure. We exposed the metastatic breast line, MDA-MB231, to physiologically achievable concentrations of five agents in long-term culture and observed favourable alterations to cell cycle, clonogenicity, apoptosis and expression of several proteins associated with EMT (Moiseeva et al unpublished data). Encouragingly, quite a

few published studies, in which single treatments with physiological concentrations of dietary agents have been used *in vitro*, also reveal significant biological activity [reviewed in 26]. Additionally a number of phytochemical effects observed *in vitro* have been validated in animal models.

Monolayer cultures are unrepresentative of *in vivo* environments, so that models where cells are grown on relevant substrata like collagen, laminin or matrigel, or as 3-dimensional spheroids may be more compatible with *in vivo* activity. We found that breast cells responded differently to I3C under such growing conditions [27]. In 3D culture (collagen 1 or spheroids) the sensitivity of MCF7 and MDA-MB-468 breast cancer cells towards I3C was increased. In MDA-MB-468 cells the expression of the EGFR and β 1-integrin was modulated by 3D culture, with cells responding differently to EGF or the EGFR inhibitor, PD153035. Phytochemical effects are often cell-type specific and so different panels of biomarkers may be required for different target tissues, or for different cancer subtypes within a single tissue. On the other hand there appears to be a certain degree of similarity in the protein targets affected by a variety of structurally unrelated phytochemicals, suggesting similar mechanisms of action (Table 1).

A detailed understanding of the effects of dietary agents (for example on growth factor signalling, EMT, cell cycle arrest and apoptosis) following extended treatment at physiologically achievable doses, and related to target tissue and cancer subtype, will help to identify useful biomarkers. Such an understanding would include identification of primary targets of phytochemicals (particular proteins such as receptors, or more general effects such as endoplasmic reticulum stress or altered redox status), an appreciation as to why healthy cells are generally more resistant and comparison of *in vitro* with *in vivo* efficacy.

Cancer-initiating stem cells

An increasing number of studies are reporting the identification of a subset of cancer stem cells within a tumour, which are thought to be responsible for the highly aggressive nature of different cancers. Breast cancer cells, grown in immune compromised mice, contained a subpopulation expressing cell surface markers [epithelial specific antigen (ESA)⁺CD44⁺CD24^{-/low}Lineage⁻]. This subset maintained the ability to form new tumours [28 Al-Hajj]. Interestingly, the basal-like breast cancer subtype has similarity with breast stem cells [20, 29], suggesting the “stem-like” characteristics of this subtype may be responsible for its aggressiveness and poor prognosis. Using a xenograft model of primary human pancreatic adenocarcinoma, Li et al [30] described a subpopulation of cancer cells

[expressing CD44, CD24 and ESA] that were 100-fold more tumorigenic than other cells from the same tumours. Similarly O'Brien et al., [31] identified a highly tumorigenic subset of colon cancer cells positive for CD133 (1 colon cancer initiating cell per 5.7×10^4 tumour cells). Clearly if biomarkers can be used reliably to identify subsets of cancer initiating cells, it will be essential to determine the efficacy of treatments against these, in preference to other phenotypes within the tumour.

Dietary agents in combined treatments

Also of increasing importance is the investigation of combinations of phytochemicals, or their use in conjunction with other therapies, to increase efficacy or decrease unwanted side effects [26, 32]. We have shown in breast cell lines that I3C exhibits enhanced efficacy in combination with src or EGFR kinase inhibitors [24] and *in vivo* I3C prevented the hepatotoxicity of trabectedin (ET743), an experimental anti-tumour drug with promising activity in sarcoma, breast and ovarian carcinomas, without compromising anti-tumour efficacy [33]. Curcumin enhances the efficacy of oxaliplatin in both p53^{positive} and p53^{mutant} colon cancer cells [34]. However, caution is required, since it has also been reported to compromise the efficacy of some chemotherapeutic drugs in human breast cancer models [35].

Proteomics

Methods which do not require a detailed knowledge of mechanisms, initially at least, offer an alternative approach to developing biomarkers and assessing chemopreventive efficacy. Mass spectrometry of biological samples offers a powerful proteomic tool for the discovery of novel biomarkers and for profiling [36]. Proteins and peptides in clinical samples reflect the intracellular activities of healthy and diseased tissue. Plasma samples subjected to mass spectrometry can provide characteristic protein profiles, when over- and under-expressed peptides are determined by pattern comparison using a variety of machine learning algorithms [37-39]. This technique should offer the possibility of monitoring plasma profiles in at risk individuals in response to a particular treatment regime. Development of these methods for cultured cells, would also provide the means to screen effects of chemopreventive agents – for example, to estimate the lowest concentration of agent that causes a change in profile; to look for dose-response in altered peaks; to compare effects of one agent in different cell types; to compare the signatures between compounds in the same cell type; and to enable identification of signature peaks. Individual proteins can be identified by correlating the sequences of tryptic peptides generated by tandem mass spectrometry (MS/MS) with sequences in protein databases.

The evolutionary algorithms with support vector machine (SVM) supervised learning enable identification of biomarkers with the highest diagnostic and prognostic potential. The effectiveness of combined treatments could also be explored. Nuclear fractions could provide enrichment for investigating changes in transcription factors and cell cycle-related proteins. In view of the importance of protein phosphorylation in proliferation, differentiation and apoptosis, development of techniques selective for phosphopeptides would provide added value [40, 41].

Conclusions

In order to fully appreciate the potential for dietary compounds in cancer prevention and bridge the gap between apparent *in vitro* efficacy and clinical use, a number of approaches are required. Attention must be paid to the use of physiologically relevant concentrations, to chronic exposure and to 3D cultures, to more accurately mimic *in vivo* situations. Validation of potential mechanisms in appropriate animal models is also important. The mechanistic biomarkers identified *in vitro* need to be verified in human tissues as being central to the carcinogenic process. Finally genomics and proteomics approaches offer novel ways of predicting clinical efficacy.

Acknowledgements

The authors are grateful to Professor Peter Farmer and Dr Don Jones for critical discussion.

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Table 1 – Proteins modulated by dietary agents

Dietary compound	Altered expression/activity
curcumin (from turmeric)	p21; p27; p38; p53; p70S6K; abl; AIF; pAkt; AP-1; ARNT; ATPase; Bad; Bax; Bcl-2; Bcl-XL; Bid; BTG2; E-cadherin; cadherin-11; caspase 3, 4, 8, 9; β-catenin; CD80/86; cdc2; cdc25C; cdc25B; CHK2; chymase II; collagenase; cyclin A/B1/D1/E; cyt c; COX-2; DR5; EGFR; EIF2α; elastase; ELK1; Erg1; ERK1/2; FLIP; c-fos; GADD153/CHOP; GADD45; Gb3 synthase; GM-CSF; GRP78; HAT; HDAC; Her2; HIF-1; HO-1; hyaluronidase; IAP1/2; ICAM-1; IFNγ; IGF-1R; IκBα; IKK; IL2/6/8/10/12; iNOS; IRAK; IRF3; JAK1/2; JNK; c-jun; LOX; MAPKAP-K1β; MDM2; MHC class II antigens; MMP2/3/9/13; mTOR; myc; MyD88; myeloperoxidase; Nag-1(PLAB); NF-κB; Notch1/3/4; Nrf2; ODC; PARP; PCNA; PERK; PGE; phase1/2 enzymes; PHK; PKC; PPARγ; pRb; src; smac; STAT1/3; survivin; SYK; TGFβ; Th1; thioredoxin reductase; TNFα; TNFR1; topoisomerase II; TRAIL; α/β-tubulin; VEGF; wee1; XIAP;
resveratrol (from red grapes, wine)	p21; p27; p38; p53; p57; p70S6K; pAkt; AP-1; APAF1; ASK1; ATM/ATR; Bad; Bak; Bax; Bcl-2; Bcl-xL; Bid; caspase 2,3,8,9; E-cadherin; VE-cadherin; β-catenin; p300/CBP; cdc2; cdc25C; CDK2/4/6/7; CHK1/2; cyclin A/D1/D2/E/G; cdc42; COX1/2; cyt c; EGFR; ER; ErbB3; ERK; FasL; c-fos; Fra1/2; GADD153/CHOP; GADD45a; GJIC; GSK3; H2A.X; Her2; HO-1; IAP1; IκBα; IKK; IL6/8; iNOS; JNK; c-jun; LOX; Mad21; MDM2; MMP2/9; NF-κB; Nrf2; ODC; PI3K; phase1/2 enzymes; PHK; PIG7; PIG8; PIG10; PKC; PPARγ; PR; PTEN; pRb; SIRT1; Sp1; src; STAT3; survivin; TBK1; tensin; topoisomerase II; TRAIL R; TRIF; VEGF; XIAP
EGCG (from green tea)	p16; p18; p21; p27; p38; p53; p130; p107; p70S6K; pAkt; AP-1; APAF1; Bad; Bax; Bcl-2; Bcl-xL; caspase 3,8,9; DNMT; E2F; E-cadherin; VE-cadherin; β-catenin; cdc2; CDK2/4/6; C/EBPα & β; COX2; cyclin A/B1/D1/E; cyt c; DP1/2; EGFR; Erg-1, ERK1/2; Fas; FasL; bFGF; FGFR; fibrinogen; fibronectin; FKHR; c-fos; GADD153/CHOP; GADD45a; Her2; hist-rich glycoprotein; HO-1; IκBα; IKK; IL6/8; iNOS; involucrin; JNK; c-jun; lamin; LOX; 67LR; Mcl-1;MDM2; MMP1/2/7/9/13/14; NF-κB; Nrf2; ODC; PDGFR; PGES-1; PI3K; phase1/2 enzymes; PKA; PKC; PRAK; RARβ; pRb; STAT3; survivin; telomerase; TIMP1; topoisomerase I; VEGF; VEGFR; vimentin; XIAP
I3C (cruciferous vegetables)	p15; p16; p21; p27; p53; pAkt; ATM; Bad; Bax; Bcl-2; Bcl-xL; BCRP/ABCG2; BRCA1; E-cadherin; caspase 3,8,9; catenins; CDK2/4/6; CHK2; COX-2; CXCR4; cyclin D1/E; cyt c; DR5; EGFR; ER; Ets; FLIP; Her2; IAP1/2; IL6/8; MDM2; MMP2/9; MUC1; Nag-1(PLAB); NF-κB; Nrf2; ODC; Pgp; phase1/2 enzymes; PI3K; PTEN; pRb; Sp1; src; STAT3; survivin; topoisomerase II; TRAF1; TRAIL R; uPA; VEGF; vimentin; XIAP
DIM (cruciferous vegetables)	p21; p27; p38; pAkt; AR; ATF2; ATF3; Bax; Bcl-2; Bcl-xL; Bid; BRCA1; E-cadherin; cadherin-11; N-cadherin; P-cadherin; caspase 3,6,7,8,9; β-catenin; cdc2; cdc25A; CDK2/4/6; CREB; cyclin D1; cyt c; DR5; EGFR; ER; ERK1/2; Fas; FLIP; GADD45; GADD153/CHOP; GRP78(BiP); Her2; IFNγ; IFNγR1; JNK; c-jun; MEK; MHC-1; mitochondrial H⁺-ATPase; MMP9; Nag-1(PLAB); NF-κB; p56/p69- oligoadenylate synthases; PCNA; phase1/2 enzymes; PI3K; PSA; Raf; Ras-GTP; smac/Diablo; Sp1/3; STAT1/3/5; survivin; TGF-α; topoisomerases IIα/IIβ/I; α/β-tubulin; uPA; VEGF

The expression, phosphorylation, activity or binding of the proteins listed is affected by five of the best-studied dietary agents - data are taken from *in vitro* studies in many cell types, but the list is not comprehensive. There are many mechanistic similarities between compounds of different chemical structures indicated in bold. We apologise to all the authors for not citing the original references owing to space restrictions. DIM – di-indolylmethane.

Biomarkers of Dietary Energy Restriction in Women at Increased Risk of Breast Cancer

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Abstract

Dietary energy restriction (DER) reduces risk of spontaneous mammary cancer in rodents. In humans, DER in premenopausal years seems to reduce risk of postmenopausal breast cancer. Markers of DER are required to develop acceptable DER regimens for breast cancer prevention. We therefore examined markers of DER in the breast, adipose tissue, and serum.

Nineteen overweight or obese women at moderately increased risk of breast cancer (life-time risk, 1 in 6 to 1 in 3) ages between 35 and 45 were randomly allocated to DER [liquid diet, 3,656 kJ/d (864 kcal/d); $n = 10$] or asked to continue their normal eating patterns ($n = 9$) for one menstrual cycle. Biopsies of the breast and abdominal fat were taken before and after the intervention. RNA was extracted from whole tissues and breast epithelium (by laser capture microdissection) and hybridized to Affymetrix GeneChips. Longitudinal plasma and urine samples were collected before and after intervention, and metabolic profiles were generated using gas chromatography-mass spectrometry.

DER was associated with significant reductions in weight [$-7.0 (\pm 2.3)$ kg] and in alterations of serum biomarkers of breast cancer risk (insulin, leptin, total and low-density lipoprotein cholesterol, and triglycerides). In both abdominal and breast tissues, as well as isolated breast epithelial cells, genes involved in glycolytic and lipid synthesis pathways (including *stearoyl-CoA desaturase*, *fatty acid desaturase*, and *aldolase C*) were significantly down-regulated.

We conclude that reduced expressions of genes in the lipid metabolism and glycolytic pathways are detectable in breast tissue following DER, and these may represent targets for DER mimetics as effective chemoprophylactic agents.

Dietary energy restriction (DER) significantly reduces spontaneous mammary tumors in laboratory rodents irrespective of the type of macronutrient restricted (1). Observational studies indicate that weight loss in premenopausal or postmenopausal women reduces the risk of postmenopausal breast cancer (2, 3). For example, in the Iowa Women's Health Study, women who lost >5% of their body weight before menopause had 40% fewer breast cancers in the postmenopausal period compared with women who continued to gain weight (2). Postmenopausal weight loss of >10 kg reduced postmenopausal

breast cancer by 60% in the Nurses' Health Study, and >5% postmenopausal weight loss in the Iowa Women's Health Study was associated with a 25% reduction in risk (2, 3). Reduction in cancer risk is also seen after weight reduction after bariatric surgery (4–7). DER is associated with diverse cellular changes, including alteration of growth factor, signaling and metabolic pathways, and reduction of cell proliferation (8, 9), in animal studies. DER favorably alters serum markers of breast cancer risk, such as insulin, leptin, sex hormone binding globulin (SHBG), and lipids, in women who are overweight or

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Grant support: Breakthrough Breast Cancer (K.R. Ong and A.H. Sims), Genesis (M. Harvie), and Biotechnology and Biological Sciences Research Council and Engineering and Physical Sciences Research Council (W.B. Dunn, D. Broadhurst, and R. Goodacre). R.B. Clarke is a Breast Cancer Campaign Research Fellow. Genesis provided funds for the Affymetrix GeneChips.

Note: Supplementary data for this article are available at Cancer Prevention Research Online (<http://cancerprevres.aacrjournals.org/>).

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doi:10.1158/1940-6207.CAPR-09-0008

obese (10–13). A recent study reported changes in the urinary and serum metabolome of long-term dietary-restricted dogs (14). Several groups have assessed the effects of energy restriction (1–3) and changes in macronutrient profiles (1, 3) on gene expression in s.c. abdominal adipose tissue. These studies were undertaken in obese men and women and were relatively short-term studies with periods of energy restriction of between 2 and 28 days (2) or of 5 (1) and 10 weeks (3). However, there has been, to our knowledge, no assessment of breast gene expression in response to DER (15–18). Of the many gene changes induced by DER, down-regulation of genes associated with fatty acid synthesis and desaturation is the most prominent.

In the study reported here, we sought to assess whether effects of DER are apparent in breast tissue of overweight and obese women at increased risk of breast cancer to begin to understand a possible molecular mechanism for DER in breast cancer prevention.

Materials and Methods

Subject selection

Nineteen premenopausal overweight or obese [body mass index (BMI) between 28 and 40 kg/m²] parous women ages between 35 and 45 y at increased risk of breast cancer (estimated lifetime risk, 1 in 6 to 1 in 3; ref. 19) were recruited from our Family History Clinic and randomized to DER or to continue normal diet (Fig. 1). Other entry criteria included a normal mammogram within 24 mo, stable or increasing weight, and sedentary lifestyle (not participating in >40 min of moderate activity per week). Exclusion criteria included already losing weight following restrictive diets; taking dietary supplements; recent use of tamoxifen; regular use of anti-inflammatory, anticoagulant, antiplatelet, or oral contraceptive medication; pregnant or planning a pregnancy; previous hysterectomy; and serious comorbid conditions such as a previous diagnosis of cancer, diabetes, ischemic vascular disease, thyroid disease, or psychiatric disorders. Seventy-eight volunteers were eligible but 59 decided not to participate, mainly after disliking a 2-d test diet or on learning the invasiveness of the protocol. Although the 59 nonparticipating women were eligible in terms of weight and other criteria, it is unknown how they compared with participating women for the other characteristics measured in Table 1. The study was reviewed by the Bolton (Lancashire) Local Research Ethics Committee (Number 05/Q1409/42). All participants provided written informed consent before participation.

Study protocol

Breast and s.c. abdominal biopsies and fasting blood and urine were collected in the follicular phase shortly after the beginning of menses for two subsequent menstrual cycles to avoid widely fluctuating hormone levels producing confounding alterations in gene expression (20). Weight and total body fat (bioelectrical impedance, Tanita TBF300a), waist, hip, and bust measurements were also measured on this occasion. Three measurements of waist, hip, and bust size were made using standardized methods to the nearest 1.00 mm, and the mean of these measurements was used. Waist was measured at the level of the umbilicus and hip at the widest point over the tronchanters. Dietary intake and physical activity for 7 d before each assessment were assessed from a 7-d diet history and physical activity recall (21, 22). Energy and macronutrient intake of reported diets were assessed using the five-diet analysis program (Compeat). After the first biopsies, participants were randomized to either DER ($n = 10$) or a control group ($n = 9$) who were advised to maintain their usual eating and exercise pattern. DER consisted of four 325 mL cans of a commercially available nutritionally complete milk shake ("Slimfast," Unilever) and 2 to 3 L of other clear low-energy, high-potassium fluids

(water, black tea, coffee, low-calorie soft drinks, or stock-based drinks). Overall, the diet provided on average 3,614 kJ/d (864 kcal/d); 26% (58 g) of energy was from protein, 62% (97 g) from carbohydrate, 12% (26 g) from fat, and 7% (6.4 g) from saturated fat (Fig. 1). Participants randomized to DER were monitored weekly by the study dieticians to encourage good compliance, whereas those on their usual diet were monitored after 2 wk to check that weight was being maintained.

Biopsy procedures

Breast. Recent craniocaudal and mediolateral oblique mammograms of each participant were reviewed to assess the pattern of breast tissue. Biopsies in women with a dense glandular breast pattern were done under radiographic guidance ($n = 7$). A single-coned craniocaudal mammogram of the breast was obtained at the time of biopsy and done with the breast immobilized under the compression device. In women with a diffusely fatty breast pattern ($n = 12$), biopsies were done without radiological guidance. After infiltration of 2% lidocaine, a small incision was made in the skin at the biopsy site through which a 14-gauge biopsy needle was inserted to a depth estimated by the operator. Between seven and nine biopsy samples were obtained through the same skin incision, although the direction of the needle was altered for each sample.

Abdominal fat. Three to five milliliters of s.c. abdominal adipose tissue were obtained by suction biopsy under local anesthesia from the anterior abdominal wall, midway between the anterior superior iliac spine and the umbilicus. The first biopsies were taken from either the left or right breast/abdominal side chosen by computer randomization (independently) and repeat biopsies on the opposite side to eliminate gene expression changes due to the healing process. One half of two separate breast cores were fixed in 4% formalin and embedded in paraffin blocks; the remaining tissue was immediately snap frozen in liquid nitrogen and stored at -80°C.

Blood measures

Hormone, glucose, and lipid assays, which may be influenced by DER, were undertaken at the Clinical Biochemistry Department of South Manchester University Hospital Foundation Trust: glucose (hexokinase/glucose-6-phosphate dehydrogenase method; Bayer Diagnostics), insulin [electrochemiluminescence immunoassay, Elecsys; Roche Diagnostics; interassay coefficient of variation (CV), 10.6%], testosterone (chemiluminescence; Chiron Diagnostics; CV, 6.5%), and SHBG (noncompetitive IRMA; IRMA-Orion Diagnostics; CV, 8.4%). Fasting insulin and glucose were combined to calculate the insulin sensitivity index using the homeostasis model assessment (23). Total serum insulin-like growth factor-I (enzyme-labeled chemiluminescent immunometric assay; Diagnostic Products Corp.; CV, 11.3%), serum testosterone (chemiluminescence), and estradiol (electrochemiluminescence immunoassay) were also measured. Colorimetric enzyme reactions were used to measure total cholesterol (CV, 3%), triglyceride (CV, 5.4%; Bayer Diagnostics), and high-density lipoprotein cholesterol (CV, 7.1%; Randox); the levels were measured spectrophotometrically by an automated Olympus AU600 analyzer. The adipokines leptin and adiponectin and markers of inflammation high-sensitivity C-reactive protein and sialic acid were determined at MRC Human Nutrition Research Laboratory (Cambridge, United Kingdom). Plasma leptin concentration was measured using an ELISA method (Quantikine Human Leptin kit, R&D Systems; CV, 10%), whereas plasma adiponectin was measured using RIA (LINCO Research, Inc.; CV, 10%). Sialic acid was assayed using a colorimetric assay (Roche Diagnostics; CV, 1.2%) adapted for use on the Hitachi 912 Clinical Analyzer (Roche Diagnostics) and C-reactive protein using a high-sensitivity, particle-enhanced turbidometric assay (CV, 4.5%; Dade Behring). Serum and plasma samples were aliquoted, stored at -70°C, and batched so that all samples from a participant were included in the same assay (24). Laboratory personnel were blinded to the sample identity. Baseline age, BMI, and body fat and changes in weight,

adiposity, diet and exercise parameters, and hormone and lipid levels were compared between groups using the Mann-Whitney test using Statistical Package for the Social Sciences version 14 (SPSS, Inc.).

Metabolomics

Serum (in 200 µL aliquots) samples were added to an internal standard solution (0.174 mg·mL⁻¹ succinic d4 acid, 0.172 mg·mL⁻¹ glycine d5, and 0.182 mg·mL⁻¹ malonic d2 acid in water) and combined with 600 µL methanol, vortexed for 15 s, and centrifuged (15 min, 13,000 × g), and the supernatant was lyophilized (HETO VR MAXI vacuum centrifuge attached to a HETO CT/DW 60E cooling trap; Thermo Life Sciences). Urine samples were treated as for the serum, except 100 µL urease solution (10 mg·mL⁻¹) was added to 75 µL aliquots incubated at 37°C for 30 min to enzymatically degrade urea and then combined with 250 µL methanol. Lyophilized samples were chemically derivatized in two stages. 50 µL of 20 mg/mL O-methylhydroxylamine solution in pyridine were added and heated at 40°C for 90 min followed by addition of 50 µL of N-acetyl-N-(trimethylsilyl)-trifluoroacetamide and heating at 40°C for 90 min. A retention index solution was added for chromatographic alignment (20 µL, 0.6 mg/mL C10/C12/C15/C19/C22 n-alkanes in pyridine). Samples were subsequently analyzed in a random order using gas chromatography-time of flight-mass spectrometry (Agilent 6890 GC coupled to a LECO Pegasus III ToF mass spectrometer) as described previously using optimized settings for serum (25). Raw data were processed using LECO ChromaTof v2.12 and its associated chromatographic deconvolution algorithm, with the baseline set at 1.0, data point averaging of 3, and average peak width of 2.5. A reference database was prepared, incorporating the mass spectrum and retention index of all metabolite peaks detected in a random selection of samples so to allow detection of all metabolites present, whether expected or not expected from the study of metabolic pathways. Each metabolite peak in the reference database was searched for in each sample, and if matched (retention index deviation, <±10; mass spectral match, >700), the peak area was reported and the response ratio relative to the internal standard (peak area-metabolite/peak area-succinic d₄ acid internal standard) was calculated. These data (matrix of N samples × P metabolite peaks) representing normalized peak lists were exported in ASCII format. The

serum and urine data (analyzed separately) were split into DER and control groups for analysis with a two-sided paired Wilcoxon signed rank test for each metabolite using the null hypothesis that the difference (DER versus normal eating habits) in metabolite level comes from a continuous, symmetrical distribution with a median value of zero (26). Each significant ($P < 0.05$) metabolite was compared with its receiver operating characteristic for differentiating DER from controls.

RNA extraction

RNA was extracted from breast tissue using a protocol devised by M. Dowsett and A. Kendall (27). Briefly, 20 to 50 mg of breast tissue were ground to a fine powder under liquid nitrogen; RNA was isolated with 600 µL of Qiazol reagent (Qiagen Ltd.), which was passed through a commercially available RNA shearing device (QiaShredder, Qiagen); and 200 µL chloroform was added, incubated at room temperature for 5 min, and centrifuged at 12,000 rpm for 15 min. The aqueous phase was removed to a clean tube and 350 µL of 70% ethanol were added. The mixture was placed in an RNeasy Micro column (Qiagen) and the protocol for the RNeasy Micro kit was followed to completion. Abdominal adipose tissue total RNA was isolated using Trizol reagent (Invitrogen) according to the method described by Chomczynski and Sacchi (28). RNA samples were purified using RNeasy Mini columns (Qiagen) according to the manufacturer's protocols. RNA concentration and quality was assessed using a NanoDrop spectrophotometer (NanoDrop Technologies) and Agilent 2100 Bioanalyzer (Agilent Technologies). All whole tissue samples yielded RNA in sufficient quantity and quality for microarray analysis. Total RNA (2 µg) from abdominal tissue was processed according to the standard Affymetrix protocol for one-cycle target labeling to produce labeled cRNA. Total RNA (10 ng) isolated from the breast tissue core biopsies was amplified using a poly(A) PCR exponential amplification method based on the techniques described by Brady et al. (29) and Iscove et al. (30), which allow very small starting amounts of total RNA to be used. Samples were then hybridized to HGU133 Plus2 GeneChips (Affymetrix).

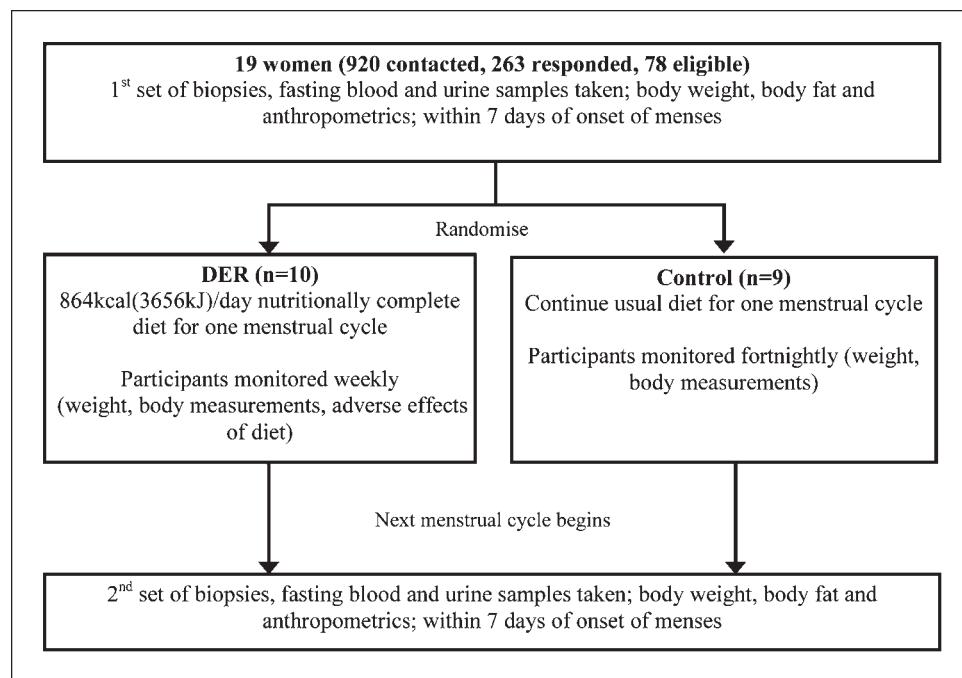


Fig. 1. Study design flowchart. Experimental design and sampling of the biopsies for the DER and control arms of the study.

Table 1. Changes in body weight, BMI, adiposity, lipid, and hormone levels in DER and control groups over the trial period

	Baseline measurements*		Change at 1 mo*		P†
	DER (n = 10)	Control (n = 9)	DER (n = 10)	Control (n = 9)	
Weight (kg)	93.3 (±10.5)	91.9 (±13.2)	-7.0 (±2.3)	0.3 (±1.5)	0.00
BMI (kg/m ²)	33.9 (±3.0)	32.6 (±2.4)	-2.5 (±0.8)	0.1 (±0.5)	0.00
Body fat (kg)	41.2 (±6.5)	40.7 (±9.0)	-4.6 (±1.5)	1.0 (±1.4)	0.00
Fat free mass (kg)	52.2 (±4.4)	51.2 (±4.3)	-2.4 (±1.3)	-0.4 (±0.9)	0.00
Body fat (%)	44.0 (±2.3)	43.8 (±3.4)	-1.9 (±1.2)	0.6 (±1.1)	0.00
Waist size (cm)	109.8 (±7.5)	104.5 (±11.6)	-6.6 (±6.0)	3.6 (±5.6)	0.00
Hip size (cm)	117.1 (±6.4)	115.0 (±8.6)	-5.8 (±2.6)	2.0 (±3.5)	0.00
Bust size (cm)	115.1 (±7.7)	111.8 (±6.3)	-4.1 (±2.6)	1.0 (±1.4)	0.00
Glucose (mmol/L)	4.4 (4.0-5.4)	4.8 (4.4-7.3)	0.0 (±0.4)	-0.1 (±1.2)	0.62
Testosterone (nmol/L)	0.9 (±0.5)	0.9 (±0.3)	0.0 (±0.3)	0.0 (±0.3)	0.90
Estrogen (pmol/L)	236.9 (124.0-1,265.0)	233.2 (138.0-996.0)	-165.9 (-906.0 to 196.0)	-96.7 (-664.0 to 458.0)	0.19
SHBG (nmol/L)	36.2 (20-138)	38.5 (28-51)	19.7 (±12.2)	0.3 (±5.1)	0.00
Cholesterol (mmol/L)	5.5 (±0.7)	4.8 (±0.9)	-1.2 (±0.5)	0.2 (±0.6)	0.00
Triglycerides (nmol/L)	1.5 (±0.5)	0.9 (±0.4)	-0.4 (±0.3)	0.1 (±0.5)	0.01
HDL (mmol/L)	1.4 (±0.2)	1.6 (±0.3)	-0.2 (±0.1)	0.0 (±0.3)	0.10
LDL (mmol/L)	3.4 (±0.7)	2.9 (±0.8)	-0.8 (±0.5)	0.2 (±0.5)	0.00
Insulin (pmol/L)	8.1 (±3.1)	8.3 (±3.3)	-2.6 (-7.4 to 3.4)	0.8 (-2.7 to 21.1)	0.14
Insulin sensitivity (μunit/mmol/L)	1.4 (0.5-2.4)	1.7 (1.0-4.8)	-0.4 (-1.3 to 0.7)	0.1 (-2.2 to 6.24)	0.32
IGF-I (μg/L)	155.3 (±35.6)	157.0 (±21.6)	-5.7 (±38.3)	5.7 (±21.5)	0.25
Leptin (ng/mL)	33.1 (±10.2)	26.9 (±13.0)	-21.0 (±6.8)	0.9 (±7.9)	0.00
Adiponectin (μg/mL)	14.5 (±6.1)	14.9 (±5.2)	-0.4 (±10.0)	-4.3 (±9.9)	0.48
Sialic acid (mg/L)	73.7 (±5.3)	72.1 (±8.3)	-2.3 (±7.5)	1.4 (±4.7)	0.13
Hs CRP (mg/L)	3.2 (±1.2)	3.2 (±1.5)	-0.3 (-3.2 to 8.5)	-0.5 (-2.6 to 4.9)	0.15

Abbreviations: Hs CRP, high-sensitivity C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; IGF-I, insulin-like growth factor-I.

*Mean (±SD) or geometric mean (range).

†P value for changes in parameters in the control group compared with changes in the DER group using Mann-Whitney test.

Laser capture microdissection

Before laser capture, frozen sections were fixed and areas of interest were stained using the Arcturus Histogene frozen section staining kit (KIT0401), as per manufacturer's instructions. The laser capture microdissection was done on the Arcturus XT System. Microdissection was done by laser pulses activating a thermoplastic polymer film attached to an inverted CapSure "cap," which then expands and surrounds the cells of interest, allowing removal. Following cell capture, RNA was extracted and isolated using the Arcturus PicoPure RNA isolation kit (KIT0202) as per manufacturer's instructions. RNA was amplified using the NuGen WT-Ovation Pico System RNA amplification kit (NuGen), labeled, and hybridized to Affymetrix U133 Plus2 GeneChips as described in full on our Web site.⁶

Microarray analysis

After scanning, array images were assessed by eye to confirm scanner alignment and the absence of significant bubbles or scratches on the chip surface. Ratios (3'/5') for glyceraldehyde-3-phosphate dehydrogenase and β-actin were confirmed to be within acceptable limits (0.86-1.38 and 0.70-1.24, respectively), and BioB spike controls were found to be present on all chips, with BioC, BioD, and CreX also pres-

ent in increasing intensity. All microarray data are MIAME compliant and accessible via MIAME VICE.⁷ Gene expression data were analyzed using packages within Bioconductor⁸ (31) implemented in the R statistical programming language. The gene expression data were normalized using the GeneChip Robust Multi-array Average algorithm (32) within the "simpleaffy" package (33) and then compared using multiclass significance analysis of microarrays (SAM; ref. 34) using the siggenes package. Baseline and repeat biopsies were paired and analyzed in the DER group and control group separately. The gene ontologies of significantly differentially expressed genes were examined using the GoMiner⁹ software (35).

Results

Ten women were randomized to DER, and 9 to their normal diet (median ages, 42.4 and 41.8 years, respectively), and median study periods were 26.2 and 26.9 days for the DER and control groups (Fig. 1). No woman withdrew from the study at any point. The biopsies were tolerated well, with no adverse side effects other than bruising at both the breast and abdominal

⁷ <http://bioinformatics.picr.man.ac.uk/vice/>

⁸ <http://www.bioconductor.org>

⁹ <http://discover.nci.nih.gov/gominer>

⁶ <http://bioinformatics.picr.man.ac.uk/mbcf/downloads/>

sites. The energy-restricted diet was well tolerated. The commonest side effects were constipation of variable degree ($n = 6$), eased in all cases by increasing intake of clear fluids, and halitosis ($n = 5$), which resolved by improving oral hygiene and was likely due to the thick texture and sugar content of the drink. There were no significant differences at baseline between the DER and control groups with respect to weight, BMI, body fat, calorie intake, dietary macronutrient composition, serum hormones, glucose, or inflammatory markers (Tables 1 and 2).

Change in anthropometry, hormones, and lipids

Compliance to the dietary regimens seemed to be good because all 10 women in the DER group lost weight (median loss of $-7.0, \pm 2.3$); in contrast, women in the control group showed minor fluctuations in weight with a median gain of $0.3 (\pm 1.5)$. Fat mass, fat free mass, waist, hip, and bust measurements decreased significantly in all of the women in the DER group; there was no change in these parameters in the control group (Table 1). As expected, the level of total and low-density lipoproteins, cholesterol, triglyceride, and leptin fell significantly and SHBG rose significantly in the DER group (Table 1). Women in both groups were sedentary and remained so throughout the study (Table 2).

Metabolomics

Of the 253 derivatized metabolite peaks detected, 83 could be definitively identified by matching the retention index (± 10) and mass spectral match (>700) of metabolite in sample to an authentic metabolite standard analyzed on the same analytic system. In addition, 49 were identified on a preliminary basis by matching (match >700) of mass spectrum only to metabolites present in available mass spectral libraries (NIST/EPA/NIH02 library and the publicly available MPI-Golm library).¹⁰ The metabolites that were significantly changed in plasma and urine for DER participants compared with controls are illustrated in Fig. 2 and were consistent with known effects of calorie restriction. A significant increase in glycolic acid in urine correlates with the reduction in glycolytic genes and increased lipid oxidation (Fig. 3). Increased urea and reduction in available amino acids, serine, and glycine in blood is concordant with the breakdown of proteins due to calorie restriction. There was also an increase in adipic acid and 3-hydroxybutanoic acid in urine following DER, suggesting increased lipid oxidation and the formation of ketone bodies. Observations of participants, however, suggested they were not ketotic, and the odor of ketones was not detectable on their breath. The low-energy diet chosen in this study was not expected to be ketogenic, containing 138 g/d of carbohydrates, which is far in excess of the minimum dietary intake of 50 g/d required to prevent ketosis (36).

Gene expression analyses

Microarrays were done on all breast and abdominal fat samples at time zero and after 1 month. In the control group, no probe sets were found to be consistently differentially expressed between baseline and repeat biopsies in either breast or abdominal adipose tissue using SAM software. Seventy-

two probe sets (representing 61 genes) were differentially expressed between baseline and repeat biopsies in the breast tissue in the DER group [with a false discovery rate (FDR) of 0.38], whereas 200 probe sets (representing 161 genes) were differentially expressed between baseline and repeat biopsies in the abdominal adipose tissue in response to DER (with a FDR of 0.01; full lists in Supplementary Tables S1 and S2). Choosing a lower FDR for the breast tissue resulted in no consistently significant genes being identified (due to greater tissue heterogeneity); conversely, a higher FDR for the abdominal tissue resulted in almost a quarter of the genes present on the array to be called significant. To ensure that the amplification step used for the breast tissue did not affect our findings, we also amplified RNA from the abdominal adipose tissue from three participants before and after DER and hybridized the samples to Affymetrix GeneChips; >90% of the top 200 probe sets identified using SAM analysis remained the same.

Many more genes were down-regulated than up-regulated with DER (56 of 61 in the breast and 113 of 161 in adipose tissue). Of the 61 genes significantly altered in the breast (Supplementary Table S1), 11 were also significantly altered in adipose tissue; several of these were represented by more than one probe set, showing reproducibility, although P values were lower for the abdominal tissue (Table 3). The most down-regulated genes in the breast [*stearoyl-CoA desaturase (SCD)*, *fatty acid desaturase 1 (FADS1)*, *transferrin*, and *aldolase C (ALDOC)*] were among those shared with adipose tissue. Other genes down-regulated in the breast were *osteonidogen*, *transmembrane protein 135*, *mitochondrial carrier triple repeat 1*, and *hexokinase 2*; the most down-regulated genes unique to adipose tissue were *ELOVL family member 6 elongation of fatty acids*, *secreted frizzled related protein2*, *cholesteryl ester transfer protein*, and *plasma and collagen type II $\alpha 1$* (Table 3).

Gene ontology and pathway analysis

Genes differentially expressed in both breast and abdominal tissue following DER were most frequently related to metabolism and energy pathways, with approximately one third of gene ontology terms common to both tissues (Supplementary Tables S3 and S4). Looking at genes within the highlighted pathways using GenMAPP,¹¹ it was clearly seen that almost all the individual genes involved in the glycolysis, tricarboxylic acid cycle, electron transport chain, and fatty acid metabolism were down-regulated in both adipose and breast tissue following DER. Many of these genes were significantly differentially expressed ($P < 0.05$). Several gluconeogenesis genes were up-regulated and genes within the fatty acid β -oxidation pathway were both up-regulated and down-regulated (summarized in Fig. 3).

Expression of epithelial genes in the breast tissue

Epithelial-specific genes (*keratin 14*, *keratin 18*, *CD24*, and *SC38GB202*) were expressed at high levels in all 38 breast samples but were low or not expressed in abdominal adipose tissue (Fig. 4), suggesting that some epithelium was present in all breast tissue hybridized to GeneChips. The expression of eight epithelial genes was also assessed by quantitative reverse

¹⁰ http://csbdb.mpimp-golm.mpg.de/csbdb/dload/dl_msri.html

¹¹ www.genmapp.org

Table 2. Dietary intake and physical activity in the DER and control groups at baseline and after the 1-mo trial period

Measurements*	Baseline measurements*		Change at 1 mo*		P†
	DER (n = 10)	Control (n = 9)	DER (n = 10)	Control (n = 9)	
Energy					
kJ	9,778.5 (\pm 2,158.5)	9,342.8 (\pm 1,606.6)	-5,829.5 (\pm 1,990.0)	-230.0 (\pm 1,374.1)	0.00
kcal	2,339.4 (\pm 516.4)	2,235.1 (\pm 384.3)	-1,394.7 (\pm 476.1)	-55.0 (\pm 328.7)	0.00
Protein (g)	85.4 (\pm 16.9)	89.8 (\pm 18.2)	-29.4 (\pm 16.9)	-3.1 (\pm 12.5)	0.00
Fat (g)	93.7 (\pm 26.6)	84.0 (\pm 17.5)	-82.7 (\pm 26.6)	-3.2 (\pm 17.2)	0.00
PUFA (g)	16.7 (\pm 6.4)	16.0 (\pm 4.0)	-14.7 (\pm 6.4)	0.1 (\pm 4.7)	0.00
MUFA (g)	30.6 (\pm 9.9)	27.8 (\pm 6.9)	-24.6 (\pm 9.9)	1.0 (\pm 5.8)	0.00
SFA (g)	34.5 (\pm 11.0)	30.9 (\pm 8.7)	-32.5 (\pm 11.0)	-2.3 (\pm 8.9)	0.00
Carbohydrates (g)	291.2 (\pm 78.6)	272.2 (\pm 67.9)	-153.2 (\pm 78.6)	1.9 (\pm 39.2)	0.00
Fiber (g)	15.7 (\pm 2.9)	13.2 (\pm 2.8)	3.9 (\pm 2.9)	-0.5 (\pm 2.6)	0.01
Alcohol (g)	10.8 (\pm 11.2)	14.2 (\pm 9.8)	-10.8 (\pm 11.2)	-2.9 (\pm 8.4)	0.09
% Energy from fat	35.8 (\pm 4.0)	33.8 (\pm 4.8)	-24.8 (\pm 4.0)	-0.5 (\pm 3.8)	0.00
% Energy from carbohydrate	46.5 (\pm 6.6)	45.6 (\pm 6.8)	13.5 (\pm 6.6)	1.3 (\pm 4.0)	0.00
% Energy from protein	14.9 (\pm 2.4)	15.9 (\pm 1.9)	11.1 (\pm 2.4)	0.0 (\pm 1.2)	0.00
% Energy from alcohol	3.2 (\pm 3.0)	4.3 (\pm 2.6)	-3.2 (\pm 3.0)	-0.6 (\pm 2.0)	0.00
Moderate activity, minutes/week (21)	77 (\pm 66)	133 (\pm 111)	-19 (-120 to 0.0)	-41 (-180 to 60)	0.63

Abbreviations: PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids.

*Mean (\pm SD or range).

†P value for changes in parameters in the control group compared with changes in the DER group using Mann-Whitney test.

transcription-PCR in the four quadrants of each breast from four women undergoing bilateral risk reduction mastectomy. Although there was some variation between sides, the differences between individuals were significantly greater, suggesting relatively lower heterogeneity between contralateral breasts in individuals (Supplementary Table S5).

Laser capture microdissection was then used to isolate breast epithelial cells from fixed frozen tissue, and sufficient RNA for GeneChip analysis was obtained from three matched samples before and after DER. The majority of expression changes for the highlighted DER genes were consistent between the microdissected epithelium and whole breast tissue for this limited number of samples. Using the MAS5 normalization method (37) to estimate whether particular probe sets are expressed (called "present," rather than absent), we determined that 93% (67 of 72) of the DER breast probe sets were called present using MAS5 normalization in at least three of the six microdissected samples. Just 2 of the 72 DER breast probe sets representing ABCC6 and LOC389393 were not called present in any of the epithelial-only samples (Supplementary Table S1). Using the same method, we also confirmed expression of >80% of the 72 DER breast genes in the ducts and lobules of at least half of the normal breast tissue samples isolated by laser capture microdissection in a study published by Turashvili et al. (Supplementary Table S1; ref. 38), suggesting that DER-regulated genes are expressed in breast epithelium.

Discussion

Because studies in rodents and observational studies in women indicate that DER reduces the risk of mammary can-

cer, we designed the study reported here to determine potential biomarkers of the effect and effectiveness of DER in women at increased risk of breast cancer (1–5). DER (~60% normal diet) was sufficient to favorably change known serum/urine biomarkers of breast cancer risk (insulin, leptin, and SHBG). Gene expression in breast tissue was also altered following DER, with some changes consistent with those in the adipose tissue. The metabolomic results seem to corroborate the changes in metabolic pathways seen at the gene expression level.

The tissue composition of the breast is variable and changes with age and during pregnancy. In parous women ages 35 to 45 years, it is estimated that the breast comprises 10% to 20% epithelium, with 60% to 80% fibroconnective tissue and 10% to 20% adipose tissue (39, 40). The proportion of adipose tissue is likely to be higher in overweight and obese women, such as those who were eligible for this study. It is not clear from our study whether the changes in gene expression common to the breast and abdominal tissue following DER affect the breast adipose tissue or breast epithelium directly. However, our limited laser capture microdissection study of pure epithelium and analysis of the RNA suggested a direct effect of DER on epithelial gene expression. A study of biopsies of normal breast tissue before and after letrozole showed multiple gene changes, but these investigators were also unable to distinguish between stromal and epithelial changes because of paucity of the latter (27). Further studies are required to establish tissue-specific gene expression changes within the breast.

The four most down-regulated genes in the breast were also down-regulated in abdominal adipose tissue, and three of the genes were related to the metabolic pathways of fat synthesis

(*SCD* and *FADS*) and glycolysis (*ALDOC*). *SCD* has been shown to be a highly down-regulated gene in s.c. adipose tissue in several DER studies (15, 16, 18). *SCD* is the gatekeeper to the formation of unsaturated fatty acids because it introduces a double bond in the 9 position in the saturated fatty

acids stearate and palmitate to form monounsaturated fatty acids oleic and palmitoleic acids. *SCD*-deficient mice are lean and have reduced lipid synthesis and enhanced lipid oxidation and insulin sensitivity (41). *SCD* knockdown reduces proliferation and Akt phosphorylation in cancer cell lines (42, 43).

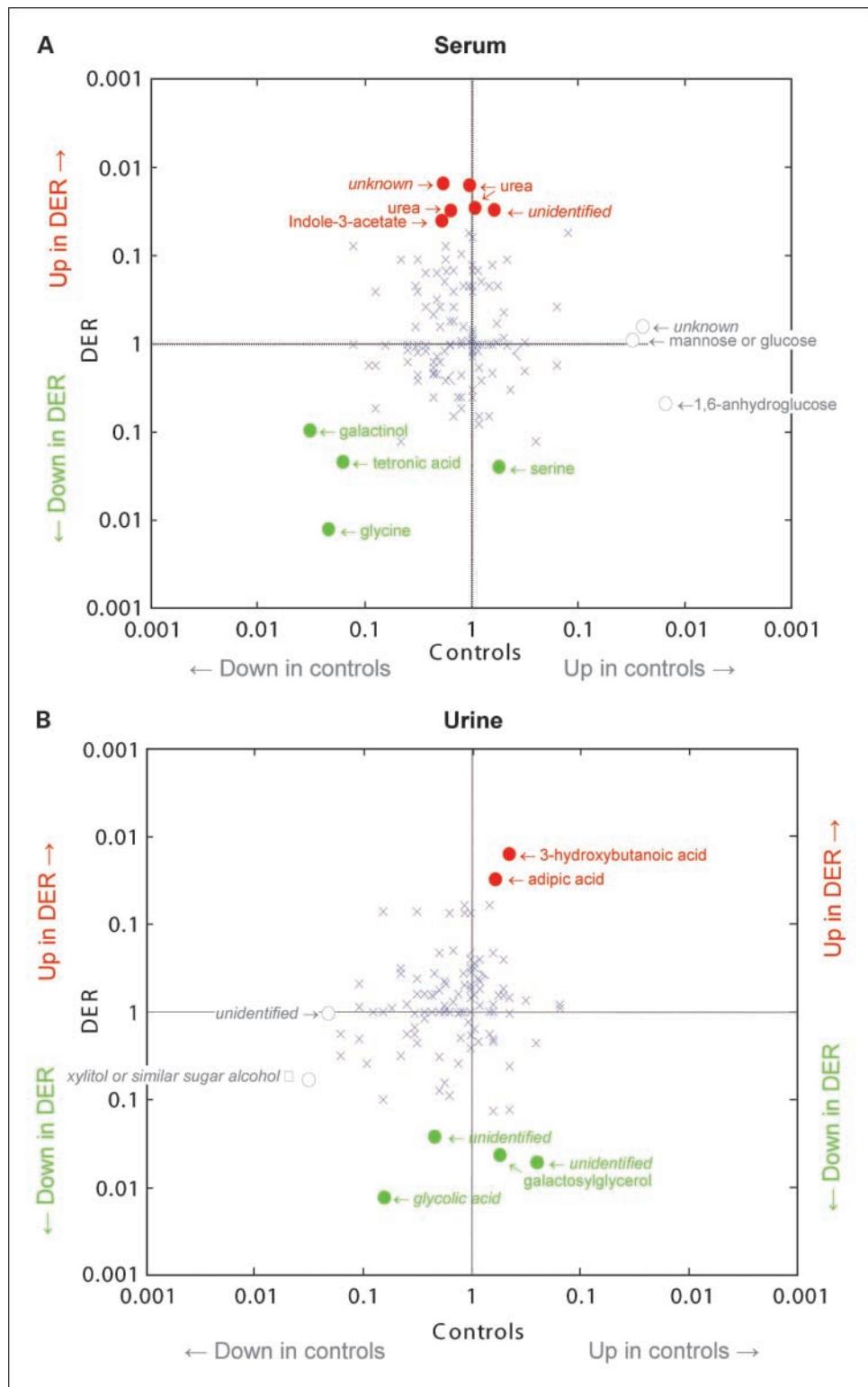


Fig. 2. Metabolomic analysis of serum (A) and urine (B) from DER and control women. •, metabolites that are significantly increased or decreased ($P < 0.05$) following the diet compared with the controls. Definitive metabolites are shown in plain text, and preliminary or unknown IDs are shown with an asterisk. Up in DER, sample population mean is higher after diet than before; Down in DER, sample population mean is lower after diet than before.

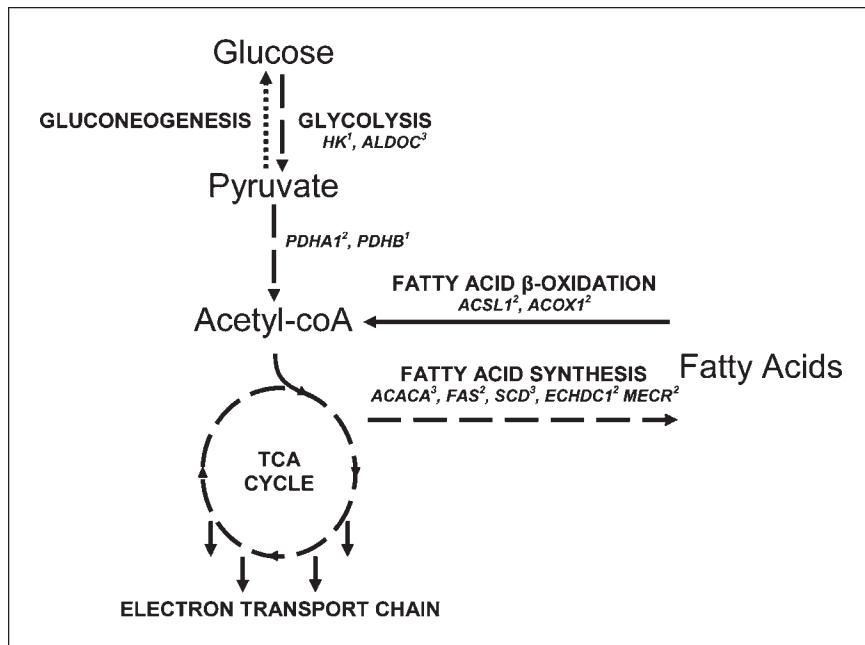


Fig. 3. Simplified representation of the glycolytic and fatty acid synthesis pathways following DER. Glycolysis, the tricarboxylic acid (TCA) cycle, electron transport chain, and fatty acid synthesis were all down-regulated (long dashes) and gluconeogenesis was up-regulated (short dashes). Genes involved with fatty acid β -oxidation were both up-regulated and down-regulated. Italics, enzymes that were down-regulated by SAM analysis in our study. Superscript numbers represent the tissue in which down-regulation occurred: 1, breast tissue; 2, adipose tissue; 3, both.

Inhibition of SCD by stearic acid reduces *N*-nitrosomethylurea-induced mammary tumor development in rats. A high unsaturated to saturated fat ratio in red cell membranes or serum phospholipids (indicating low SCD activity) is associated with reduced risk of breast cancer (44, 45). Thus, low SCD activity in the breast may not only be a marker of the effectiveness of

DER but also an indicator of reduced risk of breast cancer and a target for energy restriction mimetic agents (46).

We studied the effects of DER on a relatively homogenous group of women who were all of similar weight and age and were all parous. All biopsies, serum, and urine samples were taken at about the same day of each menstrual cycle

Table 3. Genes that were significantly differentially expressed between baseline and second biopsies in the DER group in both breast and adipose tissues using SAM analysis

Affymetrix probe set	Gene symbol	Description	Fold change (breast)	P (breast)	Fold change (adipose)	P (adipose)
211708_s_at	SCD	Stearoyl-CoA desaturase (δ -9-desaturase)	-16.7	0.00002	-4.3	0.0000026
211162_x_at	SCD	Stearoyl-CoA desaturase (δ -9-desaturase)	-8.3	0.00032	-4.3	0.0000090
208964_s_at	FADS1	Fatty acid desaturase 1	-3.7	0.00072	-2.2	0.0000004
208963_x_at	FADS1	Fatty acid desaturase 1	-3.8	0.00006	-2.0	0.0000044
200831_s_at	SCD	Stearoyl-CoA desaturase (δ -9-desaturase)	-4.2	0.00284	-3.2	0.0000371
214063_s_at	TF	Transferrin	-2.3	0.00230	-1.9	0.0000059
202022_at	ALDOC	Aldolase C, fructose-bisphosphate	-2.3	0.00015	-2.3	0.0000005
204776_at	THBS4	Thrombospondin 4	-1.8	0.00015	-2.1	0.0000551
214033_at	ABCC6	ATP-binding cassette, subfamily C (CFTR/MRP) 6	-1.8	0.00018	-1.6	0.0000360
202998_s_at	LOXL2	Lysyl oxidase-like 2	-1.8	0.00207	-1.3	0.0000353
203435_s_at	MME	Membrane metalloendopeptidase (CALLA, CD10)	-1.6	0.00003	-1.7	0.0000015
223839_s_at	SCD	Stearoyl-CoA desaturase (δ -9-desaturase)	-1.8	0.00029	-1.6	0.0000020
209600_s_at	ACOX1	Acyl-coenzyme A oxidase 1, palmitoyl	-1.6	0.00039	-1.5	0.0000024
229125_at	ANKRD38	Ankyrin repeat domain 38	-1.5	0.00255	-1.5	0.0000225
200832_s_at	SCD	Stearoyl-CoA desaturase (δ -9-desaturase)	-1.7	0.00020	-2.1	0.0000260
202709_at	FMOD	Fibromodulin	-1.3	0.00032	-1.8	0.0000296

NOTE: Two hundred probe sets representing 161 genes were significantly differentially expressed between baseline and repeat biopsies in the adipose tissue in response to DER with a FDR of 0.01. Seventy-two probe sets representing 61 genes were significantly differentially expressed between baseline and repeat biopsies in the breast tissue in the DER group at a FDR of 0.38. Full lists of differentially expressed genes in breast and adipose tissue are given in Supplementary Tables S5 and S6.

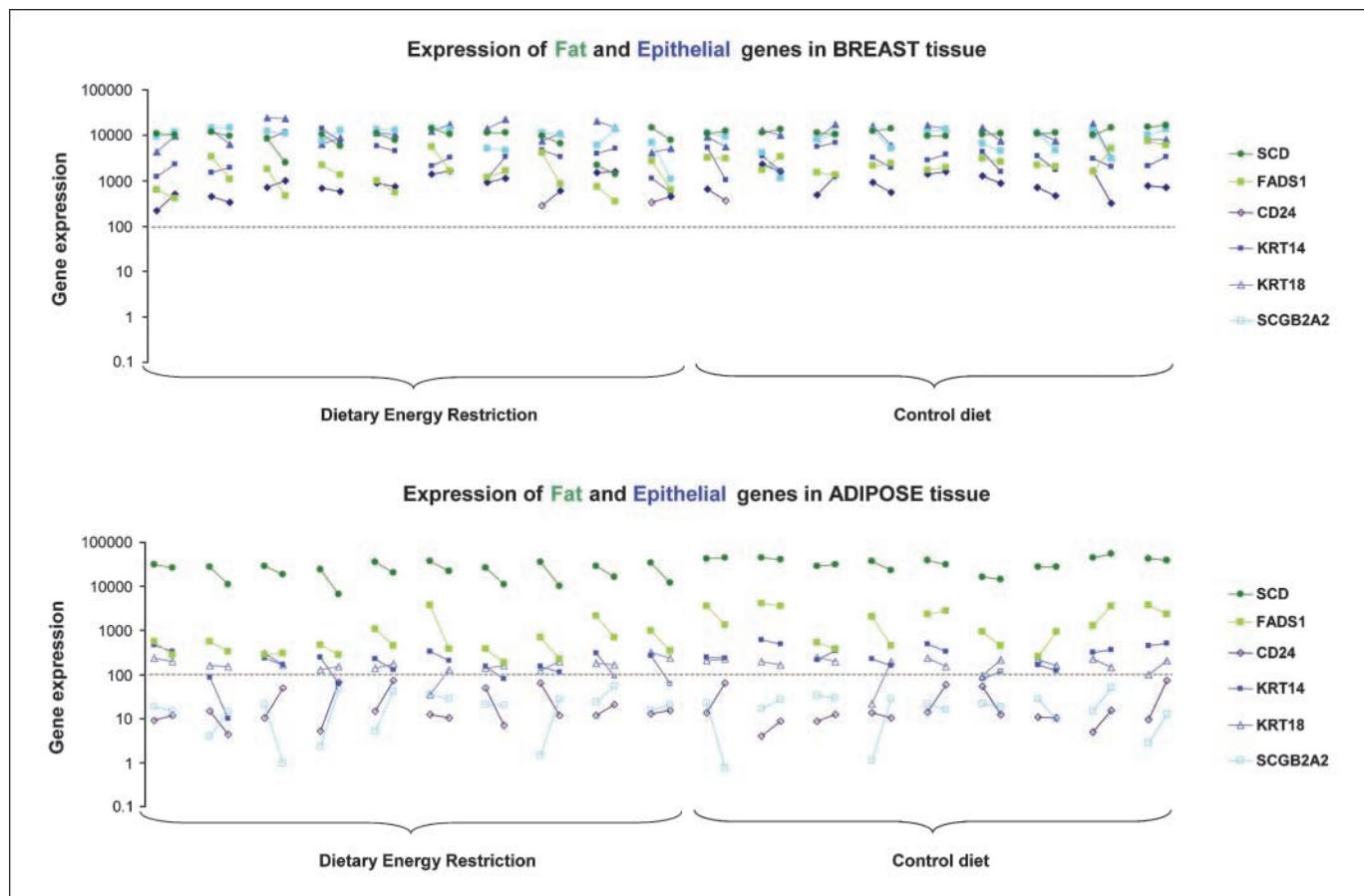


Fig. 4. Changes in the level of gene expression of fat metabolism and epithelial-specific genes in adipose and breast tissue. Level of expression using MAS5 normalization. Before (left) and after (right) measurements are shown for each patient linked with a line. Open symbols, genes that are called “absent” or “marginal.” Dashed line, the majority of genes with expression levels <100 are called absent.

under standardized, fasting conditions to limit variance. The inclusion of a control group allowed us to assess any changes in gene expression, which may be influenced by the site or timing of biopsies. The lack of changes in gene expression in the control group gives confidence that the changes observed following DER are “real” and indicates the stability of gene expression when diet and exercise are unchanged. To maximize compliance and standardize dietary change, the DER comprised a liquid meal replacement drink (Slimfast). The declines in body fat (~4.6 kg over 26 days) among the DER group are consistent with ~1,400 kcal/d energy deficit, indicating good compliance to the DER regimen (47). The clear effect of DER on body weight, fat, and lipids and leptin confirms that compliance was at a sufficient level to have metabolic effects and reflects the success of our rigorous selection process. DER is well known to cause declines in leptin (15) and SHBG (48). The decline in total and low-density lipoprotein cholesterol and triglycerides is consistent with previous studies of low-fat, energy-restricted regimens (49, 50).

The DER regimen used in our study reduced energy intake (67%) but also resulted in differential reduction of macronutrients from the woman's standard diet of calories from carbohydrate (53% reduced), protein (43% reduced),

and fat (89% reduced). It is possible that some of the changes seen in this study were due to altered composition of the diet, specifically the greatly decreased fat intake. It is known that an isocaloric dietary change of macronutrients, such as the combined requirement of carbohydrate and saturated fat for induction of SCD in humans (18), can alter urinary metabolomic patterns and gene expression in s.c. fat. However, several studies show that DER produces changes in gene expression, which are greater than changes in diet macronutrient composition (15–18). We believe that further metabolomic studies are warranted to assess relative importance between fat and carbohydrate to energy restriction. Some observational studies show a modest reduction of breast cancer risk after reduction of fat intake, but a large randomized trial of isocaloric fat reduction was not associated with risk reduction (51, 52).

Opposite breasts were sampled 1 month apart to circumvent potential inflammatory changes if biopsies had been taken from one breast only. This is a potential weakness of the study given the known heterogeneity of the breast and may account for some of the changes seen. However, no consistent differences in gene expression were seen in the controls in the biopsies taken from contralateral breasts 1 month apart, and quantitative reverse transcription-PCR assessment of eight

epithelial-specific genes from the four quadrants of each breast from four women undergoing bilateral risk reduction mastectomy suggested greater variation between individuals than between breasts.

A higher FDR (0.3) was used to identify the most consistently changed genes in the breast tissue following DER compared with the adipose tissue (0.01). We have postulated that is due to greater heterogeneity of breast tissue compared with that of the abdominal adipose. Alternatively, it could be an indication that the response to DER in breast tissue is simply more variable than the response in adipose tissue. Although the *P* values shown in Table 2 are not as low for the breast tissue as they are for the adipose tissue, they are still highly significant. Furthermore, gene ontology analysis identified many of the same pathways for both tissues and almost all genes within these were down-regulated (Supplementary Fig. S1). It would be interesting to compare gene expression in breast adipocytes, epithelium, and stroma separately; however, this approach would result in a much lower tissue to RNA yield and therefore necessitate a more invasive biopsy technique. However, we were able to do laser capture microdissection of sufficient epithelium in three before and after DER samples to obtain RNA and analyze gene expression. This revealed similar changes in genes, such as *SCD*, to those seen in whole tissue, suggesting that DER does affect breast epithelial gene expression.

Previous studies have examined the effect of DER on s.c. abdominal fat. Our findings are largely concordant with those of other human studies. Notably, fatty acid synthesis genes are down-regulated in the adipose tissue of both this study (60% DER over 1 month) and the previous Affymetrix array-based study of Dahlman et al. [30% DER over 10 weeks (15) and 50% DER over 5 weeks (18)], although this article did not specify these specific metabolism genes. Other studies have linked a 30% DER over 10 weeks with changes in the expression of genes that encode transporters and transcription factors linked to adipogenesis and mitochondrial respiration (17). Clement et al. (53) reported that ~60% of genes differentially expressed in a 60% DER study over 1 month in s.c. adipose tissue were related to fat and carbohydrate metabolism, although this group focused mainly on inflammation-related genes. A recent study reported that epithelial cells from chemical-induced mammary adenocarcinomas from Sprague-Dawley rats had increased expression of genes for glycolysis, decreased pyruvate dehydrogenase, and increased lactate dehydrogenase compared with normal epithelial cells, consistent with the Warburg effect (54). However, this pattern of gene expression was not influenced by a 40% DER. The design of this study does not enable the effects of DER on spontaneous development of breast cancer to be determined; furthermore, changes in lipid metabolism were not reported (55). Adipocytes and breast epithelial cells lie in close proximity to each other in breast tissue. Large adipocytes in obese subjects may have dysregulated secretion of factors (i.e., increased leptin and decreased adiponectin), which may promote the proliferation of transformed ductal carcinoma cells (56). DER resulted in significant reductions in serum levels of leptin (66%) but no change in serum adiponectin. Serum changes in leptin were not reflected in leptin gene expression. The dissociation between adipokine gene expression and serum levels has been noted

in earlier studies, suggesting a lack of sensitivity or post-translational effects of DER (57). There have been few metabolomic studies of DER in humans. We did not detect significant changes in glycolysis (glucose-6-phosphate and pyruvate) and tricarboxylic acid cycle metabolites (succinic acid and 2-oxoglutaric acid) despite these pathways being down-regulated at the gene expression level. However, the small sample sizes would preclude the detection of small differences in the concentrations of these metabolites.

The exact mechanism for the protective effect of DER against cancer remains unknown. Increased glycolysis and fatty acid synthesis is well described in tumors, and there is renewed interest in inhibition of these pathways not only for cancer treatment but also for prevention of cancer and other diseases (see ref. 9 for review). For example, inhibition of glycolysis with two deoxyglucose or fatty acid synthases with a compound named C75 inhibits tumor growth in rodent and human models (58, 59), whereas indirect observational evidence (increased stearic to oleic acid ratio in RBCs) is consistent with SCD activity linked to breast cancer risk (45).

This is a short-term study, and it is difficult to draw any firm conclusions about the effects of long-term DER. It is unknown which changes persist in the long-term when finally a desirable weight is achieved and weight stabilizes. In common with our study, most previous reports have assessed changes in gene expression during energy restriction and the dynamic phase of weight loss (16, 53). One report finds that most changes observed during dynamic phase of weight loss were reversed or attenuated during a 5-week weight stabilization phase (18). The beneficial changes in gene expression seen with weight loss are therefore mostly linked to energy restriction. Periods of intermittent restriction may thus be required to maintain these beneficial effects (10). Energy restriction is known to reduce cell proliferation and increase apoptotic rate in both normal tissues and cancers (60–63). The inhibition of glycolysis has been shown to impair the growth of tumor cells (64, 65), and suppression of glycolysis and fatty acid synthesis specifically in the breast epithelium may prevent breast carcinomas from developing and progressing.

In summary, we have done the first DER study to characterize gene expression changes in premenopausal normal human breast tissue. Common differentially expressed genes and pathways in breast and abdominal tissue suggests that short-term energy restriction may influence breast cancer risk at the molecular level. We have identified targets that may respond to chemoprophylactic mimetic agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank the participants for their invaluable contribution to this study; Stuart Pepper and Yvonne Hey (Cancer Research UK Affymetrix core facility) and Garry Ashton (PICR Histology Unit) for their excellent technical assistance; Dr. Twani Tuthill (Addenbrooke's Hospital, Cambridge, United Kingdom) for teaching K.R. Ong the adipose tissue biopsy technique; Rosemary Greenhalgh, Jenny Affer, and radiographers at the Nightingale Centre for their assistance with the biopsies; and Slimfast UK for their generous gift of the diet drinks.

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Cancer Prevention Research

Biomarkers of Dietary Energy Restriction in Women at Increased Risk of Breast Cancer

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Cancer Prev Res 2009;2:720-731.

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White Button Mushroom (*Agaricus Bisporus*) Exhibits Antiproliferative and Proapoptotic Properties and Inhibits Prostate Tumor Growth in Athymic Mice

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White button mushrooms are a widely consumed food containing phytochemicals beneficial to cancer prevention. The purpose of this research was to evaluate the effects of white button mushroom extract and its major component, conjugated linoleic acid (CLA) on prostate cancer cell lines in vitro and mushroom extract in vivo. In all cell lines tested, mushroom inhibited cell proliferation in a dose-dependent manner and induced apoptosis within 72 h of treatment. CLA inhibited proliferation in the prostate cancer cell lines in vitro. DU145 and PC3 prostate tumor size and tumor cell proliferation were decreased in nude mice treated with mushroom extract, whereas tumor cell apoptosis was increased compared to pair-fed controls. Microarray analysis of tumors identified significant changes in gene expression in the mushroom-fed mice as compared to controls. Gene network analysis identified alterations in networks involved in cell death, growth and proliferation, lipid metabolism, the TCA cycle and immune response. The data provided by this study illustrate the anticancer potential of phytochemicals in mushroom extract both in vitro and in vivo and supports the recommendation of white button mushroom as a dietary component that may aid in the prevention of prostate cancer in men.

BACKGROUND

The majority of common dietary constituents do not have an acute biological effect immediately after ingestion. However, when eaten daily over a lifetime, subtle, long-term effects may be observed. For this reason, cancer of the prostate, which requires many years to develop, is a prime target for prevention strategies utilizing daily factors such as dietary intake. It is estimated that the time required for the clonal outgrowth of a prostate cancer cell to develop into a 1 cm³ primary prostate cancer would be 39.4 yr, with the mean age at diagnosis occurring at 72 yr of age (1). Epidemiologic studies have suggested

that changes in lifestyle, including dietary modifications, could prevent a significant number of cancers (2–4). Of the environmental factors that affect prostate cancer development, diet has been identified as an important influence (5,6). Therefore, incorporating foods into the diet that are known to have chemopreventive properties could reduce cancer incidence and subsequently cancer-related deaths.

Several mushroom species such as *Ganoderma lucidum*, *Lentinus edodes*, *Grifola frondosa* and *Agaricus blazei* have been shown to exhibit anticancer effects such as the inhibition of cell proliferation in prostate (7,8), colon (9–11), and breast cancer cell lines (10,12). Mechanisms attributed to the anticancer activity of mushrooms include the induction of apoptosis; the inhibition of angiogenesis and prosurvival signaling pathways such as protein kinase B (AKT), extracellular-regulated kinase (ERK), nuclear factor kappa-B (NF6-B) and activating protein-1 (AP-1) (7,13,14) and modulation of the cell cycle control protein retinoblastoma (pRb) (8). Although much study has been completed on the medicinal varieties of mushroom mentioned above, studies into the anticancer effects of the common white button mushroom (*Agaricus bisporus*) are limited. Much of the research has focused on the anticancer effects of carbohydrate fractions of this species. For example, lectins isolated from the white button mushroom have been shown to increase the sensitivity of lung, colon, and glioblastoma cancer cells to chemotherapeutic drugs (15), inhibit colon cancer cell proliferation (16), and enhance cellular antioxidant defense mechanisms (17).

Previous studies in our laboratory investigated the antiaromatase activity of common vegetables that may suppress breast cancer cell proliferation. Of the 7 vegetable extracts tested, the extract of white button mushrooms was the most effective inhibitor of human placental aromatase activity (18). Our laboratory has focused on an ethyl acetate extract of the white button mushroom, which contains mainly C-18 fatty acids and simple organic and phenolic acids (19). We have previously reported that this extract effectively decreased the proliferation of breast cancer cells through the inhibition of aromatase enzyme activity.

Submitted 3 October 2007; accepted in final form 28 January 2008.

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We also identified CLA as a major component of the mushroom extract and also an active inhibitor of breast cancer cell proliferation and aromatase activity (18,19).

To determine the anticancer potential of mushroom extract in prostate cancer cell lines, the current study investigated the in vitro effects of this extract on the androgen sensitive LNCaP and androgen insensitive PC3 and DU145 prostate cancer cell lines. We also studied the in vivo chemopreventive potential of mushroom extract in two separate studies utilizing male athymic mice injected with either DU145 or PC3 prostate cancer cell lines. The information gained from this study gives us future direction for investigation into the active ingredients of mushrooms as well as their mechanisms and to the overall understanding of how inclusion of mushrooms into the diet may contribute to more effective prostate cancer prevention strategies.

MATERIALS AND METHODS

Cell Culture

LNCaP, PC3, and DU145 cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD) and grown in RPMI 1640 containing 10% fetal bovine serum in the presence of 100 U/ml penicillin and 0.1 g/l streptomycin. Cells were incubated at 37°C with 95% air and 5% carbon dioxide. All cells were kept below passage 20 and used in experiments during the linear phase of growth.

Production of Mushroom Extract

Mushroom extract was produced by chopping 60 g of fresh white button mushroom (*Agaricus bisporus*) and boiling it in water. The broth was filtered and then applied to 5 g/60 ml capacity polyamide columns (Discovery DPA-6S SPE; Supelco, Bellefonte, PA). Fractions were eluted by a step gradient (50 ml of each step) of increasing methanol to water. The 20% methanol–water fraction was rotor evaporated to dryness and then redissolved in 1 ml of water to produce the 6× mushroom extract. Therefore, 6 g of mushroom can produce 100 µl of 6× fraction.

Real-Time Proliferation Assay

Cells were plated in 16 well plates at a density of 10,000 cells/well and treated with either medium containing ethanol as vehicle control (<0.1% total) or CLA (Cayman Chemical, Ann Arbor, MI; 0–200 µM). Cell growth was monitored automatically via the ACEA RT-CES real-time proliferation machine (ACEA Biosciences, Inc., San Diego, CA), which swept the plates once an hour for up to 96 h. The machine measures electrical impedance through sensors on the bottom of the 16 well plates. Increase in impedance correlates with an increase in cell density. Media and treatments were changed after 48 h. Data are expressed as ratio of treated to untreated cells mean ± SE for 3 replications.

Apoptosis ELISA

Apoptosis was assessed utilizing the Cell Death Detection ELISA^{PLUS} Assay (Boehringer Mannheim, Indianapolis, IN). This assay is a photometric enzyme-linked immunoassay that quantitatively measures the internucleosomal degradation of DNA, which occurs during apoptosis. The assay is a quantitative sandwich-enzyme-immunoassay utilizing monoclonal mouse antibodies directed against DNA and histones that detect specifically mononucleosomes and oligonucleosomes. Quantitative measurement of the amount of internucleosomal degradation is measured photometrically at 405 nm with ABTS as substrate. Cells were plated in 60 mm dishes (Falcon, BD Biosciences, San Jose, CA) at a density of 100,000 cells/dish and allowed to attach for 24 h. Cells were treated with media control (mushroom extract is water soluble) or mushroom extract at a concentration of 20 µl/ml for 48 and 72 h. Following treatments, nonadherent cells were collected and pelleted at 200 g for 10 min. The supernatant was discarded; the cell pellet was washed with cold phosphate-buffered saline (PBS; 137 mmol/l sodium chloride, 1.5 mmol/l potassium phosphate, 7.2 mmol/l sodium phosphate, 2.7 mmol/l potassium chloride, pH 7.4) and recentrifuged. Adherent cells were washed with cold PBS, trypsinized, collected, and combined with nonadherent cells. Both live and dead cells were then counted via Trypan Blue (Pierce, Rockford, IL) exclusion, and 10,000 cells were added to the microtiter plate for all treatment groups; and apoptosis assay was performed according to the manufacturer's instructions. Absorbance was read on a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA) at 405 nm. Background values were subtracted from readings (media plus reagent, no cells) and expressed as absorbance of dye bound to antibodies bound to mononucleosomes and oligonucleosomes at 405 nm of each treated sample divided by media controls.

Annexin V Assay

Cells were plated in 100 mm dishes (Falcon, BD Biosciences, San Jose, CA) at a density of 50,000 cells/dish and allowed to attach for 72 h. Cells were treated with media control (mushroom extract is water soluble) or mushroom extract at a concentration of 20 µl/ml for 48 and 72 h. Following treatments, adherent cells were trypsinized, nonadherent cells were collected, and all cells were pelleted at 200 g for 10 min and then washed twice with cold 1 × PBS and resuspended in 1 ml of 1 × binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) at a concentration of 1 × 10⁶ cells/ml. 1 × 10⁵ cells were stained with Annexin V-FITC; BD Pharmingen, San Jose, CA) and propidium iodide (PI; Sigma-Aldrich, St. Louis, MO) for 15 min at room temperature in the dark. After adding 400 µl of 1 × binding buffer to each tube, cells were analyzed by flow cytometry within 1 h on a CyAnTM ADP 9color-UV flow cytometer (Dako, Inc., Carpinteria, CA). Controls included unstained cells, cells stained with Annexin V-FITC only, and cells stained with PI only.

Animal Experiments

Five-week-old, male BALB/c Nu-Nu, athymic mice were purchased (Charles River Laboratories). Mice were randomly divided into 6 groups with 8 mice per group. At 6 wk of age, mice were gavage fed with either 100 μ l water control or 100 μ l mushroom extract (in water). The third group was gavaged with 100 μ l water and pair fed to the 6X mushroom group (the food consumed by the 6X mushroom group was weighed and the same amount provided to the pair-fed group) to control for differences in caloric intake. Each animal received daily gavage treatment for the duration of the experiment. At 7 wk of age, mice were given two subcutaneous injections of either DU145 or PC3 cells in Matrigel (BD Biosciences, San Jose, CA). These cells were grown in RPMI 1640 with nonessential amino acids, sodium pyruvate, and Earle's salts in 10% fetal calf serum. The cells were harvested and resuspended in an equal volume of Matrigel (BD Biosciences) to a final concentration of 1×10^7 cells/0.2 ml. Body weights were monitored weekly as an indicator of the animal's overall health. At the end of 7 wk of gavage treatment, mice were euthanized; blood samples were collected; and tumors were removed, weighed, and sent for hematoxilin and eosin (H & E) histological staining through the City of Hope Pathology Department Core Facility. Tumor specimens were also stained using cleaved-caspase-3 antibody (Cell Signaling Technology, Danvers, MA) for apoptosis and Ki-67 antibody (Dakocytomation, Carpinteria, CA) staining for cell proliferation by the City of Hope Pathology Department Core Facility. Data are expressed as mean \pm SEM ($n \geq 5$).

Microarray Analysis

For microarray analysis, total RNA was extracted from 3 DU145-derived tumors from each treatment group using TRIzol reagent (Invitrogen, Carlsbad, CA). Synthesis and labeling of cRNA targets, hybridization of GeneChips, and signal detection were carried out by the Microarray Core Facility at the City of Hope. Briefly, biotinylated cRNA was generated using 5 μ g total RNA using T7 RNA polymerase. The Affymetrix GeneChip Human Genome U133A v2.0 array (HGU133A2) (Affymetrix, Santa Clara, CA) was used to define gene expression profiles from tumor samples. For microarray hybridization, the GeneChip arrays were hybridized with 15 μ g of fragmented cRNA targets and then washed. The staining was performed with streptavidin-PE. Affymetrix GeneChip images were scanned at 11- μ m resolution using a high resolution GeneChip Scanner 3000 (Hewlett-Packard).

Statistical Processing of Microarray Data

Quality assessment and statistical analysis of gene expression data was performed using the R/Bioconductor packages. To ensure the high quality of the microarray process, a set of quality assessment steps implemented in Bioconductor package "Affy-Express" were applied to the data. Raw intensity measurements

of all probe sets were converted into expression measurements using the "GCRMA" package. The "LIMMA" package was then used to identify the genes differentially expressed between mushroom-fed and water-fed samples. The genes showing altered expression were categorized on the basis of their cellular components, biological processes, molecular functions, and signal pathways using the Ingenuity Pathways Analysis (Ingenuity, Mountain View, CA) software. Significant genes were selected with a cutoff of $P < 0.01$ and log2 ratio of 1 (twofold change).

Ingenuity Pathway Analysis (IPA)

IPA is a Web-based software program that identifies the biological functions, pathways, and mechanisms most relevant to a given data set of genes. Information on individual genes is drawn from a large knowledge base of biological networks created from millions of publications (full-text articles published in scientific journals), and the networks are drawn by the Functional Analysis feature of IPA based on the connectivity of the genes.

Real-Time PCR

Trizol reagent (Invitrogen) was used for total RNA isolation. SYBR Green Supermix and iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA) were used for cDNA preparation. PCR primers for KIT were as follows: 5'GCCGACAAAA-GGAGATCTGT3' and 5'CCTTGCCACCTGGTAAGAA3'; for FH, 5'CCGCTG-AAGTAAACCAGGAT3' and 5'TCCTGA TCCAGTCTGCCATA3'; for FAS, 5'ATC-AAGGAATGCA CACTCACC3' and 5'GGTTGGAGATTAT-GAGAAC3'; for human β -actin (used as an internal control), 5'AGAAGGAGATCACTGCC-TGGCAC3' and 5'CCTGC TTCGTGATCC-ACATCTGCTG3'. Reactions were run in triplicate on the iCycler iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and results were analyzed with the software provided.

Statistical Analysis

To assess statistical significance, values were compared to controls with either Student's *t*-test or 1-way analysis of variance (ANOVA), followed by Dunnett's Multiple Range test ($\alpha = 0.05$) or 2-way ANOVA as appropriate using Prism GraphPad 4 software (GraphPad Software, Inc., San Diego, CA).

RESULTS

Effects of Mushroom Extract on Prostate Cancer Cell Proliferation

The antiproliferative activity of mushroom extract was assessed in the LNCaP, DU145, and PC3 prostate cancer cell lines. Cells were treated with mushroom extract (20 μ l/ml) for 96 h. Results from this assay showed that exposure to mushroom extract resulted in a significant, dose-dependent inhibition of cell proliferation ($P \leq 0.01$) in all cell lines tested. The magnitude

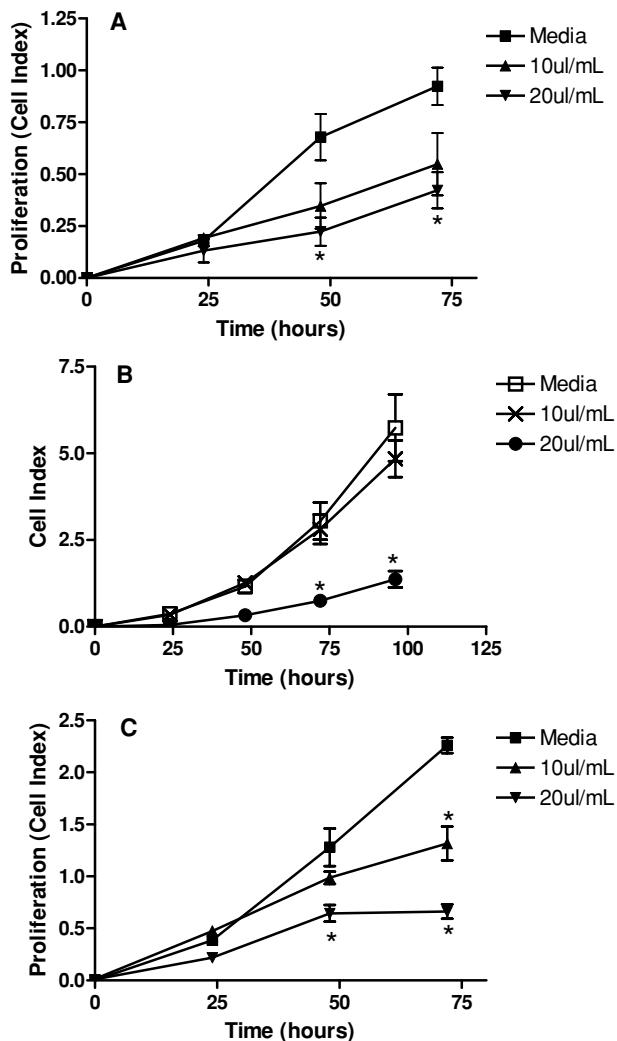


FIG. 1. Antiproliferative activity of mushroom extract on prostate cell lines. LNCaP (A), DU145 (B), and PC3 (C) cells were plated in the ACEA 16-well plate (5,000 cells/well) in media alone or treated with indicated concentrations of mushroom extract. Readings were taken every hour up to 96 h by the ACEA machine and expressed as cell index (a measure of cell number/well). Data represent means \pm SE ($n = 3$); * indicates significant difference from media control ($P \leq 0.01$). LNCaP, lymph node carcinoma of the prostate.

of response to mushroom extract was similar between cell lines (Fig. 1).

Induction of Apoptosis by Mushroom Extract

To determine whether the observed decrease in cell number after treatment with mushroom extract was due to the induction of apoptosis, the formation of mononucleosomes and oligonucleosomes was quantified using the Cell Death Detection ELISA^{PLUS} assay (Roche Diagnostics, Mannheim, Germany) and Annexin V/PI staining. Following treatment with mushroom extract (20 μ l/ml) for 48 h, a significant twofold increase in DNA fragmentation was detected in the LNCaP cells ($P \leq 0.01$) compared to untreated controls (Fig. 2A). The PC3 and DU145 cell

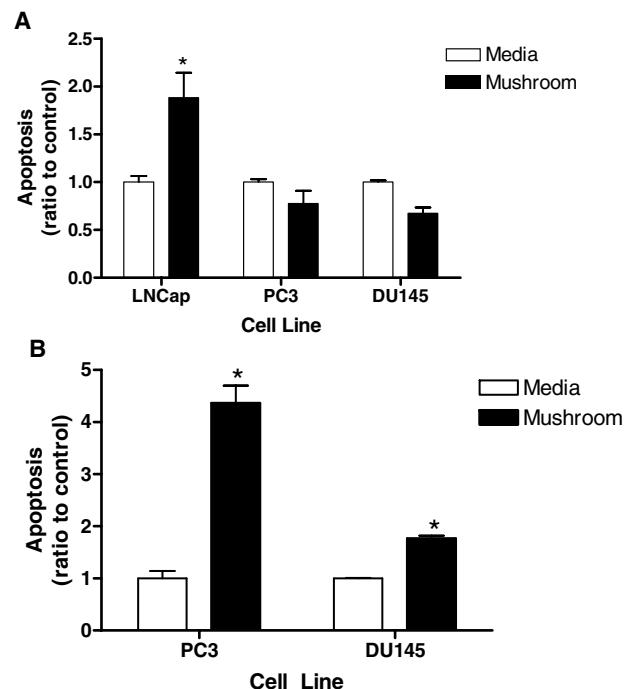


FIG. 2. Mushroom extract induces DNA fragmentation in prostate cell lines. Cells were exposed to mushroom extract (20 μ l/ml) for 48 h (A) or 72 h (B), harvested, and analyzed using the Cell Death Detection ^{PLUS} Assay. Values are means \pm SE, $n = 3$. Data are expressed as absorbance at 405 nm of each treated sample divided by vehicle controls. * indicates a significant difference ($P \leq 0.001$) compared to untreated controls.

lines showed no increase in the production of mononucleosomes and oligonucleosomes with mushroom treatment compared to untreated controls at 48 h (Fig. 2A). However, after 72 h of treatment, mushroom extract significantly induced DNA fragmentation 4.4-fold in the PC3 cells ($P \leq 0.001$) and 1.8-fold in the DU145 cells ($P \leq 0.001$; Fig. 2B).

The translocation of phosphatidylserine (PS) to the outer leaflet of the plasma membrane was determined using Annexin V-FITC/PI staining and flow cytometry. Changes in the location of cell surface markers, such as PS, are the earliest detectable features of apoptotic cells. As such, PS detection in intact cells (PI negative cells) represent an early stage of apoptosis in the cell lines tested following exposure to mushroom extract. In the LNCaP cells, Annexin V positive cells increased 4.4-fold with mushroom treatment after 48 h compared to untreated controls ($P = 0.025$). In PC3 cells treated with mushroom extract, Annexin V staining increased threefold ($P \leq 0.01$), whereas the DU145 cells exhibited a 1.4-fold increase compared to untreated controls (Fig. 3A). After 72 h of treatment with mushroom extract, PC3 cells had a twofold increase in Annexin V staining ($P \leq 0.02$), whereas DU145 cells had a 1.6-fold increase in Annexin V staining ($P \leq 0.02$; Fig. 3B). These results illustrate that the LNCaP cell line is more sensitive to the proapoptotic effects of mushroom extract compared to the

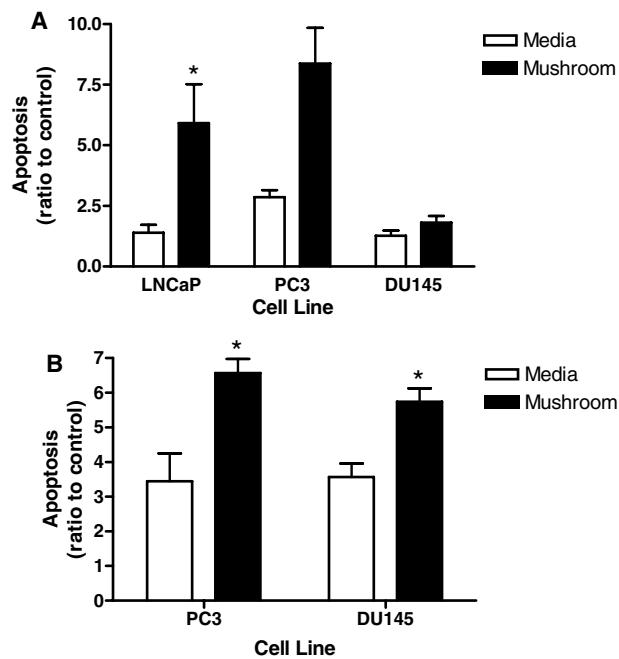


FIG. 3. Detection of phosphatidylserine externalization in prostate cells treated with mushroom extract. LNCaP, PC3, and DU145 cells were exposed to mushroom extract ($20 \mu\text{l}/\text{ml}$) for 48 h (A) and DU145 and PC3 cells were exposed to mushroom extract ($20 \mu\text{l}/\text{ml}$) for 72 h (B). Cells were stained with Annexin V-FITC and PI and analyzed on a FACS calibur flow cytometer. Data are expressed as percentage of Annexin V positive cells divided by Annexin V negative cells, mean \pm SE, $n = 3$. * indicates a significant difference ($P \leq 0.05$) compared to untreated controls.

PC3 and DU145 cell lines, which respond at a later time point. In addition, the DU145 cell line showed only a mild response to mushroom extract and is therefore more resistant than the other two cell lines. Additionally, this shows that the antiproliferative activity is distinct from the proapoptotic activity of mushroom, as all three cell lines responded equally in the proliferation assay.

Inhibition of Tumor Growth In Vivo

To evaluate the prostate cancer protective effects of white button mushroom *in vivo*, our laboratory investigated the ability of mushroom extract to inhibit PC3 and DU145-derived tumor growth in male, athymic (nu/nu) mice. The results of these animal experiments showed that the oral intake of mushroom extract significantly decreased DU145 tumor weight 44.5% ($P \leq 0.05$) (Fig. 4A) and PC3 tumor weight 68.6% ($P \leq 0.01$; Fig. 5A) compared to pair-fed control mice. There was no difference in mouse weight between treatment groups. Histological examination of DU145 tumors revealed that cell proliferation was significantly decreased 25.3% in the mushroom group compared to controls ($P \leq 0.05$; Fig. 4B), whereas the level of apoptosis between tumors from the control and mushroom extract-fed animals was increased, although this observation

was not statistically significant (Fig. 5B). Histological examination of the PC3 tumors showed that cell proliferation was significantly decreased 45% in the mushroom group compared to controls ($P \leq 0.01$; Fig. 4C), and the level of apoptosis increased 200% ($P \leq 0.001$; Fig. 5C). The LNCaP cell line did not reliably form tumors when implanted into the athymic mice, therefore we chose not to utilize that cell line for this study. The DU145 and PC3 data illustrates the ability of mushroom extract to decrease the growth and induce apoptosis of prostate cancer cells *in vivo*.

Inhibition of Prostate Cell Proliferation by Conjugated Linoleic Acid

Through GC analysis, we previously identified conjugated linoleic acid isomer 9Z, 11E as a major component in the 6X mushroom fraction (an average of 45.7% of the total extract). We also showed that 9Z, 11E CLA alone inhibits breast cancer cell proliferation through inhibition of the activity of the aromatase enzyme (19). Therefore, we tested the ability of the two common isoforms of CLA (9Z, 11E and 10E, 12Z) to inhibit prostate cancer cell proliferation. The results indicate that both isoforms of CLA significantly inhibited prostate cancer cell proliferation in a dose dependent manner (9Z, 11E with a P value ≤ 0.01 and 10E, 12Z with a P value ≤ 0.05 ; Figs. 6A–6F), suggesting that CLA may be one of the major active components in mushroom extract. However, in natural products, it is generally accepted that the overall combination of components can be more potent than any one component singled out. Further, it is possible that one of the minor components could be as active as CLA; however, in our breast cancer studies, the ethyl acetate fraction was the most potent fraction, and the major constituent was found to be CLA.

Validation of Microarray Results by Real-Time PCR

Microarray analysis results were validated by real-time PCR using primers for three of the most highly regulated and interesting genes (KIT, FAS, and FH) in tumor samples. Results showed a significant decrease in KIT expression and significant increases in the expression of FAS and FH (Figs. 7A–7C), which is in agreement with the microarray analysis results.

Network Analysis of Mushroom Responsive Genes

Genes that were regulated by mushroom extract in DU-145 tumor samples were analyzed for their functional grouping using IPA. Six networks of genes were significantly altered by mushroom extract identified by IPA in our data set (Table 1). Of these networks, 2 were of prime interest to this investigation: those grouping genes important to cell death, cellular growth and proliferation, and cellular morphology and those grouping genes important to cellular growth and proliferation, lipid metabolism, and small molecule biochemistry. An illustration of these gene networks can be found as supplemental Data 1 and 2, respectively.

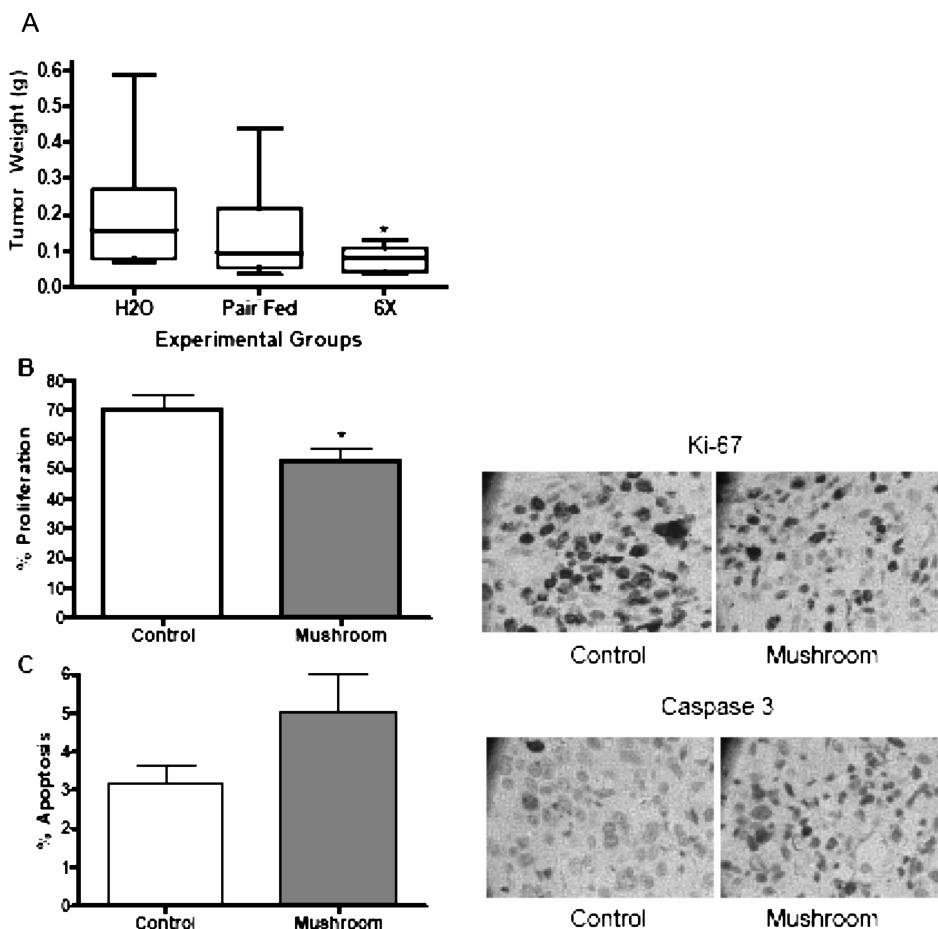


FIG. 4. In vivo effect of mushroom extract on DU145 prostate tumor growth. 6-wk-old, athymic, nude male mice were gavage fed with either 100 μ l water control or 100 μ l mushroom extract (in water). Each animal received daily gavage treatment for 7 wk. After 1 wk of gavaging, mice were given 2 subcutaneous injections of DU145 cells in Matrigel (BD Biosciences, San Jose, CA). 6 wk after injection, mice were euthanized and A: tumor weights, B: Ki-67 antibody staining for cell proliferation, and C: cleaved-caspase-3 antibody for apoptosis were evaluated. Data represent mean in each group ($n \geq 5 \pm$ standard error of the mean). * indicates statistical significance from control group ($P \leq 0.05$).

Within the gene networks, specific biological functions such as apoptosis, lipid metabolism, and immune response were altered by mushroom extract. These groupings suggest possible mechanisms of action for the effects of mushroom extract observed in the prostate tumors. Table 2 shows the gene symbol, Entrez Gene number, fold change from controls, and description of these gene groupings.

DISCUSSION

The aim of this research was to test the anticancer activity of phytochemicals in the common white button mushroom (*Agaricus bisporus*) in prostate cancer *in vitro* and *in vivo*. Our results show that mushroom extract decreased the proliferation of the prostate cancer cell lines in a dose-dependent manner compared to untreated control cells. It is important to note that mushroom extract had a similar antiproliferative effect on all three cell lines despite their differing status with regard

to androgen responsiveness. Therefore, we can conclude that the antiproliferative action of mushroom extract is mediated through an androgen-independent mechanism.

As seen in Figs. 2 and 3, differing levels of sensitivity to apoptosis induced by mushroom extract exist between the three cell lines, with the LNCaP being the most sensitive, the PC3 less sensitive, and the DU145 the least responsive to the proapoptotic effects of mushroom extract. A similar result was seen in a study utilizing an extract of *Phellinus linteus* (PL) mushroom in which LNCaP cells underwent apoptosis to a larger degree than PC3 cells after treatment with PL. This study showed that caspase 2 expression was upregulated in LNCaP cells but not PC3 cells, and inhibition of caspase 2 abolished the proapoptotic effect of PL in LNCaP cells. The proapoptotic proteins caspase 8, caspase 3, and BID were activated equally in both LNCaP and PC3 cells (20), suggesting that although apoptosis may be induced along similar pathways in DU-45, PC3, and LNCaP prostate cancer cell lines, the modulation of additional pathways in androgen

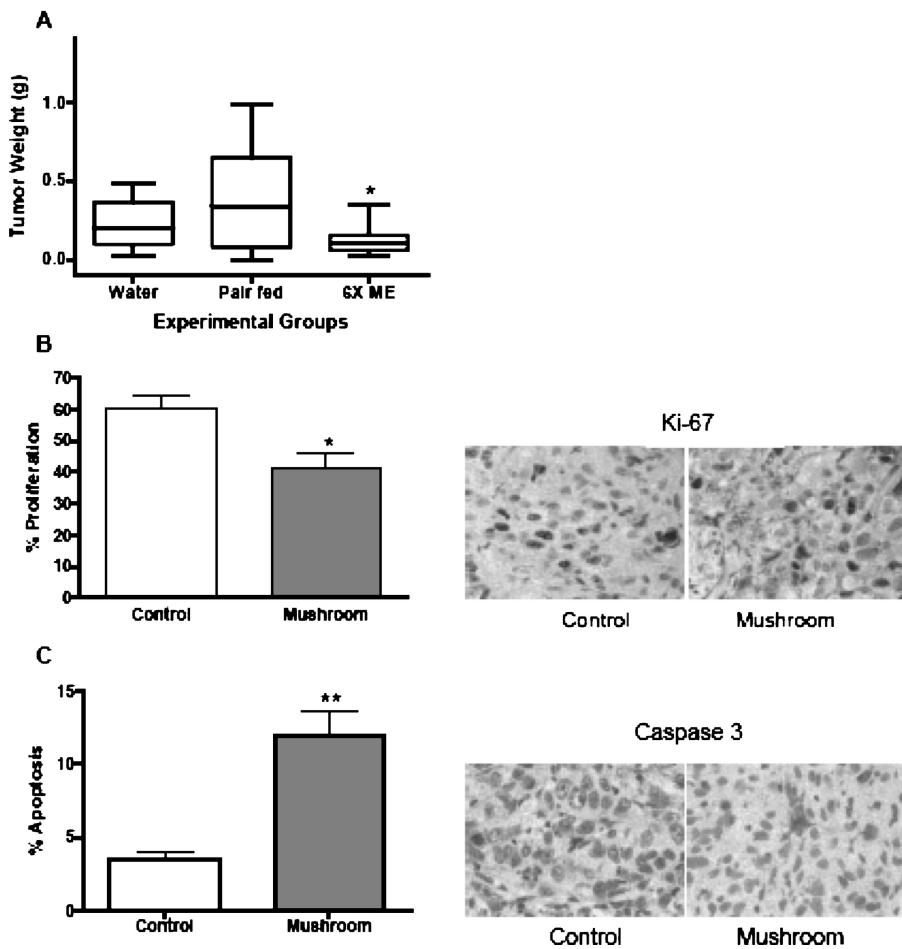


FIG. 5. In vivo effect of mushroom extract on PC3 prostate tumor growth. 6-wk-old, athymic, nude male mice were gavage fed with either 100 μ l water control or 100 μ l mushroom extract (in water). Each animal received daily gavage treatment for 7 wk. After 1 wk of gavaging, mice were given 2 subcutaneous injections of PC3 cells in Matrigel (BD Biosciences, San Jose, CA). 6 wk after injection, mice were euthanized and A: tumor weights, B: Ki-67 antibody staining for cell proliferation, and C: cleaved-caspase-3 antibody for apoptosis were evaluated. Data represent mean in each group ($n \geq 5 \pm$ standard error of the mean). * indicates statistical significance from control group ($P \leq 0.01$); ** indicates statistical significance from control group ($P \leq 0.001$).

responsive, LNCaP cells may sensitize them further and make them more susceptible to apoptosis. Future studies will be done to determine the mechanisms behind the differing response from each cell line.

Our in vivo studies illustrated that oral intake of mushroom extract suppresses the growth of androgen-independent prostate tumors in mice. This shows that the phytochemicals present in mushroom are active after oral ingestion. Further, the suppression of growth was due to inhibition of cell proliferation as measured by Ki-67 antibody staining, and induction of apoptosis was observed. This result is in agreement with the in vitro results. Our previous study (19) did not show apoptosis as a mechanism of mushroom extract in breast cancer cells lines and tumors, whereas in the current study in prostate cancer, apoptosis was observed in the prostate cell lines. It is well known that distinct cell lines within the same cancer family can respond differently to treatment (as was seen in the current study); therefore, it is

reasonable to expect that results in two different cancer types could also be dissimilar.

Results from microarray analysis suggest several mechanisms for the effect of mushroom on prostate cancer cell proliferation and apoptosis. Although the expression of many genes was affected, two genes of primary interest are FAS/APO-1 and KIT. FAS/APO-1 is a member of the tumor necrosis factor receptor superfamily. This receptor contains a death domain and plays a central role in the physiological regulation of programmed cell death (apoptosis). This gene was upregulated 2.84-fold in tumors from the mushroom group compared to pair-fed controls ($P = 0.035$). KIT encodes the human homolog of the proto-oncogene c-kit, and the protein product plays a role in the proliferation and survival of the cell. KIT gene expression was downregulated fourfold in tumors from the mushroom group compared to pair-fed controls ($P = 0.043$). These results suggest that mushroom extract may have a favorable effect on

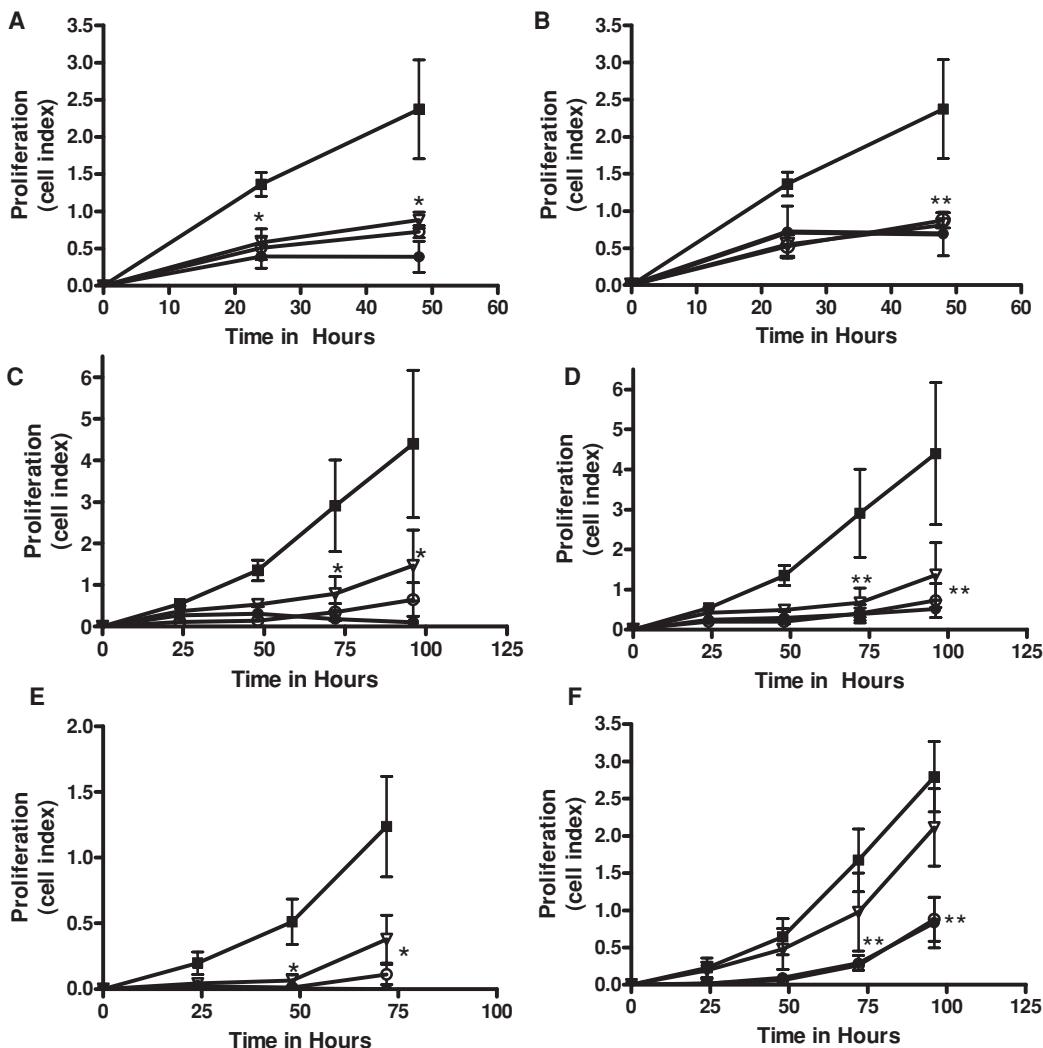


FIG. 6. CLA inhibits proliferation of prostate cancer cell lines. Cells were plated in the ACEA 16-well plate (5,000 cells/well) in media alone or treated with indicated concentrations of CLA; A: LNCaP, 9Z, 11E CLA; B: LNCaP, 10E, 12Z CLA; C: DU145 9Z, 11E CLA; D: DU145, 10E, 12Z CLA; E: PC3, 9Z, 11E CLA; F: PC3 10E, 12Z CLA. Readings were taken every hour up to 96 h by the ACEA machine and expressed as cell index (a measure of cell number/well). Data represent means \pm SE ($n = 3$). * indicates significant difference from media control ($P \leq 0.01$); **indicates significant difference from media control ($P \leq 0.05$). Symbols: ■, vehicle; ▽, 50 μ M CLA; ○, 100 μ M CLA; ●, 200 μ M CLA.

the proliferation and survival of prostate cancer cells *in vivo* through increased expression of FAS and decreased expression of KIT.

Microarray analysis results also indicate that the net effect of mushroom extract on lipid metabolism in tumor tissues could result in a decrease of diacylglycerol (DAG) and prostaglandin E2 (PGE₂) production/release while increasing ceramide and arachidonic acid release. Both ceramide and arachidonic acid are involved in proapoptotic signaling (21–23) in cancer cells. DAG production leads to the activation of PKC and the subsequent inhibition of ceramide-mediated apoptosis (24). Therefore, mushroom could also increase apoptosis through increased production of arachidonic acid (an eicosanoid) and ceramide, which is facilitated by inhibition of DAG production. Additionally, a

study by Ochoa et al. (25) found that the 9Z, 11E isomer of CLA, which was found to be prominent in mushroom extract, had significant effects on cell proliferation and apoptosis through modulation of eicosanoid biosynthesis in PC3 prostate cancer cells. Specifically, cyclooxygenase-2 (COX-2) protein expression and 5-lipoxygenase (5-LOX) mRNA expression were both decreased in cells treated with 9Z, 11E CLA, suggesting that modulation of eicosanoid biosynthesis may be one mechanism by which CLA inhibits prostate cell proliferation (25). This data is in agreement with the observed effects in the current study of CLA-rich mushroom extract in prostate cancer cells.

Arachidonic acid is converted to PGE₂ through the (COX-2) pathway, and PGE₂ production is commonly increased in tumor tissues. PGE₂ itself has been shown to promote

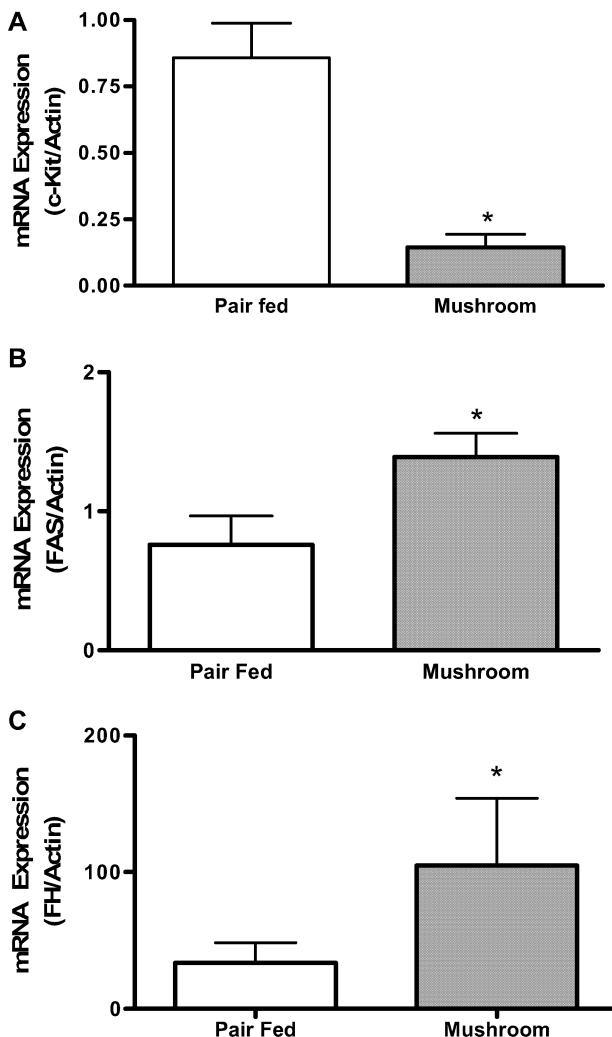


FIG. 7. Relative expression levels of c-KIT, FAS, and FH in tumor samples. mRNA expression of A: c-Kit, B: FAS, and C: FH in tumor samples was determined by quantitative, real-time PCR, and the expression of β -actin was monitored as the internal control. Data represent means \pm SE ($n = 3$); * indicates significant difference from pair-fed control ($P \leq 0.01$).

cancer progression by increasing angiogenesis through vascular endothelial growth factor (VEGF) activation, blocking apoptosis through activation of the phosphatidylinositol 3-kinase (PI3K)/AKT/peroxisome proliferator-activated receptor (PPAR) signaling pathway, increasing tumor cell proliferation through activation of the RAS/RAF/MEK/ERK signaling pathway and affecting immune suppression by increasing interleukin 10 (IL10) production (23,26). Therefore, the suppression of PGE₂ is one way in which mushroom extract may inhibit prostate tumor cell growth *in vivo*.

These effects on lipid metabolism are complimented by the inhibition of isocitrate dehydrogenase 2 (IDH2) and increased expression of FH, 2 key tricarboxylic acid (TCA) cycle proteins, which have also been shown to play a role in fatty acid metabolism and tumor angiogenesis. IDH2 expression was

downregulated threefold ($P = 0.017$), whereas FH expression was upregulated 4.3-fold ($P = 0.009$) in the mushroom-treated group compared to pair-fed controls. This is of interest due to the unique nature of normal prostate epithelial cells to secrete citrate rather than utilize it in the TCA cycle for the production of ATP. Prostate cancer cells, however, alter this balance by upregulating the citric acid cycle to produce more energy through increased ATP production (27). Therefore, mushroom extract may normalize the TCA cycle in prostate cancer cells by downregulation of isocitrate dehydrogenase expression. Citrate is converted to acetyl co-A, which is an instrumental component in fatty acid synthesis, leading back to the above-mentioned effects of mushroom treatment on this process.

With regard to FH, the expression of this enzyme was upregulated in mushroom-treated mice, showing that the citric acid cycle is not entirely inhibited; and therefore, fumarate is still produced and in need of conversion to malate. Fumarate has been shown to activate the angiogenic factor HIF1 α , therefore augmenting the cancer's ability to sustain and spread through increasing its blood supply (27). Upregulation of FH expression by mushrooms would inhibit fumarate buildup in the cell and downregulate the angiogenic response. Additionally, endothelin 1, which is a gene that is commonly upregulated in hypoxia (28), was downregulated threefold in tumor specimens from mushroom-treated mice. Therefore, through analysis of the microarray data, we can further hypothesize that mushroom decreased tumor size, tumor cell proliferation, and increased tumor cell apoptosis through its effects on the TCA cycle and fatty acid metabolism. Future studies will address this hypothesis.

Several studies have demonstrated that several mushroom species can enhance immunity through activation of natural killer (NK) cells (29) and modulation of lymphocyte number and activity (30). Studies have also shown that mushroom treatment can induce both Type 1 and Type 2 immune response (31). Evasion of the immune response is one way in which tumor cells survive; therefore, the effect of mushroom on the expression of genes important to immune function, such as was seen in the microarray analysis (Table 2), is another mechanism that could contribute to its anticancer action *in vivo*.

Identification of the phytochemicals present in mushroom extract is important to understanding its mode of action and could facilitate future studies by enabling the use of purified compounds from the extract. Similar to our previous findings in breast cancer, the current study showed that CLA inhibited the proliferation of prostate cancer cell lines. Much research has been published illustrating the antiproliferative (25,32,33) and proapoptotic (34–36) activity of CLA. CLA has also been shown to have significant effects on the immune system such as increased T-cell responsiveness and splenocyte IL-2 production in mice fed 1% CLA in the diet (37). Thus, the presence of this compound in white button mushrooms likely significantly adds to the biological activity of the whole extract.

TABLE 1
Gene Networks Regulated by Mushroom Extract in DU-145 Derived Tumors

Molecules in Network	Score	Focus Genes	Top Functions
AKR1C2, BDKRB2, COL1A1, COL3A1, DAD1, DUSP4, EDN1, EGF, EREG, HES1, HSD17B1, IGFBP3, KIT, KITLG, MAML1, MAML2, MAML3 (includes EG:55534), MARCKS, MCL1, NF1, NOTCH3, PDCD4, PPAP2B, PTPRO, RAB5A, RPS6KB2, SCNN1A, SERPINH1, SGK, SNAI2, TPM4, VEGF, WDR1, WEE1, XRCC4	29	18	Cellular growth and proliferation, lipid metabolism, small molecule biochemistry
ADCYAP1, ADFP, ADH7, AKR1B10, ALDH3A1, CBS, CEBPA, CLIC4, CRLF1, DHRS3, DMBT1, G1P3, GSTT1, HSPA4, IDH2, LCK, LITAF, MAP3K14, MAT2A, NFE2L2, NFIX, OAS2, PGD, QKI, RPS11, S100A8, S100A9, SLC1A4, SLC2A3, SOX4, SP1, TFAM, TNF, UBC, WDR48	18	13	Inflammatory disease, gene expression, cell death
ALPL, ALPP, BHLHB2, BMP2K, BUB1, CASP3, CLIC4, COL4A1, CTSW, DAPK1, DFFA, DFFB, EPRS, HIF1A, IL2, JAG2, MASK, MBP, MFAP5, NDRG1, NOTCH1, NT5E, P4HA1, PBK, PDE4B, PLOD2, PRODH, PTK9, PXN, RNF5, TGFB1, THRAP2, TP53, TP53RK, ZFHX1B	18	13	DNA replication, recombination, and repair, cancer, tumor morphology
API5, ATG5, ATG12, BAMBI, CEACAM6, CTNNB1, ENPP2, ESR2, FADD, FAS, FGF2, HIPK3, HSPA4L, ID4, IFITM1, IL15, MYOD1, NP, NPTX1, PDE4B, PGK1, PLXNA1, PTK7, PTPN13, PTPRG, RBM17, SEMA3C, SPARC, SRC, SURB7, TCF7, THBS4, TNS1, TRFP, ZNF45	18	13	Cell Death, Cellular Growth and Proliferation, Cell Morphology
ABCC2, AP1G1, AP1S1, CD44, CD53, COPA, CPB2, CSPG6, DHX9, DUSP6, EMP2, FCGRT, FXR1, HBE1, HOXA9, HRAS, ICAM3, IL6, IQGAP1, ITGB1, KIT, LGALS1, MYB, NLK, NXF1, NXT2, RANBP9, RDX, RPL27A, SPBC25, SPN, ST6GAL1, TOP2A, TYR, WSB1	18	13	Cell death, hematological disease, immunological disease
ACLY, AP2A2, AP2B1, AP2M1, AP2S1, ATP6V0E, CDC40, EPN1, EPS15, GOSR1, GOSR2, GPAA1, HRB, INS1, ITSN1, NFYB, PICALM, PTH, RAB5A, RRM2, SCAMP1, SIRPA, SLC34A1, SLC34A2, SLC39A8, SLC6A3, SNAP23, SNAP25, STX16, SYNJ1, TFG, USP10, VAMP3, VTI1B, YKT6	13	10	Cellular assembly and organization, cellular function and maintenance, cellular movement

TABLE 2
Functional Grouping of Mushroom Extract-regulated Genes

Symbol	Entrez Gene	Fold Change	Description
Apoptosis, cell death, cytotoxicity, viability, survival, proliferation, and growth			
ATG5	9474	2.22	Autophagy related 5 homolog (<i>S. cerevisiae</i>)
CEACAM6	4680	-2.22	Carcinoembryonic antigen-related cell adhesion molecule 6
CLIC4	25932	2.90	Chloride intracellular channel 4
DAPK1	1612	-2.28	Death associated protein kinase
DFFA	1676	-2.10	DNA fragmentation factor alpha polypeptide
EDN1	1906	-3.00	Endothelin 1
EGF	1950	-2.21	Epidermal growth factor
EMP2	2013	-2.20	Epithelial membrane protein 2

(Continued on next page)

TABLE 2
Functional Grouping of Mushroom Extract-regulated Genes (*Continued*)

Symbol	Entrez Gene	Fold Change	Description
FAS	355	2.84	Tumor necrosis factor (TNF) receptor family member 6
HES1	3280	-2.75	Hairy and enhancer of split 1
IGFBP3	3486	-2.31	Insulin-like growth factor binding protein 3
IL15	3600	2.41	Interleukin 15
KIT	3815	-4.00	v-kit hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
KITLG	4254	2.50	KIT ligand
LGALS1	956	2.64	Lectin, galactoside-binding soluble, 1
MCL1	4170	2.21	Myeloid cell leukemia sequence 1
NF1	4763	-2.10	Neurofibromin 1
RBM17	84991	2.10	RNA binding motif protein 1
S100A8	6279	3.49	S100 calcium binding protein A8
S100A9	6280	2.01	S100 calcium binding protein A9
SGK	6446	2.20	Serum/glucocorticoid regulated kinase
SPC25	57405	2.51	NDC80 kinetochore complex component
VEGF	7422	-2.03	Vascular endothelial growth factor
WIF1	11197	-5.03	WNT inhibitory factor 1
XRCC4	7518	2.12	X-ray repair complementing defective repair in Chinese hamster cells 4
Lipid metabolism			
BDKRB2	624	2.95	Bradykinin receptor B2
DHRS3	9249	4.21	Dehydrogenas/reductase (SDR family) member 3
EDN1	1906	-3.00	Endothelin 1
EGF	1950	-2.21	Epidermal growth factor
FAS	355	2.84	TNF receptor family member 6
KIT	3815	-4.00	v-kit hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
KITLG	4254	2.50	KIT ligand
LGALS1	1956	2.64	Lectin, galactoside-binding soluble, 1
MARCKS	4082	2.21	Myristoylated alanine-rich protein kinase C substrate
S100A	86279	3.49	S100 calcium binding protein A8
S100A	96280	2.01	S100 calcium binding protein A9
Immune response			
ALPP	250	-4.20	Alkaline phosphatase, placental
FAS	355	2.84	TNF receptor family member 6
IL15	3600	2.41	Interleukin 15
KIT	3815	-4.00	v-kit hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
KITLG	4254	2.50	KIT ligand
LGALS1	1956	2.64	Lectin, galactoside-binding soluble, 1
NF1	4763	-2.10	Neurofibromin 1
S100A8	86279	3.49	S100 calcium binding protein A8
S100A9	96280	2.01	S100 calcium binding protein A9
VEGF	7422	-2.03	Vascular endothelial growth factor

Taken together with research from other laboratories, our results support the recommendation of white button mushroom as a dietary component that may aid in the prevention of prostate cancer in men. The in vivo studies demonstrate

that oral intake of the extract is effective in the inhibition of prostate tumor growth in mice. Importantly, the dosage used in the in vivo studies is considered physiologic, as the common conversion factor from murine to human dosage is 25,

calculated on body surface area (38). In addition to the inclusion of whole mushrooms into the diet, our work with the extract and isolation of CLA from the extract suggests that purified compounds or mixtures of compounds from mushroom may have efficacy as potential dietary supplements. Future study into the mechanisms of action of mushroom extract will help us to further delineate possible roles of mushroom phytochemicals in the prevention and treatment of prostate cancer.

ACKNOWLEDGMENTS

This research was supported by the American Institute for Cancer Research Grant 05B026, National Institutes of Health Grants ES08258 and a grant from the Mushroom Council.

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Meat Intake and Mortality

A Prospective Study of Over Half a Million People

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Background: High intakes of red or processed meat may increase the risk of mortality. Our objective was to determine the relations of red, white, and processed meat intakes to risk for total and cause-specific mortality.

Methods: The study population included the National Institutes of Health–AARP (formerly known as the American Association of Retired Persons) Diet and Health Study cohort of half a million people aged 50 to 71 years at baseline. Meat intake was estimated from a food frequency questionnaire administered at baseline. Cox proportional hazards regression models estimated hazard ratios (HRs) and 95% confidence intervals (CIs) within quintiles of meat intake. The covariates included in the models were age, education, marital status, family history of cancer (yes/no) (cancer mortality only), race, body mass index, 31-level smoking history, physical activity, energy intake, alcohol intake, vitamin supplement use, fruit consumption, vegetable consumption, and menopausal hormone therapy among women. Main outcome measures included total mortality and deaths due to cancer, cardiovascular disease, injuries and sudden deaths, and all other causes.

Results: There were 47 976 male deaths and 23 276 female deaths during 10 years of follow-up. Men and women

in the highest vs lowest quintile of red (HR, 1.31 [95% CI, 1.27-1.35], and HR, 1.36 [95% CI, 1.30-1.43], respectively) and processed meat (HR, 1.16 [95% CI, 1.12-1.20], and HR, 1.25 [95% CI, 1.20-1.31], respectively) intakes had elevated risks for overall mortality. Regarding cause-specific mortality, men and women had elevated risks for cancer mortality for red (HR, 1.22 [95% CI, 1.16-1.29], and HR, 1.20 [95% CI, 1.12-1.30], respectively) and processed meat (HR, 1.12 [95% CI, 1.06-1.19], and HR, 1.11 [95% CI 1.04-1.19], respectively) intakes. Furthermore, cardiovascular disease risk was elevated for men and women in the highest quintile of red (HR, 1.27 [95% CI, 1.20-1.35], and HR, 1.50 [95% CI, 1.37-1.65], respectively) and processed meat (HR, 1.09 [95% CI, 1.03-1.15], and HR, 1.38 [95% CI, 1.26-1.51], respectively) intakes. When comparing the highest with the lowest quintile of white meat intake, there was an inverse association for total mortality and cancer mortality, as well as all other deaths for both men and women.

Conclusion: Red and processed meat intakes were associated with modest increases in total mortality, cancer mortality, and cardiovascular disease mortality.

Arch Intern Med. 2009;169(6):562-571

M EAT INTAKE VARIES SUBSTANTIALLY around the world, but the impact of consuming higher levels of meat in relation to chronic disease mortality is ambiguous.¹⁻⁶ To increase sample size, pooled

vegetarian diet are legumes, grains, and nuts. Vegetarian diets also include higher intakes of vegetables, unsaturated fats, dietary fiber, and antioxidants (carotenoids and vitamins C and E), although they contain lower amounts of iron, zinc, and vitamin B₁₂. Furthermore, other lifestyle factors, such as smoking, physical activity, and alcohol consumption among vegetarians and members of select religious groups can differ substantially from the general population.

We prospectively investigated red, white, and processed meat intakes as risk factors for total mortality, as well as cause-specific mortality, including cancer and cardiovascular disease (CVD) mortality in a cohort of approximately half a million men

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analyses of meat intake have been carried out in Seventh-Day Adventists in the United States^{1,2} and other vegetarian populations in Europe.³⁻⁶ Vegetarian diets differ from nonvegetarian diets in several respects. The main sources of protein in a

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and women enrolled in the National Institutes of Health (NIH)–AARP (formerly known as the American Association of Retired Persons) Diet and Health Study.⁷ This large prospective study facilitated the investigation of a wide range of meat intakes with chronic disease mortality.

METHODS

STUDY POPULATION

Individuals aged 50 to 71 years were recruited from 6 US states (California, Florida, Louisiana, New Jersey, North Carolina, and Pennsylvania) and 2 metropolitan areas (Atlanta, Georgia, and Detroit, Michigan) to form a large prospective cohort, the NIH-AARP Diet and Health Study. Questionnaires on demographic and lifestyle characteristics, including dietary habits, were mailed to 3.5 million members of AARP in 1995, as described in detail elsewhere.⁷ The NIH-AARP Diet and Health Study was approved by the Special Studies Institutional Review Board of the US National Cancer Institute. Completion of the baseline questionnaire was considered to imply informed consent.

DIETARY ASSESSMENT

A 124-item food frequency questionnaire (<http://riskfactor.cancer.gov/DHQ/forms/files/shared/dhq1.2002.sample.pdf>) was completed at baseline. The food frequency questionnaire collected information on the usual consumption of foods and drinks and portion sizes over the last 12 months. The validity of the food frequency questionnaire was estimated using two 24-hour recalls,⁸ and the estimated energy-adjusted correlations ranged from 0.36 to 0.76 for various nutrients and attenuation factors ranged from 0.24 to 0.68. Red meat intake was calculated using the frequency of consumption and portion size information of all types of beef and pork and included bacon, beef, cold cuts, ham, hamburger, hotdogs, liver, pork, sausage, steak, and meats in foods such as pizza, chili, lasagna, and stew. White meat included chicken, turkey, and fish and included poultry cold cuts, chicken mixtures, canned tuna, and low-fat sausages and low-fat hotdogs made from poultry. Processed meat included bacon, red meat sausage, poultry sausage, luncheon meats (red and white meat), cold cuts (red and white meat), ham, regular hotdogs and low-fat hotdogs made from poultry. The components constituting red or white and processed meats can overlap because both can include meats such as bacon, sausage, and ham, while processed meat can also include smoked turkey and chicken. However, these meat groups are not used in the same models; thus, they are not duplicated in any one analysis.

To investigate whether the overall composition of meat intake was associated with mortality, we created 3 diet types: high-, medium-, and low-risk meat diet. To form these diet variables, red and white meat consumption was energy adjusted and split into 2 groups using the median values as cut points. Individuals with red meat consumption in the upper half and white meat consumption in the lower half got a score of 1 (high-risk meat diet), those with both red and white meat consumption in the same half got a score of 2 (medium-risk meat diet), and those with red meat consumption in the lower half and white meat consumption in the upper half got a score of 3 (low-risk meat diet).

COHORT FOLLOW-UP AND CASE ASCERTAINMENT

Cohort members were followed-up from the date the baseline questionnaire was returned (beginning 1995) through Decem-

ber 31, 2005, by annual linkage of the cohort to the National Change of Address database maintained by the US Postal Service and through processing of undeliverable mail, other address change update services, and directly from cohort members' notifications. For matching purposes, we have virtually complete data on first and last name, address history, sex, and date of birth. Follow-up for vital status is performed by annual linkage of the cohort to the Social Security Administration Death Master File in the US verification of vital status, and cause of death information is provided by follow-up searches of the National Death Index (NDI) Plus with the current follow-up for mortality covered until 2005.

CAUSE-SPECIFIC CASE ASCERTAINMENT

Cancer (*International Classification of Diseases, Ninth Revision [ICD-9]* codes 140-239 and *International Statistical Classification of Diseases, 10th Revision [ICD-10]* codes C00-C44, C45.0, C45.1, C45.7, C45.9, C48-C97, and D12-D48) mortality included deaths due to cancers of the oral cavity and pharynx, digestive tract, respiratory tract, soft tissue (including heart), skin (excluding basal and squamous cell carcinoma), female genital system and breast, male genital system, urinary tract, endocrine system, lymphoma, leukemia, and other miscellaneous cancers.

Cardiovascular disease (*ICD-9* codes 390-398, 401-404, 410-438, and 440-448 and *ICD-10* codes I00-I09, I10-I13, I20-I51, and I60-I78) mortality was from a combination of diseases of the heart; hypertension without heart disease; cerebrovascular diseases; atherosclerosis; aortic aneurysm and dissection; and other diseases of the arteries, arterioles, and capillaries.

Mortality from injuries and sudden deaths (*ICD-9* codes 800-978 and *ICD-10* codes U01-U03, V01-Y09, Y35, Y85-Y86, Y87.0, Y87.1, and Y89.0) included deaths due to unintentional injury, adverse effects, suicide, self-inflicted injury, homicide, and legal intervention.

All others deaths included mortality from tuberculosis, human immunodeficiency virus, other infectious and parasitic diseases, septicemia, diabetes mellitus, Alzheimer disease, stomach and duodenal ulcers, pneumonia and influenza, chronic obstructive pulmonary disease and allied conditions, chronic liver disease and cirrhosis, nephritis, nephrotic syndrome and nephrosis, congenital anomalies, certain conditions originating in the perinatal period, ill-defined conditions, and unknown causes of death.

Total mortality is a combination of all of the aforementioned causes of deaths.

STATISTICAL ANALYSIS

A total of 617 119 persons returned the baseline questionnaire; of these, we excluded individuals who moved out of the 8 study areas before returning the baseline questionnaire (n=321), requested to be withdrawn from the study (n=829), died before study entry (n=261), had duplicate records (n=179), indicated that they were not the intended respondent and did not complete the questionnaire (n=13 442), provided no information on gender (n=6), and did not answer substantial portions of the questionnaire or had more than 10 recording errors (n=35 679). After these exclusions, we further removed individuals whose questionnaire was filled in by someone else on their behalf (n=15 760). We excluded 4849 subjects reporting extreme daily total energy intake defined as more than 2 interquartile ranges above the 75th percentile or below the 25th percentile and 140 people who had zero person-years of follow-up. After all exclusions, our analytic cohort comprised 322 263 men and 223 390 women.

We estimated hazard ratios (HRs) and 95% confidence intervals (CIs) with time since entry into the study as the underlying time metric using Cox proportional hazards regression models. Quintile cut points were based on the entire cohort, and multivariate-adjusted HRs are reported using the lowest quintile as the referent category. The violation of the proportional hazard assumption was investigated by testing an interaction between a time-dependent binary covariate, which indicated if follow-up was in the first 5 years or in the second 5 years, and the quintile terms for meat consumption. Dietary variables were energy adjusted using the nutrient density method, and meat variables in each model added up to total meat (addition model). For example, one model contained both red and white meat, while the processed meat model also contained a nonprocessed meat variable.

To address confounding, we used forward stepwise variable selection to include covariates to develop the fully adjusted model. Smoking was the largest confounder of the association between meat intake and mortality. Physical activity and education were also important covariates, but not to the same degree as smoking. The final model included age (continuous); education (<8 years or unknown, 8-11 years, 12 years [high school], some college, or college graduate); marital status (married: yes/no); family history of cancer (yes/no) (cancer mortality only); race (non-Hispanic white, non-Hispanic black, Hispanic/Asian/Pacific Islander/American Indian/Alaskan native, or unknown); body mass index (18.5 to <25, 25 to <30, 30 to <35, ≥35 [calculated as weight in kilograms divided by height in meters squared]); 31-level smoking history using smoking status (never, former, or current), time since quitting for former smokers and smoking dose; frequency of vigorous physical activity (never/rarely, 1-3 times/mo, 1-2 times/wk, 3-4 times/wk, ≥5 times/wk); total energy intake (continuous); alcohol intake (none, 0 to <5, 5 to <15, 15 to <30, ≥30 g/d); vitamin supplement user (≥1 supplement/mo); fruit consumption (0 to <0.7, 0.7 to <1.2, 1.2 to <1.7, 1.7 to <2.5, ≥2.5 servings/1000 kcal); vegetable consumption (0 to <1.3, 1.3 to <1.8, 1.8 to <2.2, 2.2 to <3.0, ≥3.0 servings/1000 kcal); and menopausal hormone therapy among women in the multivariate models.

In subanalyses, we investigated the relation between meat intake and mortality by smoking status. We used median values of each quintile to test for linear trend with 2-sided P values. We also calculated population-attributable risks as an estimate of the percentage of mortality that could be prevented if individuals adopted intake levels of participants within the first quintile. This was computed as 1 minus the ratio consisting of the sum of the estimated HR (derived from the Cox proportional hazard regression models) of each member of the cohort divided by the sum of the estimated HR for which meat exposure was assigned to the lowest or highest quintile, depending on which quintile was the ideal level of meat consumption. The population-attributable risk was multiplied by 100 to convert them to a percentage. All statistical analyses were carried out using Statistical Analytic Systems (SAS) software (SAS Institute Inc, Cary, North Carolina).

RESULTS

During 10 years of follow-up, there were 47 976 male deaths and 23 276 female deaths. In general, those in the highest quintile of red meat intake tended to consume a slightly lower amount of white meat but a higher amount of processed meat compared with those in the lowest quintile. Subjects who consumed more red meat tended to be married, more likely of non-Hispanic white ethnicity, more likely a current smoker, have a higher body mass

index, and have a higher daily intake of energy, total fat, and saturated fat, and they tended to have lower education and physical activity levels and lower fruit, vegetable, fiber, and vitamin supplement intakes (**Table 1**).

RED MEAT

There was an overall increased risk of total, cancer, and CVD mortality, as well as all other deaths in both men (**Table 2**) and women (**Table 3**) in the highest compared with the lowest quintile of red meat intake in the fully adjusted model. There was an increased risk associated with death from injuries and sudden death with higher consumption of red meat in men but not in women.

WHITE MEAT

When comparing the highest with the lowest quintile of white meat intake, there was an inverse association for total mortality and cancer mortality, as well as all other deaths for both men (Table 2) and women (Table 3). In contrast, there was a small increase in risk for CVD mortality in men with higher intake of white meat. There was no association between white meat consumption and death from injuries and sudden death in men or women.

PROCESSED MEAT

There was an overall increased risk of total, cancer, and CVD mortality, as well as all other deaths in both men (Table 2) and women (Table 3) in the highest compared with the lowest quintile of processed meat intake. In contrast, there was no association for processed meat intake and death from injuries and sudden death in either sex.

A lag analysis, excluding deaths occurring in the first 2 years of follow-up, produced results consistent with the main findings in Table 2 and Table 3. For example, the HRs for total mortality in men for red meat was as follows: second quintile HR, 1.05 (95% CI, 1.01-1.09); third quintile HR, 1.13 (95% CI, 1.09-1.17); fourth quintile HR, 1.20 (95% CI, 1.16-1.24); and fifth quintile HR, 1.30 (95% CI, 1.26-1.35). For women, the HRs were as follows: second quintile HR, 1.07 (95% CI, 1.02-1.12); third quintile HR, 1.15 (95% CI, 1.11-1.21); fourth quintile HR, 1.27 (95% CI, 1.21-1.33); and fifth quintile HR, 1.35 (95% CI, 1.28-1.42). Furthermore, we investigated our models for a violation of the proportional hazard assumption. Proportional hazard assumption was not rejected for all analyses except one, the model with red and white meat among the women for total mortality ($P=.008$). On further examination in that model of the relative HR between the first 5 years of follow-up and the second 5 years of follow-up, the red meat results were consistent between the 2 follow-up periods. However, for white meat, the second 5-year period showed less of an inverse trend compared with the first 5-year period (data not shown).

We investigated whether people who consumed a high-risk meat diet had mortality risk profiles that were different from people who consumed a low-risk meat diet. Both men and women who consumed a low-risk meat diet had statistically significant lower HRs compared with

Table 1. Selected Age-Adjusted Characteristics of the National Institutes of Health–AARP Cohort by Red Meat Quintile Category^a

Characteristic	Red Meat Intake Quintile, g/1000 kcal				
	Q1	Q2	Q3	Q4	Q5
Men (n=322 263)					
Meat intake					
Red meat, g/1000 kcal	9.3	21.4	31.5	43.1	68.1
White meat, g/1000 kcal	36.6	32.2	30.7	30.4	30.9
Processed meat, g/1000 kcal	5.1	7.8	10.3	13.3	19.4
Age, y	62.8	62.8	62.5	62.3	61.7
Race, %					
Non-Hispanic white	88.6	91.8	93.1	94.0	94.1
Non-Hispanic black	4.2	3.2	2.7	2.2	1.9
Hispanic/Asian/Pacific Islander/American Indian/Alaskan native/unknown	7.2	5.0	4.2	3.8	4.0
Positive family history of cancer, %	47.0	47.7	48.4	48.6	47.8
Currently married, %	80.8	84.4	86.1	86.7	85.6
BMI	25.9	26.7	27.1	27.6	28.3
Smoking history, % ^b					
Never smoker	34.4	30.5	28.8	27.6	25.4
Former smoker	56.5	58.1	57.5	57.1	55.8
Current smoker or having quit <1 y prior	4.9	7.6	9.9	11.4	14.8
Education, college graduate or postgraduate, %	53.0	47.3	45.1	42.3	39.1
Vigorous physical activity ≥5 times/wk, %	30.7	23.6	20.5	18.6	16.3
Dietary intake					
Energy, kcal/d	1899	1955	1998	2038	2116
Fruit, servings/1000 kcal	2.3	1.8	1.6	1.4	1.1
Vegetables, servings/1000 kcal	2.4	2.1	2.0	2.0	1.9
Alcohol, g/d	20.2	20.4	17.6	15.3	12.5
Total fat, g/1000 kcal	25.8	30.5	33.5	35.9	39.4
Saturated fat, g/1000 kcal	7.6	9.4	10.5	11.3	12.7
Fiber, g/1000 kcal	13.2	11.0	10.2	9.6	8.8
Vitamin supplement use ≥1/mo	67.3	62.1	59.1	55.8	52.0
Women (n=223 390)					
Meat intake					
Red meat, g/1000 kcal	9.1	21.2	31.2	42.8	65.9
White meat, g/1000 kcal	37.4	35.6	34.9	35.1	35.3
Processed meat, g/1000 kcal	3.8	6.4	8.7	11.3	16.0
Age, y	62.2	62.2	62.0	61.7	61.3
Race, %					
Non-Hispanic white	86.2	89.9	91.0	91.8	91.4
Non-Hispanic black	7.5	5.5	4.8	4.1	3.8
Hispanic/Asian/Pacific Islander/American Indian/Alaskan native/unknown	6.3	4.5	4.3	4.1	4.9
Positive family history of cancer, %	51.4	53.0	52.9	52.4	51.5
Currently married, %	37.2	42.4	46.3	48.8	50.7
BMI	25.6	26.6	27.1	27.7	28.4
Never received HT (women only)	46.6	463	47.1	48.1	50.5
Smoking history, % ^b					
Never smoker	45.5	44.3	43.23	42.2	40.0
Former smoker	41.8	39.5	38.1	37.0	35.4
Current smoker or having quit <1 y prior	8.8	12.7	15.3	17.7	21.2
Education, college graduate or postgraduate, %	37.1	30.7	27.7	25.6	22.7
Vigorous physical activity ≥5 times/wk, %	22.5	16.3	13.9	12.0	11.0
Dietary intake, %					
Energy, kcal/d	1526	1539	1584	1613	1646
Fruit, servings/1000 kcal	2.5	2.0	1.8	1.5	1.3
Vegetables, servings/1000 kcal	2.8	2.5	2.4	2.3	2.3
Alcohol, g/d	5.8	6.3	6.2	5.7	5.1
Total fat, g/1000 kcal	27.7	32.1	34.7	37.0	40.1
Saturated fat, g/1000 kcal	8.3	9.9	10.8	11.6	12.7
Fiber, g/1000 kcal	13.8	11.7	10.9	10.3	9.5
Vitamin supplement use ≥1/mo	72.2	68.4	66.1	63.7	58.8

Abbreviations: AARP, formerly the American Association of Retired Persons; BMI, body mass index (calculated as weight in kilograms divided by height in meters squared); HT, hormone therapy; Q, quintile.

^aData are given as mean value or percentage of participants (N = 545 653). Generalized linear models were used to estimate mean values for the continuous variables and frequencies for dichotomous proportions within each red meat intake quintile.

^bA total of 12 597 men (3.9%) and 7885 women (3.5%) had missing smoking history data.

Table 2. Multivariate Analysis for Red, White, and Processed Meat Intake and Total and Cause-Specific Mortality in Men in the National Institutes of Health–AARP Diet and Health Study^a

Mortality in Men (n=322 263)	Quintile					<i>P</i> Value for Trend
	Q1	Q2	Q3	Q4	Q5	
Red Meat Intake^b						
All mortality						
Deaths	6437	7835	9366	10 988	13 350	
Basic model ^c	1 [Reference]	1.07 (1.03-1.10)	1.17 (1.13-1.21)	1.27 (1.23-1.31)	1.48 (1.43-1.52)	<.001
Adjusted model ^d	1 [Reference]	1.06 (1.03-1.10)	1.14 (1.10-1.18)	1.21 (1.17-1.25)	1.31 (1.27-1.35)	<.001
Cancer mortality						
Deaths	2136	2701	3309	3839	4448	
Basic model ^c	1 [Reference]	1.10 (1.04-1.17)	1.23 (1.16-1.29)	1.31 (1.24-1.39)	1.44 (1.37-1.52)	<.001
Adjusted model ^d	1 [Reference]	1.05 (0.99-1.11)	1.13 (1.07-1.20)	1.18 (1.12-1.25)	1.22 (1.16-1.29)	<.001
CVD mortality						
Deaths	1997	2304	2703	3256	3961	
Basic model ^c	1 [Reference]	1.02 (0.96-1.08)	1.10 (1.04-1.17)	1.24 (1.17-1.31)	1.44 (1.37-1.52)	<.001
Adjusted model ^d	1 [Reference]	0.99 (0.96-1.09)	1.08 (1.02-1.15)	1.18 (1.12-1.26)	1.27 (1.20-1.35)	<.001
Mortality from injuries and sudden deaths						
Deaths	184	216	228	280	343	
Basic model ^c	1 [Reference]	1.02 (0.84-1.24)	0.97 (0.80-1.18)	1.09 (0.90-1.31)	1.24 (1.03-1.49)	.01
Adjusted model ^d	1 [Reference]	1.06 (0.86-1.29)	1.01 (0.83-1.24)	1.14 (0.94-1.39)	1.26 (1.04-1.54)	.008
All other deaths						
Deaths	1268	1636	1971	2239	2962	
Basic model ^c	1 [Reference]	1.13 (1.05-1.22)	1.25 (1.17-1.35)	1.33 (1.24-1.42)	1.68 (1.57-1.80)	<.001
Adjusted model ^d	1 [Reference]	1.17 (1.09-1.26)	1.28 (1.19-1.38)	1.34 (1.25-1.44)	1.58 (1.47-1.70)	<.001
White Meat Intake^e						
All mortality						
Deaths	12 521	10 442	9359	8444	7210	
Basic model ^c	1 [Reference]	0.83 (0.81-0.85)	0.77 (0.75-0.79)	0.74 (0.72-0.76)	0.74 (0.72-0.76)	<.001
Adjusted model ^d	1 [Reference]	0.92 (0.90-0.95)	0.90 (0.88-0.93)	0.90 (0.88-0.93)	0.92 (0.89-0.94)	<.001
Cancer mortality						
Deaths	4424	3647	3203	2830	2329	
Basic model ^c	1 [Reference]	0.82 (0.79-0.86)	0.74 (0.71-0.78)	0.71 (0.67-0.74)	0.68 (0.65-0.72)	<.001
Adjusted model ^d	1 [Reference]	0.91 (0.87-0.95)	0.87 (0.83-0.91)	0.85 (0.81-0.90)	0.84 (0.80-0.88)	<.001
CVD mortality						
Deaths	3521	3015	2771	2578	2336	
Basic model ^c	1 [Reference]	0.85 (0.81-0.89)	0.81 (0.77-0.85)	0.81 (0.77-0.85)	0.86 (0.81-0.90)	<.001
Adjusted model ^d	1 [Reference]	0.96 (0.91-1.00)	0.96 (0.91-1.01)	0.99 (0.94-1.04)	1.05 (1.00-1.11)	.009
Mortality from injuries and sudden deaths						
Deaths	333	266	249	219	184	
Basic model ^c	1 [Reference]	0.81 (0.69-0.95)	0.78 (0.66-0.93)	0.73 (0.62-0.87)	0.71 (0.59-0.85)	<.001
Adjusted model ^d	1 [Reference]	0.89 (0.76-1.05)	0.90 (0.76-1.06)	0.86 (0.72-1.03)	0.85 (0.70-1.02)	.11
All other deaths						
Deaths	2775	2206	1948	1722	1425	
Basic model ^c	1 [Reference]	0.79 (0.75-0.83)	0.72 (0.68-0.76)	0.68 (0.64-0.73)	0.67 (0.63-0.72)	<.001
Adjusted model ^d	1 [Reference]	0.90 (0.85-0.95)	0.88 (0.83-0.93)	0.86 (0.81-0.92)	0.86 (0.80-0.92)	<.001

(continued)

people who consumed a high-risk meat diet for all-cause, cancer, and CVD mortality, as well as all other deaths; for example, for all-cause mortality, the HR for a low-risk meat diet was 0.92 (95% CI, 0.80-0.94) for men and 0.80 (95% CI, 0.78-0.84) for women.

To further explore possible confounding by smoking, we analyzed meat intake and mortality in 2 subgroups—never smokers (15 413 deaths among 190 135 never smokers) and former/current smokers (n=52 754 deaths among 335 036 former/current smokers). For men, the risks in the fifth quintile of red meat intake for never and former smokers were as follows: for total mortality, HR, 1.28 (95% CI, 1.19-1.38), and HR, 1.25 (95% CI, 1.20-1.30), respectively; for cancer mortality, HR, 1.16 (95% CI,

1.02-1.33), and HR, 1.17 (95% CI, 1.09-1.24), respectively; and for CVD mortality, HR, 1.43 (95% CI, 1.25-1.63), and HR, 1.17 (95% CI, 1.10-1.26), respectively. In women, the risks in the fifth quintile of red meat intake for never and former/current smokers were as follows: for total mortality, HR, 1.36 (95% CI, 1.25-1.48), and HR, 1.28 (95% CI, 1.21-1.35), respectively; for cancer mortality, HR, 1.10 (95% CI, 0.95-1.27), and HR, 1.16 (95% CI, 1.06-1.27), respectively; and for CVD mortality, HR, 1.63 (95% CI, 1.38-1.93), and HR, 1.34 (95% CI, 1.18-1.51), respectively. Risks were similar for the 2 smoking categories in most instances for processed meat except for cancer mortality, for which we found a null relation for both sexes in never smokers (men: HR, 1.01 [95% CI, 0.88-1.15]; women: HR, 1.02

Table 2. Multivariate Analysis for Red, White, and Processed Meat Intake and Total and Cause-Specific Mortality in Men in the National Institutes of Health–AARP Diet and Health Study^a (continued)

Mortality in Men (n=322 263)	Quintile					<i>P</i> Value for Trend
	Q1	Q2	Q3	Q4	Q5	
Processed Meat Intake^f						
Deaths	6235	7738	9435	11 249	13 319	
Basic model ^c	1 [Reference]	1.04 (1.01-1.08)	1.13 (1.09-1.16)	1.20 (1.16-1.24)	1.30 (1.26-1.34)	<.001
Adjusted model ^d	1 [Reference]	1.01 (0.98-1.04)	1.07 (1.04-1.11)	1.12 (1.08-1.16)	1.16 (1.12-1.20)	<.001
Cancer mortality						
Deaths	2032	2784	3334	3906	4377	
Basic model ^c	1 [Reference]	1.15 (1.08-1.22)	1.22 (1.15-1.29)	1.28 (1.21-1.35)	1.32 (1.25-1.39)	<.001
Adjusted model ^d	1 [Reference]	1.07 (1.01-1.14)	1.11 (1.05-1.17)	1.14 (1.07-1.20)	1.12 (1.06-1.19)	.001
CVD mortality						
Deaths	1977	2225	2752	3255	4012	
Basic model ^c	1 [Reference]	0.94 (0.88-1.00)	1.02 (0.96-1.09)	1.08 (1.02-1.14)	1.22 (1.15-1.29)	<.001
Adjusted model ^d	1 [Reference]	0.92 (0.87-0.98)	0.99 (0.93-1.05)	1.02 (0.96-1.08)	1.09 (1.03-1.15)	<.001
Mortality from injuries and sudden deaths						
Deaths	190	201	257	273	330	
Basic model ^c	1 [Reference]	0.87 (0.72-1.07)	0.98 (0.81-1.19)	0.93 (0.77-1.13)	1.04 (0.86-1.25)	.24
Adjusted model ^d	1 [Reference]	0.88 (0.72-1.08)	0.99 (0.81-1.20)	0.93 (0.76-1.13)	1.00 (0.83-1.21)	.48
All other deaths						
Deaths	1259	1548	1896	2430	2943	
Basic model ^c	1 [Reference]	1.05 (0.97-1.13)	1.15 (1.07-1.23)	1.31 (1.22-1.41)	1.46 (1.36-1.56)	<.001
Adjusted model ^d	1 [Reference]	1.05 (0.97-1.13)	1.14 (1.06-1.23)	1.28 (1.19-1.38)	1.33 (1.24-1.43)	<.001

Abbreviations: AARP, formerly the American Association of Retired Persons; CVD, cardiovascular disease.

^aData are given as hazard ratio (95% confidence interval) unless otherwise specified.

^bMedian red meat intake based on men and women (g/1000 kcal): Q1, 9.8; Q2, 21.4; Q3, 31.3; Q4, 42.8; and Q5, 62.5.

^cBasic model: age (continuous); race (non-Hispanic white, non-Hispanic black, Hispanic/Asian/Pacific Islander/American Indian/Alaskan native, or unknown); and total energy intake (continuous).

^dAdjusted model: basic model plus education (<8 years or unknown, 8-11 years, 12 years [high school], some college, or college graduate); marital status (married: yes/no); family history of cancer (yes/no) (cancer mortality only); body mass index (18.5 to <25, 25 to <30, 30 to <35, ≥35 [calculated as weight in kilograms divided by height in meters squared]); 31-level smoking history using smoking status (never, former, current), time since quitting for former smokers, and smoking dose; frequency of vigorous physical activity (never/rarely, 1-3 times/mo, 1-2 times/wk, 3-4 times/wk, ≥5 times/wk); alcohol intake (none, 0 to <5, 5 to <15, 15 to <30, ≥30 servings/1000 kcal), vitamin supplement user (≥1 supplement/mo); fruit consumption (0 to <0.7, 0.7 to <1.2, 1.2 to <1.7, 1.7 to <2.5, ≥2.5 servings/1000 kcal); and vegetable consumption (0 to <1.3, 1.3 to <1.8, 1.8 to <2.2, 2.2 to <3.0, ≥3.0 serving/1000 kcal).

^eMedian white meat intake based on men and women (g/1000 kcal): Q1, 9.5; Q2, 18.4; Q3, 27.4; Q4, 39.4; and Q5, 64.6.

^fMedian processed meat intake based on men and women (g/1000 kcal): Q1, 1.6; Q2, 4.4; Q3, 7.4; Q4, 12.2; and Q5, 22.6.

[95% CI, 0.89-1.17]), but in former/current smokers we found higher risks (men: HR, 1.12 [95% CI, 1.05-1.19]; women: HR, 1.11 [95% CI, 1.02-1.21]). Intriguingly, there was increased risk with higher intake of white meat for CVD mortality in never smokers (men: HR, 1.24 [95% CI, 1.10-1.40]; women: HR, 1.20 [95% CI, 1.03-1.41]).

We calculated the population attributable risks, representing the percentage of deaths that could be prevented if individuals adopted red or processed meat intake levels of participants within the first quintile. For overall mortality, 11% of deaths in men and 16% of deaths in women could be prevented if people decreased their red meat consumption to the level of intake in the first quintile. The impact on CVD mortality was an 11% decrease in men and a 21% decrease in women if the red meat consumption was decreased to the amount consumed by individuals in the first quintile. The median red meat consumption based on men and women in the first quintile was 9.8 g/1000 kcal/d compared with 62.5 g/1000 kcal/d in the fifth quintile. For women eating processed meat at the first quintile level, the decrease in CVD mortality was approximately 20%. The median processed meat consumption based on men and women in the first quintile was 1.6 g/1000 kcal/d compared with 22.6 g/1000 kcal/d in the fifth quintile.

COMMENT

We examined total and cause-specific mortality in relation to meat consumption in a large prospective study. We found modest increases in risk for total mortality, as well as cancer and CVD mortality, with higher intakes of red and processed meat in both men and women. In contrast, higher white meat consumption was associated with a small decrease in total and cancer mortality in men and women.

The principal strength of this study is the large size of the cohort, which provided us the ability to investigate the relationship of many deaths (47 976 male deaths and 23 276 female deaths) within the context of a single study with a standardized protocol and a wide range of meat consumption. In contrast, other reports investigating meat intake in relation to mortality have pooled data from different studies conducted in California, the United Kingdom, and Germany because the numbers of events were limited in each study.^{1-6,9-14} The protocols and questionnaires in these studies were different, as were the populations: Seventh-Day Adventists in California and vegetarians and nonvegetarians in Europe. Pooled analyses of specialized populations with distinct healthy lifestyles are subject to unmeasured confounding. Further-

Table 3. Multivariate Analysis Red, White, and Processed Meat Intake and Total and Cause-Specific Mortality in Women in the National Institutes of Health–AARP Diet and Health Study^a

Mortality in Women (n=223 390)	Quintile					<i>P</i> Value for Trend
	Q1	Q2	Q3	Q4	Q5	
	Red Meat Intake ^b					
All mortality						
Deaths	5314	5081	4734	4395	3752	
Basic model ^c	1 [Reference]	1.11 (1.07-1.16)	1.24 (1.20-1.29)	1.43 (1.38-1.49)	1.63 (1.56-1.70)	<.001
Adjusted model ^{d,e}	1 [Reference]	1.08 (1.03-1.12)	1.17 (1.12-1.22)	1.28 (1.23-1.34)	1.36 (1.30-1.43)	<.001
Cancer mortality						
Deaths	2134	1976	1784	1687	1348	
Basic model ^c	1 [Reference]	1.07 (1.01-1.14)	1.15 (1.08-1.23)	1.34 (1.26-1.43)	1.42 (1.33-1.52)	<.001
Adjusted model ^{d,e}	1 [Reference]	1.02 (0.96-1.09)	1.06 (1.00-1.14)	1.20 (1.12-1.28)	1.20 (1.12-1.30)	<.001
CVD mortality						
Deaths	1173	1155	1101	1027	900	
Basic model ^c	1 [Reference]	1.15 (1.06-1.25)	1.32 (1.22-1.44)	1.54 (1.41-1.68)	1.82 (1.66-1.98)	<.001
Adjusted model ^{d,e}	1 [Reference]	1.13 (1.04-1.23)	1.26 (1.16-1.37)	1.39 (1.27-1.52)	1.50 (1.37-1.65)	<.001
Mortality from injuries and sudden deaths						
Deaths	129	97	74	76	61	
Basic model ^c	1 [Reference]	0.86 (0.66-1.12)	0.77 (0.58-1.03)	0.96 (0.72-1.28)	1.01 (0.74-1.37)	.88
Adjusted model ^{d,e}	1 [Reference]	0.85 (0.65-1.12)	0.75 (0.56-1.02)	0.92 (0.68-1.25)	0.94 (0.68-1.31)	.88
All other deaths						
Deaths	1178	1187	1181	1058	961	
Basic model ^c	1 [Reference]	1.18 (1.09-1.28)	1.41 (1.30-1.53)	1.58 (1.45-1.72)	1.91 (1.76-2.09)	<.001
Adjusted model ^{d,e}	1 [Reference]	1.16 (1.07-1.26)	1.35 (1.24-1.47)	1.44 (1.32-1.57)	1.61 (1.46-1.76)	<.001
White Meat Intake^f						
All Mortality						
Deaths	5006	4606	4469	4520	4675	
Basic model ^c	1 [Reference]	0.87 (0.84-0.91)	0.81 (0.78-0.84)	0.78 (0.75-0.81)	0.76 (0.73-0.79)	<.001
Adjusted model ^{d,e}	1 [Reference]	0.96 (0.92-1.00)	0.94 (0.90-0.98)	0.95 (0.91-0.99)	0.92 (0.88-0.96)	<.001
Cancer mortality						
Deaths	1887	1757	1728	1735	1822	
Basic model ^c	1 [Reference]	0.89 (0.83-0.95)	0.84 (0.78-0.90)	0.80 (0.75-0.85)	0.78 (0.73-0.83)	<.001
Adjusted model ^{d,e}	1 [Reference]	0.94 (0.88-1.01)	0.92 (0.86-0.99)	0.92 (0.86-0.98)	0.89 (0.83-0.95)	.001
CVD mortality						
Deaths	1107	1007	1090	1049	1103	
Basic model ^c	1 [Reference]	0.86 (0.79-0.93)	0.89 (0.82-0.97)	0.82 (0.75-0.89)	0.81 (0.75-0.88)	<.001
Adjusted model ^{d,e}	1 [Reference]	0.97 (0.89-1.06)	1.07 (0.98-1.17)	1.05 (0.96-1.14)	1.04 (0.96-1.14)	.19
Mortality from injuries and sudden deaths						
Deaths	89	81	92	86	89	
Basic model ^c	1 [Reference]	0.92 (0.68-1.25)	1.01 (0.75-1.35)	0.89 (0.66-1.20)	0.82 (0.61-1.10)	.17
Adjusted model ^{d,e}	1 [Reference]	0.96 (0.71-1.31)	1.09 (0.81-1.47)	0.99 (0.73-1.34)	0.91 (0.67-1.24)	.52
All other deaths						
Deaths	1319	1155	1016	1055	1020	
Basic model ^c	1 [Reference]	0.82 (0.76-0.89)	0.69 (0.64-0.75)	0.68 (0.63-0.74)	0.63 (0.58-0.68)	<.001
Adjusted model ^{d,e}	1 [Reference]	0.93 (0.86-1.01)	0.84 (0.77-0.91)	0.88 (0.82-0.96)	0.82 (0.75-0.89)	<.001

(continued)

more, recall bias and reverse causality were minimized in our study because diet was assessed prior to the diagnosis of the conditions that led to death.

There is a possibility that some residual confounding by smoking may remain; however, we used a detailed 31-level smoking history variable and repeated the analyses within smoking status strata. Within smoking subgroups, we found consistent results for red, white, and processed meat intakes; however, there were some intriguing differences that could be further investigated. We found a positive association for processed meat intake and cancer mortality among former/current smokers but not among never smokers. This may be because we were still not able to fully statistically adjust for residual confounding of smoking because people who eat processed meat may also smoke. An

additional reason could be that in addition to being exposed to *N*-nitroso compounds from processed meats, smokers inhale carcinogenic chemicals. The possible reason why there was an increased risk with white meat consumption among never smokers is not readily apparent.

Because our cohort was predominantly non-Hispanic white, more educated, consumed less fat and red meat and more fiber and fruits and vegetables, and had fewer current smokers than similarly aged adults in the US population, caution should be applied when attempting to generalize our findings to other populations,⁷ although this caution is somewhat tempered because it is unlikely that the mechanisms relating meat to mortality differ quantitatively between our study population and other white populations older than 50 years.

Table 3. Multivariate Analysis Red, White, and Processed Meat Intake and Total and Cause-Specific Mortality in Women in the National Institutes of Health–AARP Diet and Health Study^a (continued)

Mortality in Women (n=223 390)	Quintile					<i>P</i> Value for Trend
	Q1	Q2	Q3	Q4	Q5	
	Processed Meat Intake ^g					
All mortality						
Deaths	5624	5133	4525	4181	3813	
Basic model ^c	1 [Reference]	1.13 (1.09-1.17)	1.20 (1.15-1.25)	1.35 (1.29-1.40)	1.49 (1.43-1.56)	<.001
Adjusted model ^{d,e}	1 [Reference]	1.07 (1.03-1.12)	1.11 (1.06-1.15)	1.20 (1.15-1.25)	1.25 (1.20-1.31)	<.001
Cancer mortality						
Deaths	2283	2035	1722	1550	1339	
Basic model ^c	1 [Reference]	1.08 (1.02-1.15)	1.10 (1.04-1.18)	1.21 (1.13-1.30)	1.28 (1.19-1.37)	<.001
Adjusted model ^{d,e}	1 [Reference]	1.03 (0.97-1.10)	1.02 (0.96-1.09)	1.10 (1.02-1.17)	1.11 (1.04-1.19)	.001
CVD mortality						
Deaths	1245	1132	1039	973	967	
Basic model ^c	1 [Reference]	1.13 (1.04-1.22)	1.25 (1.14-1.35)	1.41 (1.29-1.54)	1.69 (1.55-1.84)	<.001
Adjusted model ^{d,e}	1 [Reference]	1.08 (0.99-1.17)	1.15 (1.05-1.25)	1.24 (1.13-1.35)	1.38 (1.26-1.51)	<.001
Mortality from injuries and sudden deaths						
Deaths	118	115	71	71	62	
Basic model ^c	1 [Reference]	1.22 (0.94-1.59)	0.91 (0.67-1.23)	1.10 (0.82-1.50)	1.18 (0.86-1.62)	.52
Adjusted model ^{d,e}	1 [Reference]	1.21 (0.93-1.57)	0.89 (0.65-1.21)	1.06 (0.78-1.45)	1.10 (0.80-1.53)	.83
All other deaths						
Deaths	1265	1174	1101	1055	970	
Basic model ^c	1 [Reference]	1.16 (1.07-1.26)	1.32 (1.22-1.44)	1.54 (1.42-1.68)	1.72 (1.58-1.87)	<.001
Adjusted model ^{d,e}	1 [Reference]	1.11 (1.02-1.20)	1.22 (1.12-1.32)	1.35 (1.24-1.47)	1.39 (1.27-1.51)	<.001

Abbreviations: AARP, formerly the American Association of Retired Persons; CVD, cardiovascular disease.

^aData are given as hazard ratio (95% confidence interval) unless otherwise specified.

^bMedian red meat intake based on men and women (g/1000 kcal): Q1, 9.8; Q2, 21.4; Q3, 31.3; Q4, 42.8; and Q5, 62.5.

^cBasic model: age (continuous); race (non-Hispanic white, non-Hispanic black, Hispanic/Asian/Pacific Islander/American Indian/Alaskan native, or unknown); and total energy intake (continuous).

^dAdjusted model: basic model plus education (<8 years or unknown, 8-11 years, 12 years [high school], some college, or college graduate); marital status (married: yes/no); family history of cancer (yes/no) (cancer mortality only); body mass index (18.5 to <25, 25 to <30, 30 to <35, ≥35 [calculated as weight in kilograms divided by height in meters squared]); 31-level smoking history using smoking status (never, former, current), time since quitting for former smokers, and smoking dose; frequency of vigorous physical activity (never/rarely, 1-3 times/mo, 1-2 times/wk, 3-4 times/wk, ≥5 times/wk); alcohol intake (none, 0 to <5, 5 to <15, 15 to <30, ≥30 servings/1000 kcal); vitamin supplement user (≥1 supplement/mo); fruit consumption (0 to <0.7, 0.7 to <1.2, 1.2 to <1.7, 1.7 to <2.5, ≥2.5 servings/1000 kcal); and vegetable consumption (0 to <1.3, 1.3 to <1.8, 1.8 to <2.2, 2.2 to <3.0, ≥3.0 serving/1000 kcal).

^eHormone therapy included in models for women.

^fMedian white meat intake based on men and women (g/1000 kcal): Q1, 9.5; Q2, 18.4; Q3, 27.4; Q4, 39.4; and Q5, 64.6.

^gMedian processed meat intake based on men and women (g/1000 kcal): Q1, 1.6; Q2, 4.4; Q3, 7.4; Q4, 12.2; and Q5, 22.6.

Furthermore, the population-attributable risks in our cohort may be conservative estimates because red and processed meat consumption may be higher in the general population than in our cohort.

The inherent limitations of measurement error in this study are similar to those of any nutritional epidemiologic study that is based on recall of usual intake over a given period. We attempted to reduce measurement error by adjusting our models for reported energy intake.¹⁵ The correlations for red meat consumption assessed from the food frequency questionnaire compared with two 24-hour recall diaries were 0.62 for men and 0.70 for women, as reported previously by Schatzkin et al.⁷ The problem of residual confounding may still exist and could explain the relatively small associations found throughout this study despite the care taken to adjust for known confounders.

Overall, we did not find statistically significant association between meat consumption and deaths from injury and sudden deaths in most instances. The relative HRs of meat consumption with the other causes of death (total, cancer, and CVD mortality) were similar in magnitude in some cases to those of deaths from injury and sudden deaths; however, the number of deaths from in-

jury and sudden deaths was less than the other causes of deaths, and thus the HRs were generally not statistically significant. We observed a higher risk with the category that included "all other deaths"; this is a broad category with many heterogeneous conditions (eg, diabetes mellitus, Alzheimer disease, stomach and duodenal ulcers, chronic liver disease, cirrhosis, nephritis, nephrotic syndrome, and nephrosis), some of which may be positively related to meat intake.

There are various mechanisms by which meat may be related to mortality. In relation to cancer, meat is a source of several multisite carcinogens, including heterocyclic amines and polycyclic aromatic hydrocarbons,¹⁶⁻²¹ which are both formed during high-temperature cooking of meat, as well as N-nitroso compounds.^{22,23} Iron in red meat may increase oxidative damage and increase the formation of N-nitroso compounds.²⁴⁻²⁷ Furthermore, meat is a major source of saturated fat, which has been positively associated with breast²⁸⁻³⁰ and colorectal cancer.³¹

In relation to CVD, elevated blood pressure has been shown to be positively associated with higher intakes of red and processed meat, even though the mechanism is unclear, except that possibly meat may substitute for other beneficial foods such as grains, fruits, or vegetables.³² Mean

plasma total cholesterol, low-density lipoprotein cholesterol, very-low-density lipoprotein cholesterol, and triglyceride levels were found to be decreased in subjects who substituted red meat with fish.^{33,34} Vegetarians have lower arachidonic, eicosapentaenoic, and docosahexaenoic acid levels and higher linoleate and antioxidant levels in platelet phospholipids; such a biochemical profile may be related to decreased atherogenesis and thrombogenesis.³⁴⁻³⁶

Red and processed meat intakes, as well as a high-risk meat diet, were associated with a modest increase in risk of total mortality, cancer, and CVD mortality in both men and women. In contrast, high white meat intake and a low-risk meat diet was associated with a small decrease in total and cancer mortality. These results complement the recommendations by the American Institute for Cancer Research and the World Cancer Research Fund to reduce red and processed meat intake to decrease cancer incidence.³¹ Future research should investigate the relation between subtypes of meat and specific causes of mortality.

Accepted for Publication: October 24, 2008.

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Author Contributions: Drs Sinha and Cross had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All authors have given full approval to the final manuscript. **Study concept and design:** Sinha, Cross, and Graubard. **Acquisition of data:** Sinha and Schatzkin. **Analysis and interpretation of data:** Sinha, Cross, Graubard, Leitzmann, and Schatzkin. **Drafting of the manuscript:** Sinha, Cross, and Graubard. **Critical revision of the manuscript for important intellectual content:** Sinha, Cross, Graubard, Leitzmann, and Schatzkin. **Statistical analysis:** Sinha, Graubard, and Leitzmann. **Obtained funding:** Schatzkin. **Administrative, technical, and material support:** Cross and Schatzkin.

Financial Disclosure: None reported.

Funding/Support: This research was supported in part by the Intramural Research Program of the NIH, National Cancer Institute (NCI).

Additional Contributions: Adam Risch, Leslie Carroll, MA, and Dave Campbell from Information Management Services Inc, and Traci Mouw, MS, from NCI, assisted in data management. We are indebted to the participants in the NIH-AARP Diet and Health Study for their outstanding cooperation. Cancer incidence data from the Atlanta metropolitan area were collected by the Georgia Center for Cancer Statistics, Department of Epidemiology, Rollins School of Public Health, Emory University. Cancer incidence data from California were collected by the California Department of Health Services, Cancer Surveillance Section. Cancer incidence data from the Detroit metropolitan area were collected by the Michigan Cancer Surveillance Program, Community Health Administration. The Florida cancer incidence data used in this report were collected by the Florida Cancer Data System under contract to the Department of Health (DOH)

(the views expressed herein are solely those of the authors and do not necessarily reflect those of the contractor or the DOH). Cancer incidence data from Louisiana were collected by the Louisiana Tumor Registry, Louisiana State University Medical Center in New Orleans. Cancer incidence data from New Jersey were collected by the New Jersey State Cancer Registry, Cancer Epidemiology Services, New Jersey State Department of Health and Senior Services. Cancer incidence data from North Carolina were collected by the North Carolina Central Cancer Registry. Cancer incidence data from Pennsylvania were supplied by the Division of Health Statistics and Research, Pennsylvania Department of Health, Harrisburg (the Pennsylvania Department of Health specifically disclaims responsibility for any analyses, interpretations, or conclusions).

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Correction

Error in Text. In the Original Investigation titled "Oregonians' Reasons for Requesting Physician Aid in Dying" by Ganzini et al, published in the March 9 issue of the *Archives* (2009;169[5]:489-492), an error occurred in the text on page 490. In the first paragraph of the "Results" section, the last sentence should have appeared as follows: "At death, 18 (44%) had received a prescription for medication under the ODDA, and 9 (22%) died by lethal ingestion." Online versions of this article on the *Archives of Internal Medicine* Web site were corrected on March 9, 2009.

Cellular Proliferation, Apoptosis and Angiogenesis: Molecular Targets for Nutritional Preemption of Cancer

Cindy D. Davis, Nancy J. Emenaker, and John A. Milner

Malignant cells are characterized by abnormal signaling pathways involving proliferation, apoptosis, and angiogenesis. These cancer centric pathways are known to be modified by several bioactive dietary components, although admittedly there are inconsistencies in the response. The response is dependent on the amount and duration of exposure to the dietary component and the cell type. While caution should be exercised when extrapolating *in vitro* data to *in vivo* conditions, such studies do provide valuable insights into plausible mechanisms. Significant gene-nutrient and nutrient-nutrient interactions may contribute to the uncertainty of the response to foods and/or their components. One of the challenges is the identification of which process(es), either singly or in combination, is/are most important in leading to a dietary-mediated phenotypic change. The dearth of controlled intervention studies that have investigated molecular targets for nutritional preemption in humans make firm dietary recommendations difficult. Until more definite information surfaces, a balanced but varied diet is most prudent.

Semin Oncol 37:243-257. Published by Elsevier Inc.

Evidence continues to mount that altering dietary habits is an effective and cost-efficient approach both for reducing cancer risk and for modifying the biological behavior of tumors. The World Cancer Research Fund/American Institute of Cancer Research has estimated that cancer is 30% to 40% preventable by appropriate food and nutrition, regular physical activity, and avoidance of obesity.¹ On a global scale, they have estimated that yearly about 3 to 4 million cancer cases might be prevented by adherence to their recommendations.¹

While optimizing the intake of specific foods and/or their bioactive components seems to be a practical, noninvasive, and cost-effective strategy for reducing the cancer burden, this is far from simple.² The complexity of the problem is evident in the literally thousands of dietary compounds consumed each day.^{2,3} For example, it is estimated that humans consume more than 5,000 individual flavonoids, yet only a few have been examined for their cancer protective effects.²

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0270-9295/- see front matter

Published by Elsevier Inc.

doi:10.1053/j.seminoncol.2010.05.001

Furthermore, the lack of quantitative information about some known food constituents limits the ability to decipher which are most important. Unfortunately, since many bioactive food components remain largely uncharacterized, confusion can arise about the true role of a food as a determinant of health and disease prevention. Interactions, both synergistic and antagonistic, between food components may explain why isolated components do not always behave identically to intact foods.⁴ Finally, the composition of the entire diet (pattern) may influence the magnitude of the response to a bioactive food component and ultimately cancer.⁴

Predictive, validated and sensitive biomarkers, including those that (1) evaluate "intake" (exposure) to a specific food or bioactive component; (2) assess one or more specific biological responses (effect); and (3) predict individual "susceptibility" as a function of nutrient-nutrient interactions and genetics, are fundamental to evaluating who will benefit most or be placed at risk from dietary interventions. These biomarkers must be readily accessible, easily and reliably assayed, and predictive of a key process(es) involved in cancer.

In addition to identifying bioactive dietary components, it is critical to define the amount of the specific bioactive component that is needed to achieve concentrations in target tissues that will lead to a phenotypic change. The response to a food is determined not only

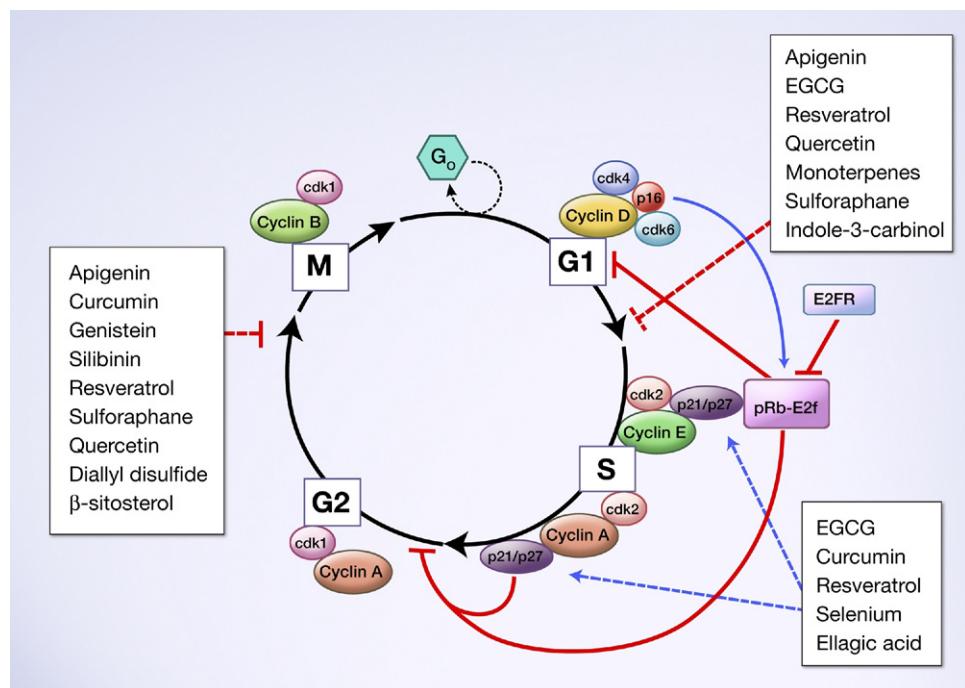


Figure 1. The various phases of the cell cycle (G₁, S, G₂, and M), and the different cyclins and their cyclin-dependent kinases (Cdks) that control progression through the cell cycle. Different dietary components act at different cell cycle checkpoints. Some dietary components have been shown to have multiple molecular targets that can affect different phases of the cell cycle. The specific molecular targets may depend on the specific type of cell being treated and the concentration being used. Arrows indicate activation and blocked lines indicate inhibitory effects.

by the effective concentration of the bioactive food component(s) reaching the target tissue but also the amount of the target requiring modification. Thus, this threshold response to foods and their components can vary substantially from individual to individual. Unfortunately, most of current knowledge about food components arises from cell culture studies and often with nonphysiological concentrations. Thus, extrapolating such data to humans needs is problematic, if not impossible.⁵

All of the major signaling pathways, which are deregulated in cancer, and which have been examined as targets for cancer prevention, can be modified by one or more dietary components. These include, but are not limited to, carcinogen metabolism, DNA repair, cell proliferation, apoptosis, inflammation, immunity, differentiation, and angiogenesis.⁶⁻¹³ Since multiple pathways may be influenced simultaneously, it is a daunting challenge to determine which is most important in determining cancer risk or tumor behavior.¹⁴ Determining the site of action can be further complicated by a response which is cell type-dependent and influenced by a potential myriad of nutrient-nutrient and nutrient-gene interactions.

CELL PROLIFERATION

One of the hallmarks of cancer is aggressive proliferation of cells. In normal cells, proliferation reflects a

finely controlled balance between growth-promoting and growth-inhibiting signals. However, cancer cells often acquire the capability of not only generating their own growth signals, but also becoming insensitive to growth-suppressing signals.¹⁵ Several proteins are recognized to be critical for cell cycle regulation including the cyclins, cyclin-dependent kinases (CDKs), CDK inhibitors (CDKIs), regulatory proteins (retinoblastoma [Rb] and p53), and the E2F transcription factor (Figure 1). The progression of the cell cycle from one phase to the next is regulated by sequential activation and inactivation of “check points” that monitor the cell’s status.¹⁶ These “check points” are mechanisms whereby the cell actively halts progression through the cell cycle until it can ensure that an earlier process, such as DNA replication or mitosis, is complete. In response to DNA damage, checkpoints can also trigger the induction of necessary repair genes or cause the cells to undergo programmed cell death or apoptosis. The DNA damage checkpoint arrests cells in the G₁, S, or G₂ phase depending on the cell cycle status of the cell at the time damage was incurred.¹⁷

Mutations in genes that control the cell cycle are common in human cancer. Rb and p53 are the two main cell cycle control proteins frequently targeted in tumorigenesis. Alterations occurring in each depend on the tumor type¹⁸; virtually all human tumors deregulate either the Rb or p53 pathway, and many times both proteins are influenced simultaneously.¹⁸

Table 1. Examples of Bioactive Dietary Components That Inhibit Cell Proliferation*

Dietary Agent	Molecular Target/Mechanism	Cell Line(s) Concentration	Reference
EGCG (epigallocatechin gallate)	↓ cyclin D, ↓ cyclin E, ↓ CDK1, ↓ CDK2, ↓ CDK4, ↑ pRB, ↑ p53	MDA-MB-231 50–80 µg/mL	19
Genistein	↓ cyclin B1, ↓ Cdc25c, ↓ Cdc2, ↓ cyclin B, ↑ p21	MDA-MB-231 0–20 µmol/L	20
Curcumin	↓ cyclin A, ↓ CDK1, ↑ p21	T-24 bladder cells 0–40 µmol/L	21
Apigenin	↓ cyclin D, ↑ p27	DU-45, PC-3 0–40 µmol/L	22
Diallyl disulfide	↑ cyclin B, ↓ CDK1, ↑ p53	HCT-116 0–400 µmol/L	23
Resveratrol	↑ cyclin A, ↑ cyclin B, ↑ cyclin E, ↑ CDK2	SW-480 30 µmol/L	24
Quercetin	↓ cyclin B, ↓ CDK1, ↑ p21, ↑ pRB	SK-BR3, MDA-MB-453, MD-MBA-231 0–10 µmol/L	25
Sulforaphane	↓ CDK4, ↓ cyclin D, ↑ p21	DU-145 <1 µmol/L	26
Silymarin	↓ cyclin D, ↓ cyclin E, ↓ cyclin A, ↓ cyclin B, ↓ CDK2, ↓ Cdc2	PC-3, LNCap 90 µmol/L	27
Ellagic acid	↓ CDK2, ↑ p53, ↑ p21	T-24 bladder cells 1–50 µmol/L	28
Methylseleninic acid	↓ cyclin A, ↓ CDK2, ↓ CDK1, ↑ p21	PC-3 1–10 µmol/L	29
Sesamin	↓ cyclin D, ↓ cyclin A	MCF-7 0–100 µmol/L	30

*Note that this table just contains select examples.

Many bioactive dietary components can inhibit specific phases of the cell cycle (Figure 1, Table 1). The effects can either be direct or indirect.³¹ For example, epigallocatechin gallate (EGCG) has been found to directly inhibit CDKs in various cancer cells (prostate, lung, and skin),¹⁵ or indirectly by inducing the expression of *p21* and *p27* genes and inhibiting the expression of cyclin D1 and Rb phosphorylation; all this is done in a dose- and time-dependent manner.^{32,33} Tumor cells are generally more sensitive to the growth depression caused by food components than are normal cells. For example, Park et al³⁴ reported that EGCG induced a dose-dependent inhibition of cell growth via a G₀-G₁ phase arrest in human osteosarcoma cells (MG-3 and Saos-2) but did not influence normal rat osteoblasts.

Indole-3-carbinol (I3C) has recently been reported to be a natural elastase enzymatic inhibitor and thus disrupts cyclin E protein processing in some neoplastic cells.³⁵ This inhibition of cyclin E protein processing by I3C enhances the occurrence of 50-kd cyclin E and less of the lower molecular weight cyclin E typically occurring in some tumors. This change leads to a downregulation of CDK2 kinase activity and G₁ cell cycle arrest in human breast cancer cells. Moreover, the authors observed that siRNA ablation of elastase production mim-

icked the cell cycle blockage caused by I3C, demonstrating that the loss of elastase activity is sufficient to cause growth arrest.

Some dietary components may have multiple molecular targets that can affect different phases of the cell cycle. Resveratrol, a polyphenol found at high concentrations in red wine and grapes, inhibits cell cycle progression at different stages depending on the cell examined.^{36,37} Treatment of LNCaP and PC-3 prostate cancer cells with resveratrol (19–150 µmol/L) reduced the expression of cyclin D1, E, and CDK4, as well as reducing cyclin D1/CDK4 kinase activity, and resulted in a G₁/S phase cell cycle arrest.³⁶ It should be noted that animal and human studies often find plasma resveratrol concentrations around 1 to 2 µmol/L, raising issues about the physiological relevance of these findings.³⁸ In contrast, in HL-60 cells, resveratrol arrested cells at the S/G₂ phase via an overexpression of cyclins A and E without modification of p21 expression.³⁷

Sulforaphane is another dietary component that can modulate different phases of the cell cycle. In DU-145 prostate cancer cells, sulforaphane at concentrations less than 1 µmol/L induces a G₀/G₁ block via downregulation of the expression of cyclin D1 and CDK4.³⁹

Sulforaphane at concentrations of 15 $\mu\text{mol/L}$ induces cell cycle arrest at the G₂/M phase by increasing expression of cyclin B1 in human colon and breast cells.⁴⁰ In HT-29 cells, this G₂/M phase arrest was a consequence of maintaining the cdc2 kinase in its active dephosphorylated form, and was associated with phosphorylation/activation of Rb.⁴¹ In contrast, 20 $\mu\text{mol/L}$ sulforaphane treatment of either prostate PC-3 cells or bladder UM-UC-3 cells causes reduced expression of cyclin B1.^{42,43} Sulforaphane treatment of UM-UC-3 cells also results in S phase arrest via reduced expression of cyclin A.⁴³

The cell cycle response caused by many bioactive food components remains largely unexplored. It is likely that for many the response may be similar to what occurs when genistein is added to cultures such that low exposures (0.5–1 $\mu\text{mol/L}$) increase proliferation, whereas higher concentrations (50 $\mu\text{mol/L}$) depress cell growth.⁴⁴ Collectively, several studies demonstrate that the ability of a bioactive dietary component to alter cell proliferation depends on the specific cell examined, as well as the concentration of the test component.

Combinations of dietary components, rather than individual agents, offer an exciting opportunity for maximizing the response. EGCG ($\geq 50 \mu\text{mol/L}$) and γ -tocotrienol ($\geq 25 \mu\text{mol/L}$) have been reported to cause approximately 33% and 58% inhibition of cell proliferation of MCF-7 cells, respectively, when added independently.⁴⁵ However, when each was provided at 10 $\mu\text{mol/L}$ in combination, there was a significant and additive 70% reduction in cell proliferation.⁴⁵ This suppression was associated with downregulation of CDK4, cyclin D1, and E2F. Similarly, 0.5 $\mu\text{mol/L}$ resveratrol, quercetin, and catechin had no effect on MDA-MB-231 cell proliferation when added individually, but when combined at 0.5 $\mu\text{mol/L}$ each, cell proliferation was blocked in vitro.⁴⁶ Furthermore, using *in situ* imaging technologies it was discovered that combining these dietary polyphenols reduced the growth of breast cancer xenografts in a nude mouse model.⁴⁶

Although cell culture models are useful in obtaining mechanistic insights, the observations obtained with the *in vitro* models must be verified *in vivo* at physiologically relevant concentrations. One of the dietary flavonoids that has been examined both *in vitro* and *in vivo* is apigenin. This flavonoid is widely distributed in fruits and vegetables, including onions, parsley, celery, and oranges. *In vitro* studies with prostate cancer cells found that apigenin causes a G₀/G₁ cell cycle arrest via decreases in total Rb protein and its phosphorylation, and decreased cyclin D1, D2, and E expression, as well as their regulatory partners CDK2, 4, and 6.⁴⁷ Similarly, studies in mice have shown that apigenin resulted in a dose-dependent downregulation of the growth of prostate cancer cell xenografts that was associated with decreased phosphorylation of Rb and the protein ex-

pression of cyclins D1, D2, and E, and CDK2, 4, and 6.⁴⁸ These effects were observed at intakes of 20 μg per mouse per day, which is similar to the median intake of apigenin in humans of 40 to 50 mg/d.⁴⁹

Curcumin is another dietary component whose *in vitro* molecular targets are influenced *in vivo*. Bioavailability data suggest that *in vitro* studies with curcumin in the 10 $\mu\text{mol/L}$ range or below are physiologically relevant to humans.³⁸ Treatment of either LNCaP or PC-3 prostate cancer cells with 10 $\mu\text{mol/L}$ curcumin inhibited cyclin D1 and cyclin E and induced the CDK inhibitors p16, p21, and p27.⁵⁰ In rats, gavage administration of curcumin (200 or 600 mg/kg) inhibited diethylnitrosamine-induced hepatic hyperplasia, possibly by increasing p21 expression and decreasing the expression of cyclin E and cdc2.⁵¹ Unfortunately, few bioactive food components have been systematically examined in both controlled and physiologically relevant *in vitro* and *in vivo* studies.

Combinations of dietary components may also be more efficacious *in vivo*. For example, in an *in vitro* model of oral cancer, EGCG blocked cells in the G₀/G₁ phase, while curcumin blocked in the G₂/M phase of the cell cycle. The combination appeared to be better than either provided alone.⁵² Similarly, while tea or curcumin individually decreased the number and volume of dimethylbenzanthracene-induced oral tumors in hamsters, only the combination decreased the proliferation index of squamous cell carcinoma.⁵³ While these combination studies are interesting, the dearth of studies that have examined blends of bioactive food components is troubling and clearly an area that deserves additional investigations.

Human intervention studies have documented that some dietary components, such as calcium or low-fat dairy foods^{54,55} or dietary fiber,⁵⁶ can suppress colonic or rectal epithelial cell proliferation *in vivo*. Unfortunately, the molecular targets by which these dietary components influence proliferation remain to be determined. A biomarker that is often used to measure cell proliferation in humans is Ki-67. Recent studies have demonstrated that neither the combination of 2 g/d calcium and/or 800 IU vitamin D affected Ki-67 expression in normal colon mucosa⁵⁷; neither prebiotics (resistant starch), probiotics (*Bifidobacterium lactis*), or synbiotics (their combination) altered Ki-67 expression in rectal samples⁵⁸; and the combination of a low-fat, high-fruit and -vegetable diet with weight loss (−314 kcal, −12.2% energy, +1.8 servings/d fruits and vegetables, and −4.0 kg weight) did not change Ki-67 expression in Barrett's esophagus.⁵⁹ The paucity of data in humans that critically evaluate the effects of dietary components on cell proliferation likely relates to the difficulty in obtaining tissues noninvasively. Additional studies are needed to identify surrogate tissues that

may be predictive of the response observed in target tissues.

APOPTOSIS

Apoptosis, interchangeably referred to as programmed cell death, is a key pathway for regulating homeostasis. In Greek apoptosis literally means “falling away.” It is a natural, organized process that accounts for approximately 3 billion cell deaths in the human body every minute. It helps maintain a natural balance between cell death and cell renewal by destroying excess, damaged, or abnormal cells. Apoptosis is one of the most potent defenses against cancer since this process eliminates potentially deleterious, mutated cells. Triggers for apoptosis induction include DNA damage, disruption of the cell cycle, hypoxia, detachment of cells from their surrounding tissue, and loss of trophic signaling.⁶⁰ It is characterized by cell shrinkage,

chromatin condensation, and fragmentation of the cell into compact membrane-enclosed structures, called “apoptotic bodies” that are engulfed by macrophages and removed from the tissue in a controlled manner.⁶¹ These morphological changes are a result of characteristic molecular and biochemical events occurring in the cell, most notably the activation of proteolytic enzymes. Proteolytic cleavage of proenzymes is an important step leading to caspase activation, which in turn is amplified by the cleavage and activation of other downstream caspases in the apoptosis cascade. Caspases are a family of cysteinyl aspartate-specific proteases involved in apoptosis and subdivided into initiation (8, 9, and 10) and executioner (3, 6, and 7) caspases. There are two main pathways of apoptosis: the extrinsic pathway (death receptor pathway) and the intrinsic pathway (mitochondrial pathway), which are activated by caspase-8 and caspase-9, respectively (Figure 2). A critical common element to both pathways is the

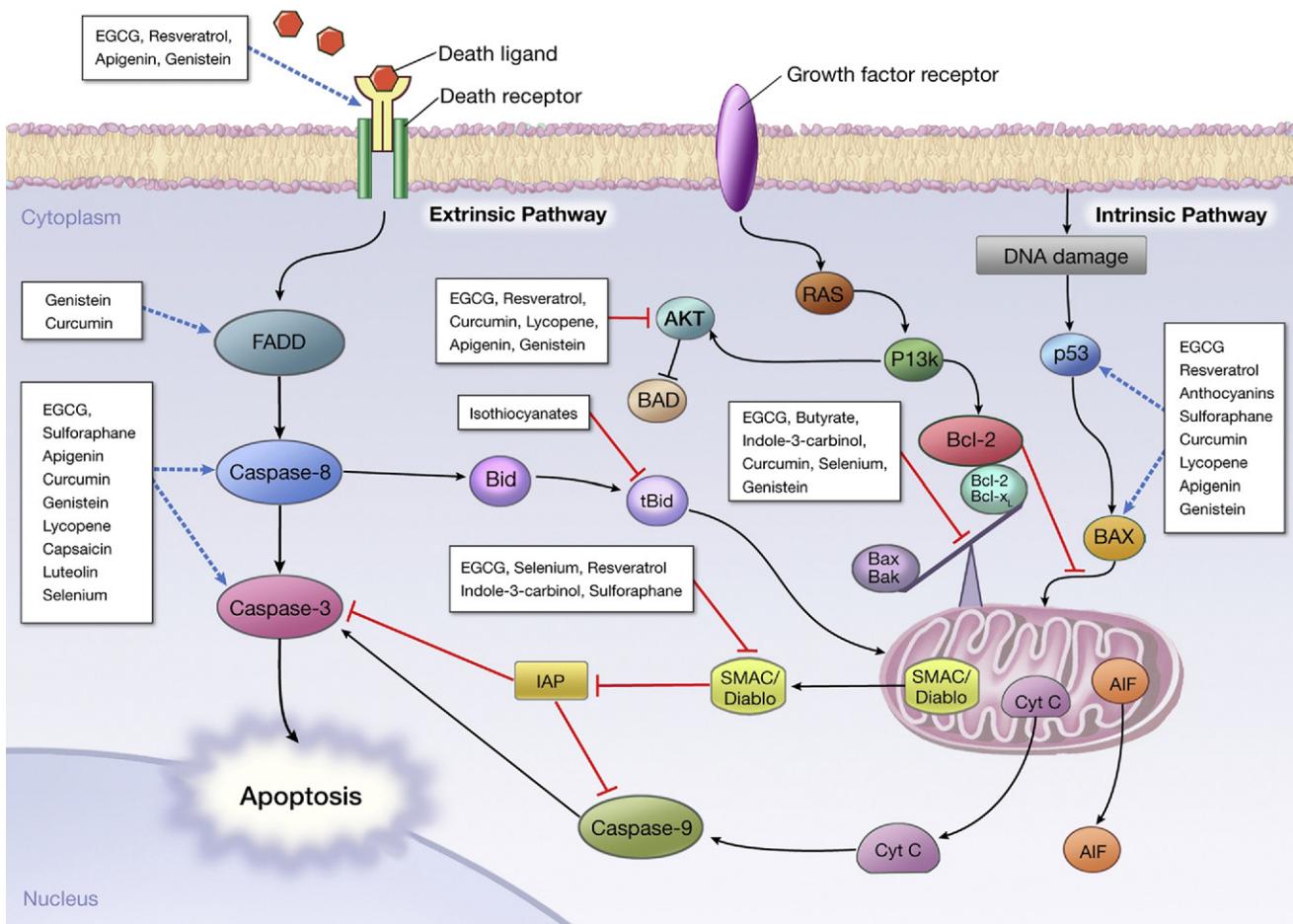


Figure 2. The two main pathways of apoptosis. The extrinsic pathway is activated at the cell surface when a specific ligand binds to its corresponding cell-surface death receptor and activates caspase-8. The intrinsic or mitochondrial pathway occurs when there is release of mitochondrial proteins leading to the activation of caspase-9. Both pathways converge in the activation of caspase-3. Different dietary components can affect different molecular targets associated with apoptosis. Some dietary components can act on multiple molecular targets depending on the specific type of cell being treated and the concentration being used. Arrows indicate activation and blocked lines indicate inhibitory effects.

Table 2. Examples of Bioactive Dietary Components That Induce the Extrinsic/Death Receptor Pathway of Apoptosis*

Dietary Agent	Molecular Target/Mechanism	Cell Line(s)/Concentration	Reference
Lupeol	Increased Fas receptor and FADD protein expression	LNCaP, CRW22Rv1 1–30 $\mu\text{mol/L}$	62
Sulforaphane	Increased Fas ligand and cleavage of caspase-8 and caspase-3	MDA-MB-231 15–25 $\mu\text{mol/L}$	63
Curcumin	Increased TRAIL-induced apoptosis via inhibition of NF- κ B	LNCaP, PC-3 10–30 $\mu\text{mol/L}$	64
Diindolylmethane	Increased TRAIL-induced apoptosis via downregulation of c-FLIP; increased caspase-8 cleavage	HepG2, HT-29 20 $\mu\text{mol/L}$	65
EGC or EGCG	Caspase-8 activation and proteolytic cleavage of Bid	SH-SY5Y 50 $\mu\text{mol/L}$	66
Genistein	Decreased expression of TNF ligand and receptor family	SPC-A-A 20 $\mu\text{mol/L}$	67

*Note that this table just contains selected examples. Abbreviations: FADD, Fas (TNFRSF6)-associated death domain; TRAIL, tumor necrosis factor-inducing ligand; EGCC, epigallocatechin-3 gallate; TNF, tumor necrosis factor.

involvement of caspase-3, which results in cleavage and inactivation of key cellular proteins including the DNA repair enzyme poly(ADP-ribose) polymerase (PARP). In addition, mitogenic and stress response pathways are involved in the regulation of apoptotic signaling.

Compelling evidence exists that bioactive dietary components can trigger apoptosis through numerous intracellular molecular targets in both apoptotic pathways *in vitro* (Figure 2, Tables 2 and 3). Distinct from the apoptotic events in the normal physiological process, which are mediated mainly by the interaction between death receptors and their relevant ligands,⁷⁹ many bioactive dietary components appear to induce apoptosis through the mitochondria mediated pathway by activating *p53* and its target genes. For example, EGCG induces the expression of *p53* and its target *BAX* in prostate cancer cells with wild-type *p53* but not with inactive *p53*.⁸⁰ Similarly, resveratrol induces apoptosis only in cells expressing wild-type *p53* but not in *p53*-deficient cells.⁸¹ Many dietary compounds can induce oxidative stress, which downregulates anti-apoptotic molecules such as Bcl-2 or Bcl-x and upregulates pro-apoptotic molecules such as Bax or Bak.⁸²

Many dietary agents appear to exhibit some degree of specificity for neoplastic cells, while sparing normal cells. For example, 25 to 50 $\mu\text{mol/L}$ quercetin significantly inhibited the growth of highly aggressive PC-3 and moderately aggressive DU-145 prostate cancer cell lines but did not affect less aggressive LNCaP cells or normal fibroblasts.⁸³

The concentration tested in cell culture studies determines whether the agent is functioning nutritionally

or pharmacologically. For example, the concentrations of EGCG needed to significantly downregulate anti-apoptotic proteins and induce programmed cell death *in vitro* (20 $\mu\text{mol/L}$) are considerably greater than the physiological concentrations that could be obtained by typical tea consumption.⁸⁴ Consumption of 6 to 7 cups of green tea per day (\sim 30 mg/kg/d EGCG) would generate a plasma EGCG concentration of about 1 $\mu\text{mol/L}$.⁸⁴ However, to achieve higher plasma concentrations, EGCG supplements are needed.⁸⁵ In contrast, in humans, consumption of onions and applesauce with peel resulted in peak plasma concentrations of quercetin of 225 ± 43 and 331 ± 7 $\mu\text{mol/L}$, respectively,⁸⁶ which are significantly greater than concentrations needed to induce apoptosis *in vitro* (30–100 $\mu\text{mol/L}$).⁸⁷

Another important consideration is that combining dietary components may promote additive and synergistic effects on apoptosis induction. For example, resveratrol and quercetin combinations additively activated caspase-3 and cytochrome c release in a human pancreatic cell line.⁸⁸ Also, quercetin and ellagic acid (at 5 and 10 $\mu\text{mol/L}$ each), work synergistically to induce apoptosis in human leukemia MOLT-4 cells.⁸⁹

Selenium and vitamin E are yet another example of synergistic benefits of combining food components on induction of apoptosis in human prostate cancer cells.⁹⁰ This synergy was accounted for primarily by selenium and vitamin E modifying distinct signaling pathways of caspase activation. Selenium activated caspases-1 and -12, whereas vitamin E activated caspase-9. Thus selenium and vitamin E in combination may activate multiple molecular targets for apoptosis

Table 3. Examples of Bioactive Dietary Components That Induce the Intrinsic/Mitochondrial Pathway of Apoptosis*

Dietary Agent	Molecular Target/Mechanism	Cell Line(s)/Concentration	Reference
Curcumin	Mitochondrial swelling and collapse of the mitochondrial membrane potential	HepG2 0–40 µg/mL	68
Sulforaphane	Collapse of mitochondrial membrane potential, activation of caspase-3, downregulation of Bcl-2	U937 0–4 µmol/L	69
Beta-carotene	Loss of mitochondrial membrane potential, increased cytochrome c release, activation of caspase-9	HL-60, HT-29, SK-MEL-2 0–20 µmol/L	70
Genistein	Decreased Bcl-2 and increased Bax mRNA and protein expression	SG7901 cells injected into nude mice 0.5–1.5 mg/kg diet	71
EGCG	Decreased Bcl-2 and PARP cleavage and increased Bax protein expression	MDA-MB-231 50 µg/mL	72
Apigenin	Increased cytochrome c release and activation of caspase-3 and -9; decreased Bcl-2 and increased Bax protein	22Rv1 10–80 µmol/L	73
Luteolin	Activation of caspases-3 and -9 and PARP cleavage	SCC-4 0–100 µmol/L	74
Diallyl disulfide	Decreased mitochondrial membrane potential, cytochrome c and Smac into the cytosol, decreased Bcl-2 and some BIRC proteins, activation of caspases-9 and -3	T98G and U87MG 100 µmol/L	75
Selenium	Decreased mitochondrial membrane potential, release of cytochrome c into the cytosol, activation of caspases-9 and -3	LNCaP 2.5 µmol/L	76
Resveratrol	Cleavage of immature caspase-3 into active fragments (p12, p17 and p20), increased caspase-3 activity and PARP cleavage	MD-MB-231 50 µmol/L	77
Luteolin	Increased p63 and PIG3. Activation of caspase-9 and caspase-3	KYSE-510 10–80 µmol/L	78

*Note that this table just contains selected examples.

induction, the endoplasmic reticulum stress/cytokine signaling pathway and mitochondrial pathway, respectively. By targeting the entire battery of initiator caspases, selenium and vitamin E in combination may act in a cooperative fashion to “switch on the full force of the apoptotic machinery.”⁹⁰ These studies suggest that combinations of bioactive food components with different sites of action can potentially induce synergistic apoptotic effects.⁹¹

Studies in human subjects have documented that dietary components can induce apoptosis. Thirty-two patients diagnosed by biopsy with prostate cancer were given tomato sauce pasta entrées (30 mg lycopene per day) for 3 weeks before prostatectomy.⁹² Tomato sauce consumption resulted in a significant increase in the percentage of apoptotic cells in benign prostatic hyperplasia (from 0.66 ± 0.13% to 1.38 ±

0.31%) and in carcinomas (0.84 ± 0.13% to 2.76 ± 0.58%). This increase was associated with decreased Bax expression in the carcinomas.⁹² Similarly, 18 men with prostate cancer who consumed 160 mg/d of red clover-derived isoflavones, containing a mixture of genistein, daidzein, formononetin, and biochanin A, prior to prostatectomy had a significantly higher percentage of apoptotic cells (1.14%) compared to un-supplemented controls (0.24%).⁹³ Dietary components also have been shown to modify apoptosis in the rectal epithelium of human subjects. Patients with adenomas in the highest versus lowest tertile of calcium intake (<420 v >739 mg/d) had 3.4 times higher odds of elevated apoptotic scores (mean number of apoptotic cells per crypt).⁹⁴ While these data are intriguing, additional human studies are needed to determine whether other dietary components, at physiologically

or pharmacologically relevant concentrations, modulate specific apoptotic molecular targets.

ANGIOGENESIS

Angiogenesis is a normal physiological process where new blood vessels grow from pre-existing ones. While angiogenesis occurs during growth and wound healing, it can also indicate that a tumor has possibly changed from a benign to a malignant state. While enhanced angiogenesis promotes tumor growth by increasing oxygen and nutrient delivery, accumulating evidence suggests that this process occurs early in cancer development and thus occurs as a result of signals arising from transformed cells. Angiogenesis in malignant tumors is a complex process that involves the tight interplay of cancerous cells and their micro-environment including surrounding endothelial cells, phagocytes and their pro- and inhibitory secreted factors. Premalignant and malignant cells both exhibit increasing levels of cellular and molecular angiogenic dysregulation, advancing their malignant phenotypes. This contributes to their ability to produce pro-angiogenic molecules, as well as to the ability of the surrounding stroma to initiate pathologic angiogenesis by paracrine microenvironmental influences acting upon endothelial cells. Endothelial cells are stimulated and are attracted to the site where the new blood supply is needed by various chemoattractants including growth

factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and insulin-like growth factor-1 (IGF-1), and by inflammatory molecules including interleukin (IL)-8, COX-2 and inducible nitric oxide synthase (iNOS).^{95,96} Chemotactic migration is potentiated by the degradation of extracellular matrix components.⁹⁷ This is accomplished via matrix-metalloproteinases (MMPs).^{98,99} MMPs include collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, and MMP-7) and elastase (MMP-12).¹⁰⁰

The expression of MMPs is predominately regulated by the AP-1 transcription complex, which can be activated by several mechanisms involving growth factors, cell-cell interactions, and interactions among cells and matrix.¹⁰¹ In addition, there are natural MMP inhibitors (tissue inhibitors of metalloproteinases, TIMPs), that are also involved in regulating the activation and activity of these enzymes. MMPs are involved in many physiological processes involving matrix remodeling, and appear to be critical in angiogenesis, tumor cell invasion, and metastasis. In addition to removing physical barriers to migration through degradation of the extracellular matrix, MMPs can modulate cell adhesion, and generate extracellular matrix degradation products that are chemotactic for endothelial cells.

Inhibition of angiogenesis serves two functions: (1) it limits tumor size by restricting oxygen and nutrients,

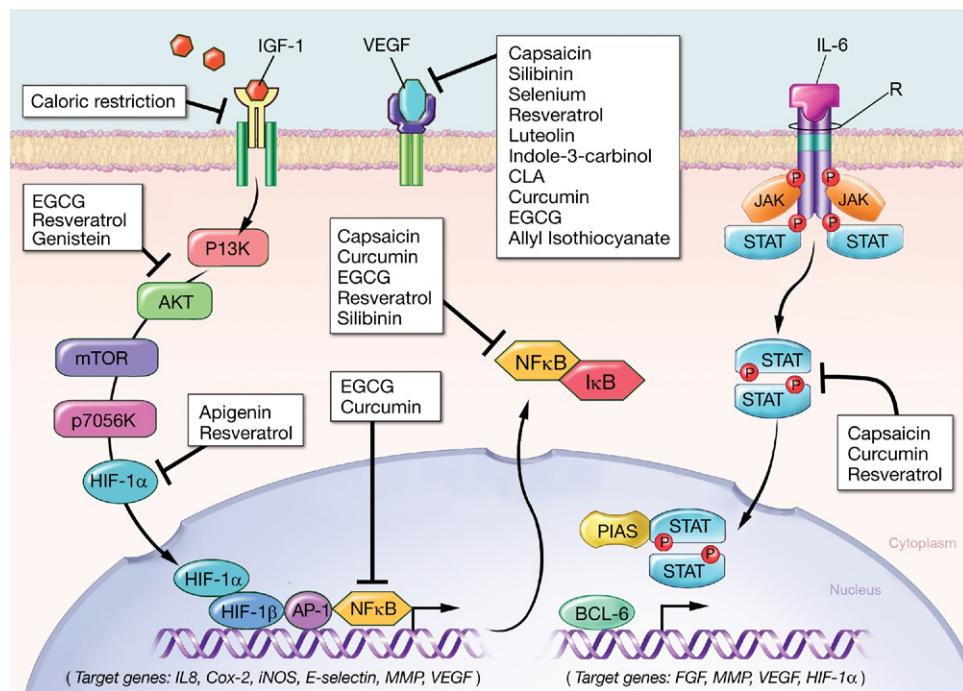


Figure 3. Angiogenesis. Endothelial cells are stimulated and are attracted to the site where a new blood supply is needed by various growth factors. Different dietary components can affect different molecular targets associated with angiogenesis. Some dietary components can act on multiple molecular targets depending on the specific type of cell being treated and the concentration being used. Arrows indicate activation and blocked lines indicate inhibitory effects.

Table 4. Examples of Bioactive Dietary Components That Inhibit Angiogenesis^{*102-104}

Dietary Agent	Effect on Angiogenesis
Allyl Isothiocyanate	Downregulates VEGF and proinflammatory cytokines (IL-1 β , IL-6, and TNF α), upregulates IL-2 and TIMP
Apigenin	Inhibits HIF-1 α and VEGF expression in human ovarian cancer cells Inhibits in vitro angiogenesis.
Berry extracts	Inhibition of VEGF expression in human keratinocytes
Capsaicin	Inhibits VEGF-induced chemotactic motility and capillary-like tube formation of primary endothelial cells
Conjugated linoleic acid	Inhibits angiogenesis in vitro and in vivo by suppression of formation of microcapillary networks Suppression of both serum and mammary gland VEGF concentrations in breast cancer model
Curcumin	Downregulates transcript levels of VEGF and bFGF and suppresses VEGF, MMP-2 and MMP-9 expression, NF- κ B, COX-2, and MAPKs activity Inhibits microvessels density in tumor xenografts
EGCG	Inhibits growth and survival of endothelial cells Suppresses tumor vasculature in tumor xenograft models Suppresses ERK1/2 activity and inhibits VEGF expression and secretion in cancer cells Suppresses MMP-2/9 expression and activation in the TRAMP model, along with inhibition of COX-2, iNOS and NF- κ B in other tumor models
Genistein	Downregulates MMP-9 and upregulates TIMP-1 Suppresses endothelial cell proliferation, migration, and invasion Inhibits VEGF and COX-2 expression and suppresses VEGF-induced tyrosine phosphorylation of receptor kinases
Grape seed procyanidins	Inhibits growth, survival, migration and matrigel invasion of HUVEC Decreases VEGF expression and microvessel density in tumor xenograft model
Indole-3-carbinol	Decreases VEGF and Flk-1 expression in endothelial cells. Inhibits growth of HUVEC and inhibits in vitro angiogenesis in matrigel Decreases iNOS expression
Inositol hexaphosphate	Decreases VEGF expression and microvessel density in tumor xenograft model Inhibits growth, survival, migration and matrigel invasion of HUVEC
Luteolin	Inhibition of VEGF-induced proliferation of HUVEC
Phenylethyl isothiocyanate	Inhibits VEGF secretion and lowers VEGF-R expression Lowers survival rate of HUVEC cells, inhibits capillary-like tube formation and migration of HUVEC cells
Quercetin	Inhibits MMP-2 and MMP-9 secretion from tumor cells and suppresses endothelial cell proliferation, migration and tube formation
Resveratrol	Inhibits capillary-like tube formation by HUVEC and capillary differentiation and VEGF binding to HUVEC Decreases iNOS and VEGF expression
Retinoic acid	Inhibits responsiveness of endothelial cells to angiogenic growth factors
Selenium	Suppresses VEGF expression, lowers microvessel density and inhibits gelolytic activity of MMP-2 in rat mammary carcinoma Initiates apoptotic death in HUVEC cells
Silibinin	Inhibits growth and survival of endothelial cells via disrupting VEGF and IGF-1 signaling Inhibits MMP-2 expression and tube formation in HUVEC Inhibits VEGF secretion from human cancer cells Decreases iNOS and VEGF expression and microvessel density in mouse lung tumors

*Additional information can be obtained from recent reviews on dietary modification of angiogenesis.¹⁰²⁻¹⁰⁴

and (2) it decreases the opportunities for metastatic cells to enter the circulatory system. Several dietary components have surfaced as inhibitors of angiogenesis in various animal and cell culture models (Figure 3, Table 4).¹⁰²⁻¹⁰⁴ Specifically, they appear to possess a

wide range of angiopreventive properties by modifying pro-angiogenic stimuli, including inflammation,¹⁰⁵⁻¹¹⁶ cytokine and growth factor production,^{111,117-122} endothelial cell function,¹²³⁻¹²⁶ and/or intracellular and extracellular communications.¹²⁷⁻¹³¹

During inflammation, nuclear factor (NF)- κ B and STAT3 stimulate production of pro-angiogenic cytokines and growth factors by infiltrating host immune cells. These growth factors and cytokines (eg, IL-6, IL-8, and VEGF) can then trigger vascular endothelial cell proliferation and migration. Some bioactive food components appear to reduce inflammatory-induced angiogenesis by one or more mechanisms, including inhibition of pro-inflammatory NF- κ B and STAT3 signaling pathways. Preclinical evidence suggests capsaicin,^{108,109} resveratrol,¹¹⁰ and curcumin^{111,112} target both NF- κ B and STAT3 signaling pathways for blockade, while other evidence suggests some bioactive food components may inhibit either the NF- κ B or STAT3 pathway but not both.¹¹¹ Curcumin treatment reduced IL-6, IL-8, IL-1 β , and tumor necrosis factor (TNF) α mRNA expression levels by upregulating mitogen-activated protein kinase phosphatase-5 activities in primary human prostatic epithelial cells and prostate cancer cell lines.¹¹⁸ Clinical studies have shown reduced STAT3 phosphorylation in peripheral blood mononuclear cells from pancreatic cancer patients receiving 8 g/d of oral curcumin.¹¹²

Bioactive food components (eg, flaxseed lignans, black raspberries, and capsaicin) may reduce growth factor expression levels such as VEGF. Enterodiol and enterolactone, two major mammalian lignan metabolites from flaxseed, have been reported to reduce extracellular VEGF expression by approximately 50% and to significantly reduce microvesseal density in an MCF-7 xenograph mouse model of premenopausal breast cancer.¹¹⁹ Feeding a 5% freeze-dried black raspberry powder (BRB) reduced carcinogen-induced VEGF-C mRNA expression from the 2.4-fold increase observed in control animals treated with nitrosomethylbenzylamine (NMBA) alone down to a 1.1-fold increase in animals treated with NMBA plus BRB. This decrease was associated with suppression of esophageal endothelial microvessel density (22.6 ± 2.6 vessels/cm in NMBA plus BRB compared to 53.7 ± 5.6 vessels/cm in NMBA-treated control animals).¹²⁰ Suppression of in vivo VEGF secretion by bioactive food components may relate to their ability to inhibit H₂O₂-induced VEGF expression.¹²²

The proliferation and migration of vascular endothelium is fundamental to angiogenesis and is modified by several growth factors and cytokines that trigger these pro-angiogenic processes. Preclinical evidence suggests that some bioactive food components significantly inhibit endothelial cell proliferation and migration in both in vitro and in vivo models of angiogenesis. Capsaicin inhibited VEGF-induced cell proliferation of human umbilical vein endothelial cells (HUVEC) by downregulating cyclin D1 expression and DNA synthesis in a dose-dependent manner.¹²³ Capsaicin also exerted a dose-dependent inhibitory effect on VEGF-induced endothelial cell migration, capillary tube

formation, and VEGF-induced phosphorylation of AKT and endothelial NOS (eNOS).¹²³ This is intriguing since the AKT/nitric oxide pathway is necessary for the VEGF-induced vessel generation that results from endothelial cell migration, proliferation and tube formation.

Dietary components also may play an important role as modifiers of intracellular and extracellular communication. Hypoxia inducible factor (HIF)-1 α is a transcription factor that responds to changes in available oxygen to promote the formation of new blood vessels. Resveratrol inhibits HIF-1 α and VEGF cellular protein expression levels by downregulating AKT and MAPK pathways and induces HIF-1 α proteosomal degradation through the 26S proteasome system in A2780/CP70 and OVCAR-3 human ovarian cancer cells.¹²⁹ Similar effects with resveratrol and EGCG have been observed in human tongue squamous cell carcinomas (SCC-9) and hepatoma (HepG2) cells.^{130,131}

While supplementation with specific bioactive components has been shown to inhibit angiogenesis, experimental findings provide strong evidence that energy restriction can suppress tumor angiogenesis in prostate tumor models as well as in an orthotopic mouse brain tumor model involving intracerebral implantation of a syngeneic malignant mouse astrocytoma.^{132,133} A recent study in rats implanted with prostate cancer cells demonstrated that a 40% caloric restriction reduced serum concentrations of IGF-1 by 35%, VEGF mRNA by 30%, and secreted VEGF protein by 33% compared to control animals.¹³⁴ Moreover, in vitro studies with the prostate cancer cells demonstrated dose- and time-dependent stimulation of VEGF expression by IGF-1. These results suggest that dietary restriction reduces endocrine and prostate tumor autocrine/paracrine IGF-1 expression, which contribute to reduced VEGF expression and signaling, to inhibit tumor angiogenesis associated with prostate tumorigenesis.¹³⁴

While many dietary components have the ability to inhibit tumor angiogenesis, this process is fundamental also to healing of wounds, acute injury, and chronic damages of the gastrointestinal mucosa. Thus, potential deleterious effects might arise from strategies aimed at suppressing cancer-related angiogenesis. Undeniably, a better understanding of the differential effects of dietary components on tumor versus physiological angiogenesis is critical.

SUMMARY

Dietary behavior is one of the most important and modifiable determinants of cancer risk and tumor behavior. Overwhelming evidence documents that a variety of dietary components can markedly influence a number of key intracellular targets that are associated with cancer. Unraveling the role of bioactive food components is complicated by the multiple steps in the

cancer process which can be modified simultaneously, including cell proliferation, apoptosis, and angiogenesis, the number of bioactive food components and the importance of dosage and timing in determining the magnitude of the response. Undeniably, greater attention needs to be given to the dose and duration of exposures needed to bring about a biologically relevant response without creating ill consequences. Intriguing evidence with combinations of food components suggest the benefits may be far greater than when provided individually.

It is clear that not all individuals respond equally to the intake of foods or their specific bioactive food components.¹³⁵ Unquestionably, bioactive food components can help maintain normal cell function as well as serve as a deterrent to the behavior of abnormal (cancerous) cells. Possibly both are critical for maximum cancer prevention. Regardless, a better understanding of genetic factors, including those associated with nutrigenetic, epigenetic, transcriptomic, proteomic and metabolomic activities, is essential to determine who will benefit most or might be placed at risk due to dietary intervention strategies. Greater knowledge about the molecular targets for bioactive food components that builds on “omics” technologies will help us to understand why some individuals appear to be more responsive to dietary change than others.

Several bioactive food components serve as regulators of gene expression and/or modulate gene products. At least some evidence suggests the response may relate to the ability of these compounds to serve as prooxidants rather than antioxidants.^{136,137} Transcriptomic profiles following a bolus challenge of a food component may provide important clues about the merits of dietary change and plausible mechanisms that underlie the beneficial or adverse effects of dietary components. Periodic transcriptomic or metabolomic profiles should also assist with the identification of genes and related events markers that are altered by food components in the pre-and post-disease states.

Unraveling the multitude of interactions among the more than 25,000 bioactive food components with key cancer-related events makes for a daunting challenge. Nevertheless, deciphering the role of dietary habits is fundamental to optimizing health and ultimately disease prevention. Access to this information will help resolve the inconsistencies within the literature and provide clues to strategies to assist individuals in preventing cancer.

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Decreased Growth of Human Prostate LNCaP Tumors in SCID Mice Fed a Low-Fat, Soy Protein Diet With Isoflavones

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Abstract: Epidemiological studies suggest that high intake of dietary fat is a risk factor for the development of clinical prostate cancer. Soy protein has also been proposed to play a role in the prevention of prostate cancer, and one of the isoflavones in soy protein, genistein, inhibits the growth of human prostate cancer cell lines in vitro. This study was designed to evaluate whether altering dietary fat, soy protein, and isoflavone content affects the growth rate of a human androgen-sensitive prostate cancer cell line (LNCaP) grown in severe-combined immunodeficient (SCID) mice. SCID mice were randomized into four dietary groups: high-fat (42.0 kcal%) + casein, high-fat (42.0 kcal%) + soy protein + isoflavone extract, low-fat (12.0 kcal%) + casein, and low-fat (12.0 kcal%) + soy protein + isoflavone extract. After two weeks on these diets, the mice were injected subcutaneously with 1×10^5 LNCaP tumor cells and placed in separate cages (1 mouse/cage) to strictly control caloric intake. Isocaloric diets were given 3 days/wk, and tumor sizes were measured once per week. The tumor growth rates were slightly reduced in the group that received the low-fat + soy protein + isoflavone extract diet compared with the other groups combined ($p < 0.05$). In addition, the final tumor weights were reduced by 15% in the group that received the low-fat + soy protein + isoflavone extract diet compared with the other groups combined ($p < 0.05$). In this xenograft model for prostate cancer, there were statistically significant effects on tumor growth rate and final tumor weight attributable to a low-fat + soy protein + isoflavone extract diet.

Introduction

In the United States, prostate cancer is the most commonly diagnosed cancer in men as well as the second leading cause of male cancer-related deaths (1). It is estimated that in 1999 there will be 179,300 new prostate cancer cases and 37,000 men will die from the disease (1). Although latent or clinically insignificant prostate cancer occurs in a

large proportion and at equal rates in autopsy studies among men of Asian countries and the United States (approx 30% of men >50 yr old), the incidence of clinically significant prostate cancer is 15-fold higher in the United States (1–4). Furthermore, Chinese and Japanese men who immigrate to this country have an increased incidence and mortality from prostate cancer compared with Chinese and Japanese men in their native country (5–8). These epidemiological studies suggest that environmental factors associated with Western culture may promote the development of clinical prostate cancer. Two such dietary factors that have been implicated are dietary fat and soy protein. A number of epidemiological studies have shown a positive association between intake of dietary fat and the incidence and mortality from prostate cancer (8–12). Furthermore, a recent study by Wang and co-workers (13) showed that androgen receptor-positive human prostate cancer cells (LNCaP) had greater tumor weights and growth rates in nude mice fed a diet high in polyunsaturated fat (40.5 kcal%) than in mice fed diets lower in fat content (21.2, 11.6, and 2.3 kcal%) (13).

One of the major differences between Asian and American diets is the consumption of soy-based foodstuffs. Coward and colleagues (14) reported the average consumption of soy protein in Taiwan to be 35 g/day per capita (14). The beneficial effects of soy have been attributed to the isoflavones found in highest content in soy, e.g., genistein and daidzein (15,16). Genistein and daidzein, and their β -glucoside conjugates, are present in soybeans in concentrations of up to 3 mg/g, and the average isoflavone intake in the Asian culture is estimated to be 39 mg/day (17,18). The average American eats, at most, a few milligrams of isoflavones per day (15). The decrease in prostate cancer mortality in Japanese men compared with those in the United States may be attributed, in part, to the high soy protein content in the Japanese diet (16). Whereas a number of investigators have shown that genistein inhibits the growth of human prostate cancer cell lines *in vitro*, no experiments

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have evaluated the effect of soy protein and isoflavones on the growth of human prostate cancer *in vivo*. This study was specifically designed to evaluate whether altering dietary fat, soy protein, and isoflavone content affects the implantation rate and growth rate of a human androgen-sensitive prostate cancer cell line (LNCaP) grown in severe-combined immunodeficient (SCID) mice.

Methods

Cells and Reagents

The androgen receptor-positive LNCaP cell line was obtained from the American Type Culture Collection (Rockville, MD). The LNCaP cells were grown in RPMI 1640 medium supplemented with L-glutamine, 10% fetal bovine serum (Omega Scientific, Tarzana, CA), and penicillin-streptomycin in an atmosphere of 5% CO₂ and air at 37°C.

Animals

Sixty-six male CB17 beige SCID mice (8 wk old) were obtained from the Department of Laboratory Animal Medicine Facility at the University of California, Los Angeles, which is accredited by the American Association for Accreditation of Laboratory Animal Care. The mice were initially housed five per cage for two weeks while they received their respective diets, then, after tumor inoculation, mice were housed one per cage. The cages were kept in a sterile and pathogen-free environment. Cages, bedding, and water were autoclaved before use. The feeding receptacles were in the top of the cages, so caloric intake could be monitored and new feedings given without the cage being opened. Strict sterile technique was

used whenever the cages, mice, and feed were handled. Approval for the experiments was obtained from the University of California, Los Angeles, Chancellor's Animal Research Committee, and all animals were cared for in accordance with the Institutional Guidelines.

Diets

The ingredients of the four experimental diets (prepared by Dyets, Bethlehem, PA) are listed in Table 1. Corn oil was used as the source of fat, and the fat calories from corn oil were balanced with carbohydrate calories from cornstarch. The protein source was soy protein isolate (Supro 670 HG, donated by Protein Technologies International, St. Louis, MO) or casein. The soy protein isolate (Supro 670 HG) contains the following isoflavones: 0.078% conjugated genistein, 0.107% unconjugated genistein, 0.037% conjugated daidzein, 0.052% unconjugated daidzein, 0.006% conjugated glycinein, and 0.009% unconjugated glycinein by weight. The soy isoflavone extract (PTI-G2535, donated by Protein Technologies International, St. Louis, MO) contains the following unconjugated isoflavones: 43.7% genistein, 21.8% daidzein, and 3.4% glycinein by weight. The total of isoflavones in each soy protein-containing diet was 0.18% by weight. The diets were radiated to obtain sterility. During the experiments, the diets were stored at 5°C.

Feeding Protocol

After tumor injection, mice were housed one mouse per cage to allow us to give the mice specific quantities of food and to accurately measure the caloric intake of the mice during the experiment. Three times a week the mice were given

Table 1. Ingredients of Experimental Diets in Grams^a

Ingredient	Diet			
	Low-fat + casein	Low-fat + soy protein + isoflavones	High-fat + casein	High-fat + soy protein + isoflavones
Corn oil	50	50	175	175
Casein	200		200	
Soy protein		200		200
Isoflavone extract		1.81		1.38
Cornstarch	398	395	163	161
Sucrose	117	118	117	118
Dextrose ^b	132	132	58	58
Cellulose	50	50	50	50
AIN-93G-MX mineral mix ^c	35		35	
AIN-93M-MX mineral mix ^c		35		35
AIN-93-VX vitamin mix ^c	10	10	10	10
L-Cysteine	3	2.5	3	2.5
L-Methionine	1.6	2.5	1.6	2.5
Choline bitartrate	2.5	2.5	2.5	2.5
Total grams	999.1	999.31	815.1	815.88

a: Four experimental diets were formulated containing varying amounts of corn oil as a source of fat and soy protein or casein as the source of protein. Isoflavones are primarily from isoflavone extract and, to a lesser extent, from soy protein.

b: Depolymerized cornstarch used to pelletize diets.

c: See Reference 19.

fresh food. Each time the mice received fresh food, the caloric intake from the prior feeding period was determined. This was done by subtracting the amount of feed remaining in the feeding receptacle from the amount available to each mouse at the beginning of each feeding period. The average caloric intake (from the prior feeding period) of all the mice was calculated, and this amount was given to each mouse for the subsequent feeding period. With this protocol, caloric intake was reduced for the mice with the highest intakes, while mice with lower caloric intakes were given the opportunity to increase their caloric consumption. This technique provided approximately equal caloric intake for all four groups of mice (see **Results**).

Design of Experiments

LNCaP cells were harvested from subconfluent cultures after incubation for one minute with 0.25% trypsin and 0.02% EDTA. After suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum, the live cells were stained with trypan blue dye and counted manually with a microscope and hemocytometer. The LNCaP cells were resuspended in ice-cold Matrigel (Collaborative Biomedical Products, Bedford, MA) at a final concentration of 1×10^5 cells/0.2 ml Matrigel. Each mouse was given a subcutaneous inoculation of 1×10^5 tumor cells in the lateral abdomen with a disposable syringe and a 26-gauge needle. An initial experiment with 20 animals (5 in each dietary group) was performed by injecting 1×10^6 tumor cells in 0.2 ml of Matrigel per animal. When the tumors in these animals reached 1.0–1.5 cm³, they caused the animals in all four dietary groups to become cachectic. This occurred in all animals at approximately seven weeks after tumor inoculation. Therefore, 1×10^5 cells in 0.2 ml of Matrigel were used for subsequent experiments to allow tumor growth over a longer time period to evaluate the effect of the diets.

Two successive experiments were performed: the first with five animals in each of the four dietary groups and the second with six or seven animals in each of the four dietary groups. The experiments were run separately because of limited space and resources in our SCID mouse facility to accommodate our one mouse per cage experimental design. The data from both experiments were used for the statistical analysis. All animals were randomly assigned to one of the four dietary groups (high-fat + casein, high-fat + soy protein + isoflavones, low-fat + casein, or low-fat + soy protein + isoflavones) and maintained on this diet for two weeks before tumor cell injection. All animals were kept on an isocaloric diet, as described in **Feeding Protocol**. Once the tumors became palpable, the dimensions were recorded weekly through Week 12 of the experiment. Animal weights were also recorded weekly. The tumor volumes were calculated using the following formula: length × width × height × 0.5236 (20). Thirteen weeks after the initiation of the experiment, all mice were sacrificed. Blood was collected and centrifuged, and the serum was frozen at -70°C. The tumors were dissected, weighed, and frozen in liquid nitrogen.

Determination of Serum Prostate-Specific Antigen and Testosterone

Serum prostate-specific antigen (PSA) levels were determined using an enzyme immunoassay kit (American Qualex Antibodies, San Clemente, CA). Total serum testosterone was determined using enzyme-linked immunosorbent assays (KMI Diagnostics, Minneapolis, MN). All the above assays were performed in duplicate. The interassay variability was <5.0%.

Statistical Analysis

A linear regression model was used to test the differences of body weight, caloric intake, final tumor weight, PSA levels, and testosterone levels among the four diet groups. Because this study consisted of two successive experiments, "experiment" was also considered a covariate and, together with baseline body weight and average weekly caloric intake, was included in the linear regression model. Baseline body weight and average weekly caloric intake did not show any significant association with tumor weight and PSA and testosterone levels. Therefore, "experiment" was the only covariate included in the final model. Growth rate was assessed by repeatedly measuring tumor volumes over time. A random coefficient model was developed to correlate tumor volume, time (week), diet, and other covariates (experiment and baseline body weight). Covariates that did not show significant association with tumor volume were excluded from the model. Log-rank test was used to test the time to palpable tumor among the four diet groups. The above analyses were also carried out comparing each individual dietary group with the other three dietary groups combined.

Results

Forty-three (94%) of the 46 mice developed measurable tumors: 10 (10/12) in the high-fat + casein group, 11 (11/11) in the high-fat + soy protein + isoflavone group, 12 (12/12) in the low-fat + casein group, and 10 (10/11) in the low-fat + soy protein + isoflavone group. There was no significant difference in caloric intake among the animals in the four groups (Figure 1). In addition, there was no significant difference in the body weights among the groups (Figure 2).

When the final tumor weight and tumor growth rate were compared among the four dietary groups, there were no significant differences. However, the final tumor weights in mice fed the low-fat + soy protein + isoflavone diet were 15% less than the final tumor weights of the animals in the other three groups combined (high-fat + casein, high-fat + soy protein + isoflavone, and low-fat + casein) ($p = 0.0499$, general linear model) (Table 2). In addition, the growth rate of the tumors in the mice fed the low-fat diet combined with soy protein and isoflavones was lower than that in the other three groups combined (Figure 3) ($p = 0.0382$, mixed model). There was no difference among the four groups in

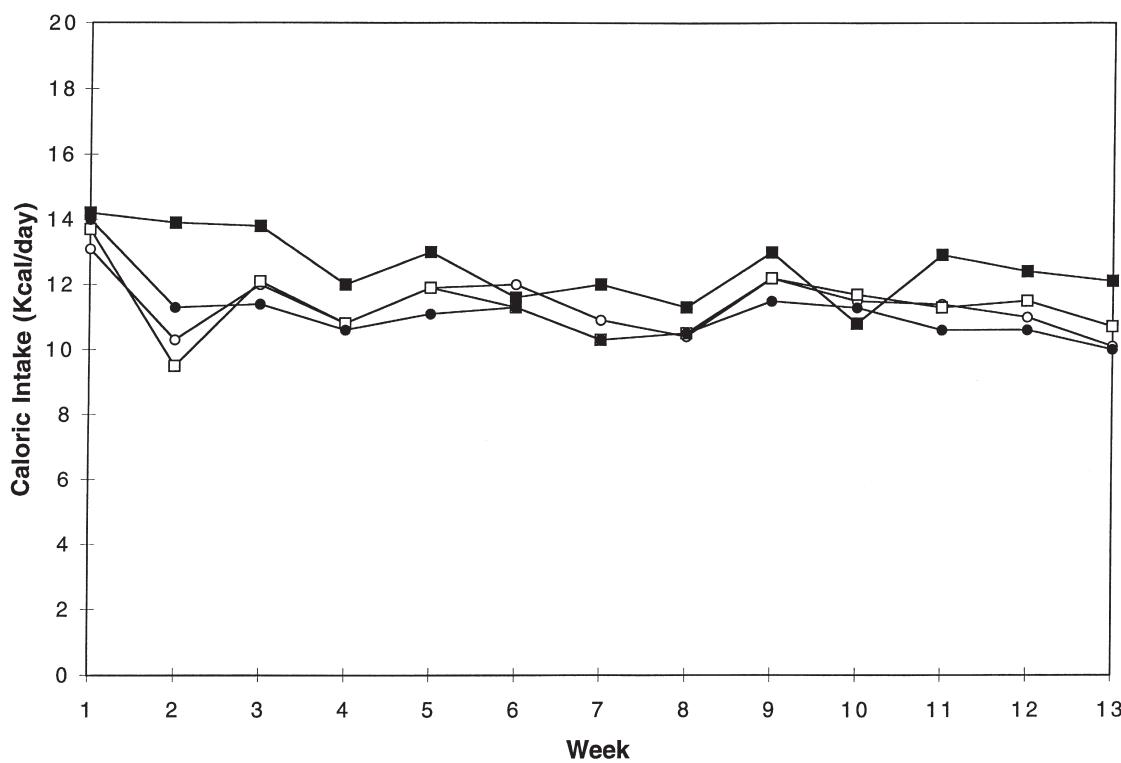


Figure 1. Caloric intake per week. Mice were housed 1/cage to allow for strict control and monitoring of caloric intake. There was no significant difference in caloric intake among groups. Filled circles, high-fat + casein; filled squares, high-fat + soy protein + isoflavones; open circles, low-fat + casein; open squares, low-fat + soy protein + isoflavones.

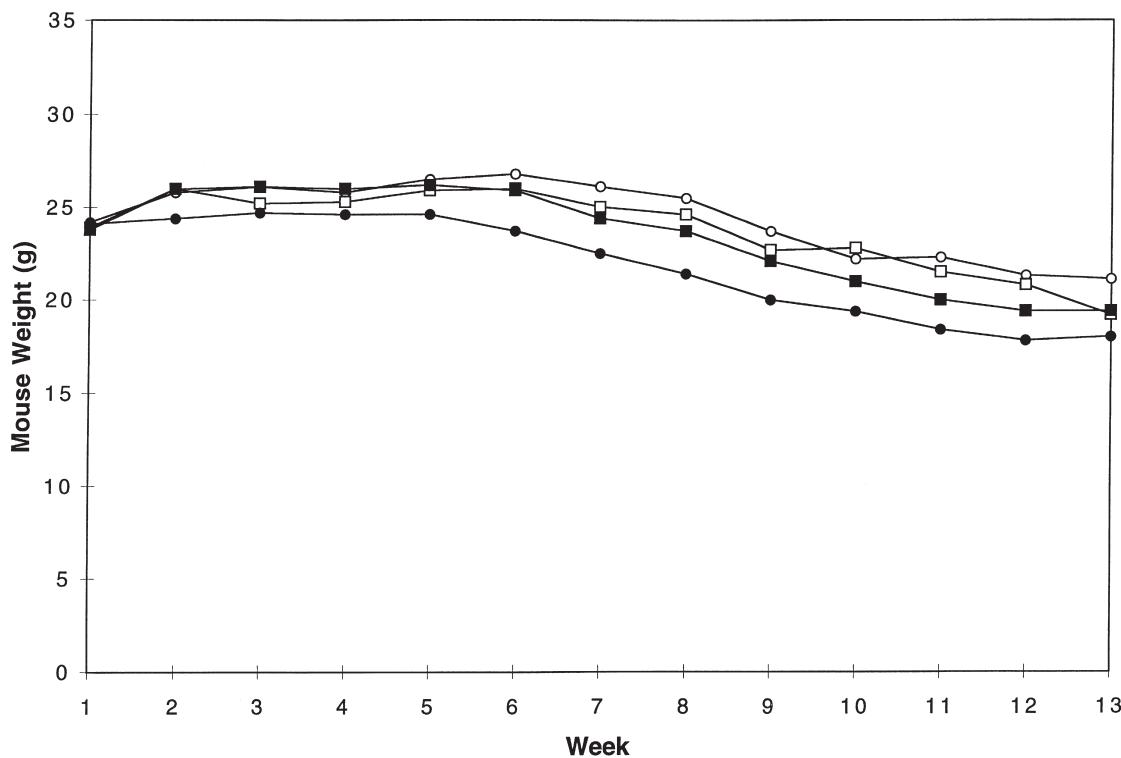


Figure 2. Mouse weights (minus tumor weights) per week. There was no significant difference in mouse weights among groups. Symbols as in Figure 1.

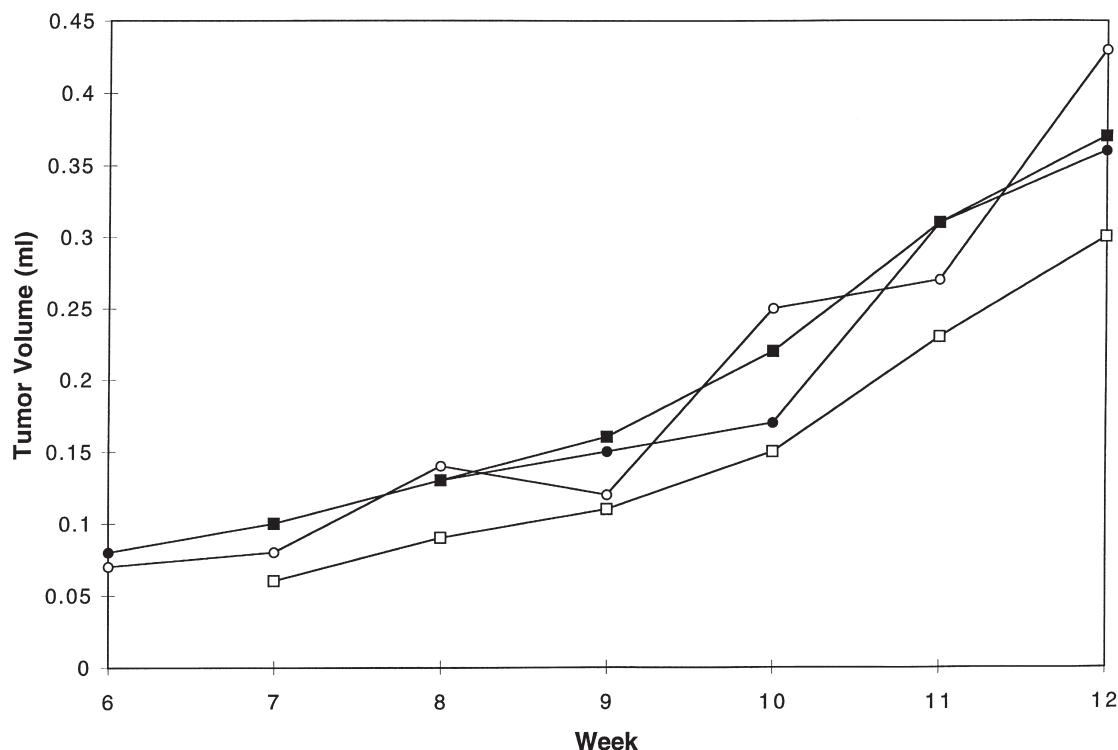


Figure 3. LNCaP tumor growth per week. Mice fed low-fat + soy protein + isoflavone diet had significantly slower-growing tumors than other dietary groups combined ($p = 0.0382$). Symbols as in Figure 1.

Table 2. Final LNCaP Tumor Weights and Time From Tumor Injection to Palpable Tumor^{a,b}

Dietary Group	n	Tumor Weight, g	Time to Palpable Tumor, wk
High-fat + casein	9	0.86 ± 0.21	8.30 ± 0.30
High-fat + soy protein + isoflavones	11	0.89 ± 0.19	8.33 ± 0.24
Low-fat + casein	12	0.81 ± 0.18	7.90 ± 0.18
Low-fat + soy protein + isoflavones	10	0.72 ± 0.16*	8.25 ± 0.16

a: Values are means ± SE; n, number of mice.

b: Statistical significance is as follows: *, significantly less than other groups ($p = 0.0499$).

time to developing palpable tumors (Table 2). The testosterone levels at 13 weeks were 70% lower in mice fed the low-fat + soy protein + isoflavone diet than in the other groups, but this was not statistically significant (Table 3). There was no difference in PSA levels between the four groups at the termination of the experiment (Table 3).

Discussion

In this study, we evaluated the effect of dietary fat, soy protein, and soy isoflavones on the growth of human prostate cancer cells in a xenograft SCID mouse model. We found that combining a low-fat diet (12 kcal%) with soy protein (20 kcal%) and isoflavones (0.18% by weight) reduced the tumor growth rate and the final tumor weights compared with the other diets in our study.

In a prior study, Wang and co-workers (13) showed a significant reduction in the growth of LNCaP tumors in nude mice fed diets lower in fat (21.2, 11.6, and 2.3 kcal%) compared with mice fed a high-fat diet (40.5 kcal%). In our study, there was a trend toward decreased tumor weights in the low-fat + casein group compared with the high-fat + casein group, although this did not reach statistical significance. It may be that, with a larger sample size, this difference between the low-fat and high-fat groups may have reached statistical significance. An alternative explanation for the differences in the results of our study and those of Wang and co-workers may be explained by differences in the experimental designs. In our study, the one animal per cage design allowed for an isocaloric feeding protocol. In the study by Wang and co-workers, there were multiple animals per cage, and therefore caloric intake was not strictly controlled or monitored. It is possible that the marked differ-

Table 3. Serum PSA and Testosterone Levels^a

Dietary Group	PSA, ng/ml	Testosterone, ng/ml
High-fat + casein	44.3 ± 12.8	1.49 ± 1.26
High-fat + soy protein + isoflavones	48.0 ± 9.5	1.09 ± 0.5
Low-fat + casein	48.6 ± 10.9	1.8 ± 0.98
Low-fat + soy protein + isoflavones	51.8 ± 11.8	0.42 ± 0.03

a: Values are means ± SE. Tests were performed at completion of experiment. PSA, prostate-specific antigen. There were no significant differences among groups.

ence between the low-fat and high-fat groups observed by Wang and co-workers may be due to a difference in caloric intake, since higher energy intake may lead to increased tumor growth (21). An alternative explanation may relate to the animal model that we used. Whereas nude mice (used by Wang and co-workers) possess B cell immunity, SCID mice lack T cell, B cell, and natural killer cell immunity. It may be that nutrition is interacting with the immune system in the nude mouse to cause more dramatic differences in tumor size.

The role of soy protein and isoflavones in the prevention and treatment of prostate cancer remains largely undefined and is actively being studied at a number of centers. Research has focused on the isoflavone genistein and its effects on prostate cancer cell lines. Genistein has been shown to inhibit the growth of human prostate cancer cell lines in tissue culture and in histoculture (22,23). In addition, genistein also inhibits proliferation and induces apoptosis of prostate cancer cell lines (24). Soy protein also contains nonisoflavone substances with anticancer properties (Bowman-Burke inhibitor), which may also be responsible for the growth-inhibitory effect seen in the present study (25).

In this study, altering the fat, soy, and isoflavone content had no effect on the PSA levels. PSA is a well-known marker for prostate cancer progression that correlates with tumor growth and metastases (26). Because the low-fat diet high in soy protein and isoflavone caused only a 15% reduction in the final tumor weight in this experiment, this effect may not be large enough to cause a significant change in the serum PSA level. Alternatively, the diet may have inhibited the growth of a population of the tumor cells that do not produce PSA. In the present study, the mice fed the low-fat diet high in soy protein and isoflavones had a 70% decrease in total testosterone levels, although this did not reach statistical significance. Wang and co-workers (13) also found that low-fat diets failed to reduce serum testosterone levels. Although several studies found a correlation between low-fat diets and circulating androgen levels, further investigation is required in this area (27,28). Low-fat diets may cause a reduction in bioavailable testosterone (free testosterone) levels by increasing sex hormone-binding globulin levels, although further investigations are needed in this area as well (29).

In conclusion, in this xenograft model for prostate cancer, there were statistically significant effects on tumor growth rate and final tumor weight attributable to a low-fat diet combined with soy protein and isoflavones.

Acknowledgments and Notes

The authors thank Dr. Bill Go and Don Yowell for advice and suggestions during the course of the experiment. This study was supported in part by The Green Family Foundation, by National Cancer Institute Grants CA-42710-11 and CA-42710-12, and by the Department of Veterans Affairs. All work was completed at the University of California, Los Angeles (Los Angeles, CA) and at the West Los Angeles Veterans Administration Medical Center (Los Angeles, CA). Address reprint requests to Dr. William J. Aronson, UCLA Dept. of Urology, Box 951738, Los Angeles, CA

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Submitted 16 March 1999; accepted in final form 15 July 1999.

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The New England Journal of Medicine

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Volume 330

APRIL 14, 1994

Number 15

THE EFFECT OF VITAMIN E AND BETA CAROTENE ON THE INCIDENCE OF LUNG CANCER AND OTHER CANCERS IN MALE SMOKERS

THE ALPHA-TOCOPHEROL, BETA CAROTENE CANCER PREVENTION STUDY GROUP*

Abstract *Background.* Epidemiologic evidence indicates that diets high in carotenoid-rich fruits and vegetables, as well as high serum levels of vitamin E (alpha-tocopherol) and beta carotene, are associated with a reduced risk of lung cancer.

Methods. We performed a randomized, double-blind, placebo-controlled primary-prevention trial to determine whether daily supplementation with alpha-tocopherol, beta carotene, or both would reduce the incidence of lung cancer and other cancers. A total of 29,133 male smokers 50 to 69 years of age from southwestern Finland were randomly assigned to one of four regimens: alpha-tocopherol (50 mg per day) alone, beta carotene (20 mg per day) alone, both alpha-tocopherol and beta carotene, or placebo. Follow-up continued for five to eight years.

Results. Among the 876 new cases of lung cancer diagnosed during the trial, no reduction in incidence was observed among the men who received alpha-tocopherol (change in incidence as compared with those who did not, -2 percent; 95 percent confidence interval, -14 to 12 percent). Unexpectedly, we observed a higher incidence of lung cancer among the men who received beta caro-

tene than among those who did not (change in incidence, 18 percent; 95 percent confidence interval, 3 to 36 percent). We found no evidence of an interaction between alpha-tocopherol and beta carotene with respect to the incidence of lung cancer. Fewer cases of prostate cancer were diagnosed among those who received alpha-tocopherol than among those who did not. Beta carotene had little or no effect on the incidence of cancer other than lung cancer. Alpha-tocopherol had no apparent effect on total mortality, although more deaths from hemorrhagic stroke were observed among the men who received this supplement than among those who did not. Total mortality was 8 percent higher (95 percent confidence interval, 1 to 16 percent) among the participants who received beta carotene than among those who did not, primarily because there were more deaths from lung cancer and ischemic heart disease.

Conclusions. We found no reduction in the incidence of lung cancer among male smokers after five to eight years of dietary supplementation with alpha-tocopherol or beta carotene. In fact, this trial raises the possibility that these supplements may actually have harmful as well as beneficial effects. (*N Engl J Med* 1994;330:1029-35.)

PREVIOUS studies have suggested that higher intakes of vitamin E (alpha-tocopherol) and beta carotene may be associated with a reduced risk of lung cancer. In particular, epidemiologic studies have linked the intake of vegetables rich in beta carotene with a lower risk of cancer (especially lung cancer) and have suggested that certain micronutrients are inhibitors of cancer.^{1,2} The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study was a randomized, double-blind, placebo-controlled primary-prevention trial undertaken to determine whether supplementation with alpha-tocopherol, beta carotene, or both would reduce the incidence of lung cancer in male smokers. A secondary outcome of interest was the incidence of other cancers. Lung cancer was deemed a

particularly appropriate target for this trial because of its high incidence, its generally poor prognosis, and the existence of a well-defined high-risk population (i.e., smokers).³ In this report we describe the initial overall results of the study, which was conducted in Finland as a joint project of the National Public Health Institute of Finland and the U.S. National Cancer Institute.

METHODS

Study Design

The rationale, design, and methods of the study, the characteristics of the participants, and the measures of compliance have been described in detail elsewhere.⁴ Briefly, the participants (n = 29,133) were male smokers who were 50 through 69 years old at entry; they were recruited from the total male population of this age group in 14 geographic areas in southwestern Finland (n = 290,406). The participants were randomly assigned to one of four supplementation regimens: alpha-tocopherol alone (n = 7286), alpha-tocopherol and beta carotene (n = 7278), beta carotene alone (n = 7282), or placebo (n = 7287). Thus, a total of 14,564 men received alphatocopherol, and 14,560 received beta carotene. The daily dose of alpha-tocopherol was 50 mg and that of beta carotene, 20 mg. Follow-up continued for 5 to 8 years (median, 6.1), until death or April 30, 1993, with a total of 169,751 person-years contributed by

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Supported by a contract (NO1-CN-45165) with the National Cancer Institute.

*The participants in the study group are listed in the Appendix.

the surviving participants. This study was approved by the institutional review boards of the participating institutions, and all subjects provided informed consent before randomization.

Eligibility

Participants were recruited in 1985 through 1988 from the respondents to a postal survey ($n = 224,377$) who lived in the designated study region. To be eligible, they had to be smokers (five or more cigarettes per day at entry), 50 to 69 years old, and willing to give informed written consent. Potential participants with a history of cancer or serious disease limiting their ability to participate, those taking supplements of vitamin E, vitamin A, or beta carotene in excess of predefined doses, and those being treated with anticoagulant agents were excluded. Before their enrollment, the participants were interviewed at 1 of 14 local study centers to obtain details of their medical, dietary, smoking, and occupational histories and information about other risk factors for cancer. Each participant's dietary intake of alpha-tocopherol and beta carotene was estimated from the diet-history questionnaire⁵; levels of alpha-tocopherol and beta carotene were measured in serum samples by high-performance liquid chromatography.⁶ Participants identified after randomization as ineligible ($n = 113$) were equally distributed among the four intervention groups; they included men with preexisting cancer other than nonmelanoma skin cancer ($n = 64$), men with lung cancer identified on the base-line chest film ($n = 33$), users of vitamin supplements in excess of the study limits ($n = 15$), and 1 nonsmoker.

Randomization and Blinding

The participants at each of the 14 study sites were randomly assigned to one of the four intervention groups. Treatment assignments were based on a two-by-two factorial design that permitted assessment of the effects of the two supplements independently. Thus, half the participants received alpha-tocopherol ($n = 14,564$) and half did not ($n = 14,569$). Similarly, half received beta carotene ($n = 14,560$) and half did not ($n = 14,573$). The proportion of participants who reported yellowing of the skin at any time during active follow-up was 34 percent in the two groups that received beta carotene, as compared with 7 percent in the groups given no beta carotene; persistent yellowing of the skin (during two thirds or more of the follow-up visits) was reported by 8.8 percent of the participants who received beta carotene, as compared with 0.3 percent of those who did not. Participants and all study staff involved in the ascertainment of end points and the assignment of final diagnoses remained blinded to the participants' treatment assignments throughout the trial.

Delivery of Supplements and Assessment of Compliance

The study agents were formulated as synthetic *dl*-alpha-tocopheryl acetate (50 percent powder) and synthetic beta carotene (10 percent water-soluble beadlets); all formulations were colored with quinoline yellow. Capsules were packaged in coded blister-pack wallets in calendar format provided by Hoffmann-LaRoche (Basel, Switzerland). All participants took a single capsule daily. The participants received a new supply of capsules at each of their threecyearly follow-up visits. Visits began in April 1985 for some participants and were concluded in April 1993 for all. Compliance was assessed by counts of the remaining capsules at each visit, by measurement of serum alpha-tocopherol and beta carotene levels after three years of supplementation, and by measurements in random serum samples throughout the study.⁴

Assessment of End Points

Cases of lung cancer were identified through the Finnish Cancer Registry.⁷ All cases known to have been diagnosed up to April 30, 1993, are included in this report. To enhance the ascertainment of cases, a chest film was obtained at a study visit every 28 months and at each participant's exit from the study. For various reasons, the final chest film was not available for 494 of the surviving men. There were no differences among the intervention groups in the proportion of exit chest films available for analysis or in the reasons why no film was obtained. All diagnostic information for each case

of lung cancer was reviewed by the Clinical Review Committee for confirmation and staging. Clinical diagnoses were based on histologic features in 77 percent of the cases, on cytologic analysis alone in 15 percent, and on clinical data alone in 8 percent.

Cancers other than lung cancer were also identified through the Finnish Cancer Registry, with medical records reviewed by clinicians at the central study office.

Monitoring of Safety and Efficacy

Possible side effects of the interventions were assessed at each follow-up visit by means of a questionnaire covering symptoms and an interview focusing on illnesses since the most recent visit that had led to a visit to a doctor or to hospitalization. Information on morbidity unrelated to cancer was also obtained from the Finnish National Hospital Discharge Registry. Deaths ($n = 3570$) were identified from the National Death Registry, a branch of Statistics Finland. The underlying cause of death was coded by trained nosologists using the *International Classification of Diseases*, ninth revision (ICD-9), and reviewed at the study coordinating center; the death certificate was not available for four participants. In 91 percent of all deaths, the cause was based on the autopsy findings (54 percent), the inpatient diagnosis, or both.

A data and safety monitoring committee was convened twice annually throughout the study to review its progress and integrity and to evaluate unblinded data relevant to safety and efficacy.

Statistical Analysis

Analyses of trial results focused on estimating the overall effect of the two supplements on the incidence of cancer and on mortality due to cancer or other causes. Analyses were based on the intention-to-treat principle; that is, follow-up and case ascertainment continued regardless of whether participants continued in the trial. We tested for an interaction between the effects of alpha-tocopherol and beta carotene by means of a proportional-hazards model.⁸

Kaplan-Meier cumulative-incidence plots and two-sided nominal P values derived from the unweighted log-rank statistic⁸ are presented for each intervention separately: alpha-tocopherol as compared with no alpha-tocopherol, and beta carotene as compared with no beta carotene. The effect of intervention is expressed as the percentage change in the incidence of an end point and its 95 percent confidence interval. Computations of confidence intervals were based on the binomial distribution, derived from conditioning on the number of cases and adjustment of probabilities for the number of person-years of follow-up in the two comparison groups.^{8,9}

The preliminary data on cancers other than lung cancer are presented in the form of counts and rates of incidence according to intervention group. Two or more of the five primary cancers in a single participant were counted as separate cases in each category, but were counted only once within each category (even in the category "other cancers"). Thus, the cancer counts are not mutually exclusive. Cases of carcinoma in situ of the lung ($n = 6$) and basal-cell carcinoma of the skin ($n = 217$) were excluded from the analysis. Cause-specific data on deaths are presented in the form of counts and mortality rates in mutually exclusive cause-of-death categories according to intervention group. The categories are based on the following ICD-9 codes: cancer (140 through 208), ischemic heart disease (410 through 414), hemorrhagic stroke (430 through 432), ischemic stroke (433 through 436 and 438), other cardiovascular disease (390 through 405, 415 through 429, 437, and 440 through 459), injuries and accidents (800 through 999), and other causes (001 through 139, 210 through 389, and 460 through 799). Only cases in which cancer was the underlying cause of death were included among the deaths due to cancer.

RESULTS

Characteristics of the Participants

At study entry, the men in the cohort averaged 57.2 years of age, smoked an average of 20.4 cigarettes daily, and had smoked for an average of 35.9 years.

There were no differences among the intervention groups with respect to any characteristic or risk factor for lung cancer that we evaluated at base line (Table 1) or during follow-up, except those directly related to supplementation. A total of 6131 participants stopped smoking during the trial; the numbers who quit in the various intervention groups differed by less than 26. Similarly, 9061 participants left the study for any reason, including death; the groups differed in the number of such dropouts by less than 37.

Lung Cancer and Base-Line Alpha-Tocopherol and Beta Carotene Levels

When the placebo group was divided according to quartiles with regard to the base-line serum alpha-tocopherol or beta carotene concentration, the incidence of lung cancer was higher among the subjects in the lowest quartile group than among those in the highest (incidence per 10,000 person-years, lowest vs. highest quartile group: alpha-tocopherol, 56.8 vs. 41.8; beta carotene, 53.3 vs. 43.1). There was, moreover, an inverse association between dietary intake of alpha-tocopherol and beta carotene at base line and the risk of lung cancer during the trial (incidence per 10,000 person-years, lowest vs. highest: alpha-tocopherol, 61.4 vs. 40.6; beta carotene, 47.9 vs. 39.9).

Compliance

Compliance, estimated on the basis of residual-capsule counts, was excellent, with four out of five active participants taking more than 95 percent of their capsules. In addition, there were no differences in capsule consumption among the intervention groups (median percentage of capsules taken, 99.0 percent in each). Participants receiving active treatment accounted for 86 percent of the total follow-up, whereas the remaining 14 percent was contributed by men who died or dropped out and therefore did not consume capsules. Compliance with intervention was confirmed by the substantial increases in serum alpha-tocopherol and beta carotene concentrations in the groups receiving the active agents, whereas the levels changed little in those who did not receive the agents (Table 2).

Incidence of Lung Cancer and Mortality

A total of 876 newly diagnosed cases of lung cancer and 564 deaths due to lung cancer were identified in the entire cohort. There was no evidence of an interaction between the two supplements in their effect on lung cancer (incidence per 10,000 person-years: alpha-tocopherol alone, 47.3; alpha-tocopherol and beta carotene, 55.3; beta carotene alone, 57.2; and placebo, 47.7; likelihood-ratio test for interaction: chi-square = 0.04, P = 0.84). Our findings regarding the incidence of lung cancer and mortality from that disease according to intervention are shown in Figures 1, 2, and 3. For alpha-tocopherol recipients, the small reduction in incidence (2 percent) during the entire trial was not statistically significant (P = 0.8 by the log-rank test). Among the men who received beta

Table 1. Median Base-Line Characteristics of the Participants, According to Whether They Received Alpha-Tocopherol and Beta Carotene.*

CHARACTERISTIC	ALPHA-TOCOPHEROL	NO ALPHA-TOCOPHEROL	BETA CAROTENE	NO BETA CAROTENE
No. of subjects	14,564	14,569	14,560	14,573
Age (yr)	57.2	57.1	57.3	57.0
Cigarettes smoked/day	20	20	20	20
Years of smoking	36	36	37	36
Serum cholesterol (mmol)	6.2	6.2	6.2	6.2
Body-mass index†	26.0	25.9	26.0	26.0
Total energy intake (kcal/day)	2,725	2,715	2,717	2,722
Total fat intake (g/day)	117.7	116.9	117.4	117.2
Alcohol intake (g/day)	11.1	10.9	10.9	11.1

*This was a two-by-two study, with a total of 29,133 participants. The numbers with data on the dietary-intake variables are as follows: alpha-tocopherol, 13,536; no alpha-tocopherol, 13,575; beta carotene 13,521; and no beta carotene, 13,590.

†The weight in kilograms divided by the square of the height in meters.

carotene, an excess cumulative incidence of lung cancer was observed after 18 months and increased progressively thereafter, resulting in an 18 percent difference in incidence by the end of the study (95 percent confidence interval, 3 to 36 percent; P = 0.01) between the participants who received beta carotene and those who did not. The results were essentially identical when the analysis was restricted to men who had no yellowing of the skin or to those with lung cancers detected radiographically during the study. Mortality due to lung cancer was also apparently higher in the groups that received beta carotene than in those that did not (P = 0.08). No difference associated with the presence or absence of beta carotene supplementation was observed in the case fatality rate or in the length of time from diagnosis to death.

The six cases of carcinoma in situ that were excluded from these analyses were distributed as follows: three each among participants who received alpha-tocopherol and those who did not, and two cases among participants who received beta carotene and four among those who did not. There was one new

Table 2. Serum Concentrations of Alpha-Tocopherol and Beta Carotene before and after Supplementation, According to Intervention.*

INDEX AND GROUP	NO. OF SUBJECTS	MEDIAN	20TH PERCENTILE	80TH PERCENTILE
<i>milligrams per liter</i>				
Alpha-tocopherol level				
At base line				
Alpha-tocopherol	14,472	11.5	9.3	14.2
No alpha-tocopherol	14,469	11.4	9.3	14.1
At three years				
Alpha-tocopherol	11,332	17.3	14.3	21.1
No alpha-tocopherol	11,258	12.4	10.2	15.1
Beta carotene level				
At base line				
Beta carotene	14,460	0.17	0.10	0.29
No beta carotene	14,460	0.17	0.10	0.29
At three years				
Beta carotene	11,276	3.0	1.6	4.5
No beta carotene	11,314	0.18	0.10	0.30

*To convert values for alpha-tocopherol to millimoles per liter, multiply by 2.322. To convert values for beta carotene to millimoles per liter, multiply by 1.863.

case of lung cancer among the 113 participants excluded after randomization; the man was assigned to receive alpha-tocopherol.

Other Cancers

A total of 1415 first cancers other than lung cancer were identified in 1331 subjects during the trial (basal-cell carcinoma of the skin was excluded, as were second cancers at a given site). Figure 2 shows the number of first cancers and their incidence, according to intervention group, at the five most common sites and at all other sites combined. The participants who received alpha-tocopherol had fewer cancers of the prostate and colorectum than those who did not receive alpha-tocopherol, whereas more cancers of the bladder, stomach, and other sites combined were diagnosed in the participants who received this supplement. The participants who received beta carotene had more cancers of the prostate and stomach and fewer cases of other cancers than those who did not receive beta carotene. There were two cancers other than lung cancer (melanoma and astrocytoma) among the participants who were excluded after randomization.

Mortality

Altogether, 3570 deaths occurred during the trial. Among participants receiving alpha-tocopherol, there were fewer deaths caused by ischemic heart disease and ischemic stroke than there were among those who did not receive alpha-tocopherol, but more deaths due to cancers other than lung cancer or due to hemorrhagic stroke (Fig. 3). Overall mortality was 2 percent higher in the alpha-tocopherol groups than in the groups that received no alpha-tocopherol (95 percent confidence interval, -5 to 9 percent; $P = 0.6$). There were more deaths due to lung cancer, ischemic heart disease, and ischemic and hemorrhagic stroke among recipients of beta carotene (Fig. 3). Overall mortality was 8 percent higher among the participants who received beta carotene than among those not given beta carotene (95 percent confidence interval, 1 to 16 percent; $P = 0.02$).

DISCUSSION

Our results provide no evidence of a beneficial effect of supplemental vitamin E (alpha-tocopherol) or beta carotene in terms of the prevention of lung cancer. In fact, men who received beta carotene were found to have lung cancer more frequently than those who did not receive beta carotene. These results are sufficiently strong that it is highly unlikely that 20 mg of beta carotene per day confers any material protective effect against lung cancer among smokers over a period of about six years.

The lack of reduction in the incidence of lung cancer among the men given supplemental beta carotene may be explained by bias, an inadequate duration of supplementation, the use of the wrong dose, or an inappropriate study population. Bias can be discount-

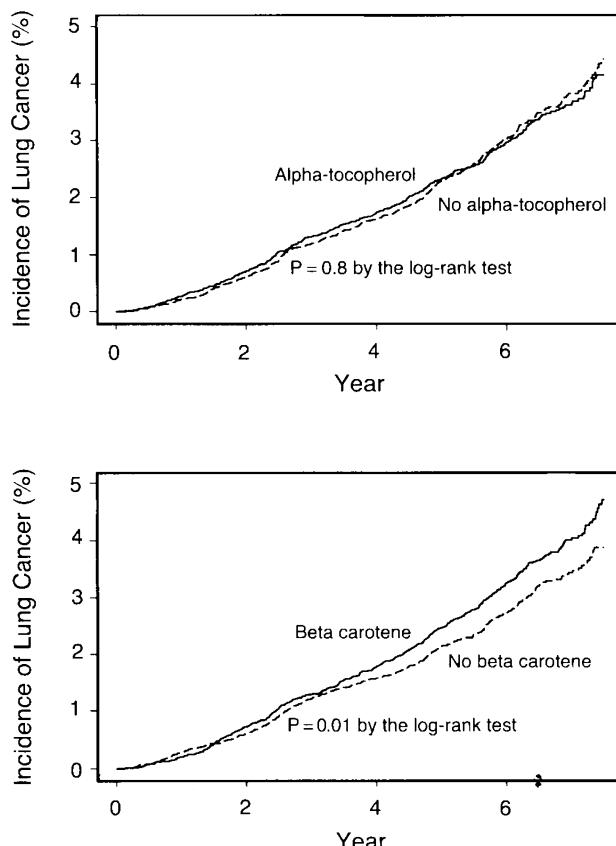


Figure 1. Kaplan-Meier Curves for the Cumulative Incidence of Lung Cancer among Participants Who Received Alpha-Tocopherol Supplements and Those Who Did Not (Upper Panel) and among Participants Who Received Beta Carotene Supplements and Those Who Did Not (Lower Panel).

Data are shown only through 7½ years of follow-up because of the small numbers of participants beyond that time.

ed, since the intervention groups were balanced in terms of all the relevant characteristics we studied. The study population was large, and case ascertainment was essentially complete. In addition, the men in the various intervention groups sought treatment at virtually the same time for lung cancer, as measured by the length of time from diagnosis to death, and even for such minor problems as yellowing of the skin. Moreover, analyses of the incidence of lung cancer that were restricted to participants who did not report yellowing of the skin or to cases diagnosed on the chest film obtained at the study examination yielded results similar to those for the entire cohort; this similarity of results essentially rules out bias caused by self-selection or by differences in diagnostic procedures.

It is plausible that the intervention period was too short to inhibit the development of cancers resulting from a lifetime of exposure to cigarette smoke and other carcinogens. Beta carotene may not be the active cancer-inhibiting component of the fruits and vegetables identified as protective in observational studies, or the intake of beta carotene may be only a nonspecific marker for lifestyles that protect against cancer.

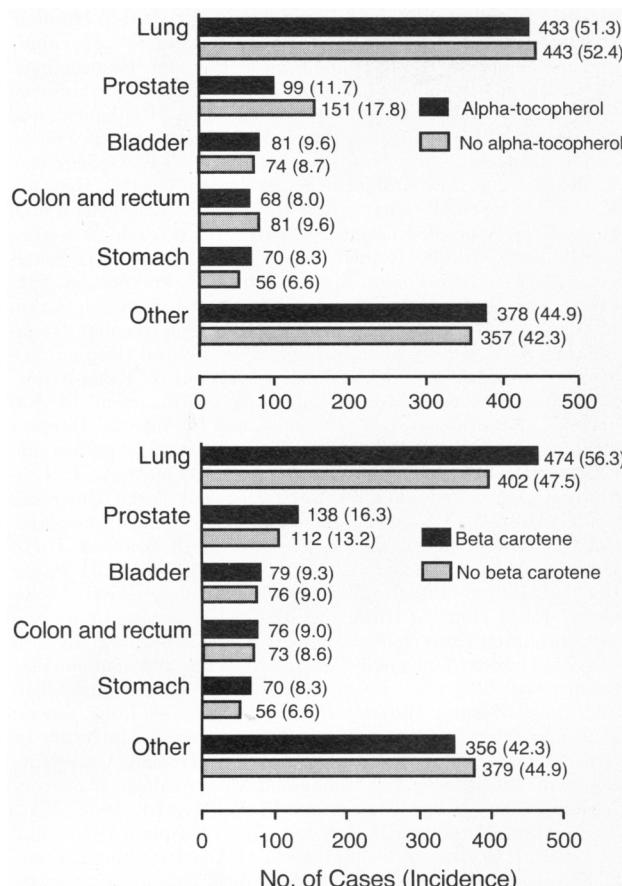


Figure 2. Number and Incidence (per 10,000 Person-Years) of Cancers, According to Site, among Participants Who Received Alpha-Tocopherol Supplements and Those Who Did Not (Upper Panel) and among Participants Who Received Beta Carotene Supplements and Those Who Did Not (Lower Panel).

Although it is conceivable that the dose we used was too low, this seems unlikely, since that dose exceeded by many times the dietary intake of beta carotene in epidemiologic studies that found a strong inverse association between the consumption of carotene-rich foods and the incidence of lung cancer.^{10,11} Finally, study findings regarded as showing supplementation to be beneficial or harmful may occur by chance.

The lack of benefit of beta carotene is particularly surprising given the substantial and consistent epidemiologic evidence of an association between a higher beta carotene intake and a lower incidence of lung cancer,¹¹⁻¹⁵ including the results of the cohort-based analysis in this study. Furthermore, a recent large trial in China found a significant reduction in mortality due to cancer among persons whose diets were supplemented daily with the combination of beta carotene (15 mg), alpha-tocopherol (30 mg), and selenium (50 µg) for 5½ years.¹⁶

We also observed no beneficial effect of alpha-tocopherol on the incidence of lung cancer or on mortality due to this disease. At the start of the trial, the *a priori* evidence that alpha-tocopherol prevented

lung cancer was less substantial than that for beta carotene, and since then little additional evidence has been accumulated.¹⁷⁻¹⁹ Possible explanations for the lack of effect are similar to those for beta carotene, although the relatively low dose and the short duration of supplementation merit greater consideration in the case of alpha-tocopherol. Furthermore, we observed no interaction between alpha-tocopherol and beta carotene in their effect on the incidence of lung cancer.

The apparently protective effect of alpha-tocopherol against prostate cancer and, to a lesser extent, against colorectal cancer is intriguing. Although there was little or no evidence linking alpha-tocopherol to the incidence of cancers at either of these sites when the trial started, limited observational data consistent with these findings have now been published.²⁰⁻²² Although these results are suggestive, many comparisons with these two agents were made in these analyses, increasing the possibility that some of the apparent benefits may have occurred by chance alone. Additional data from the continued follow-up

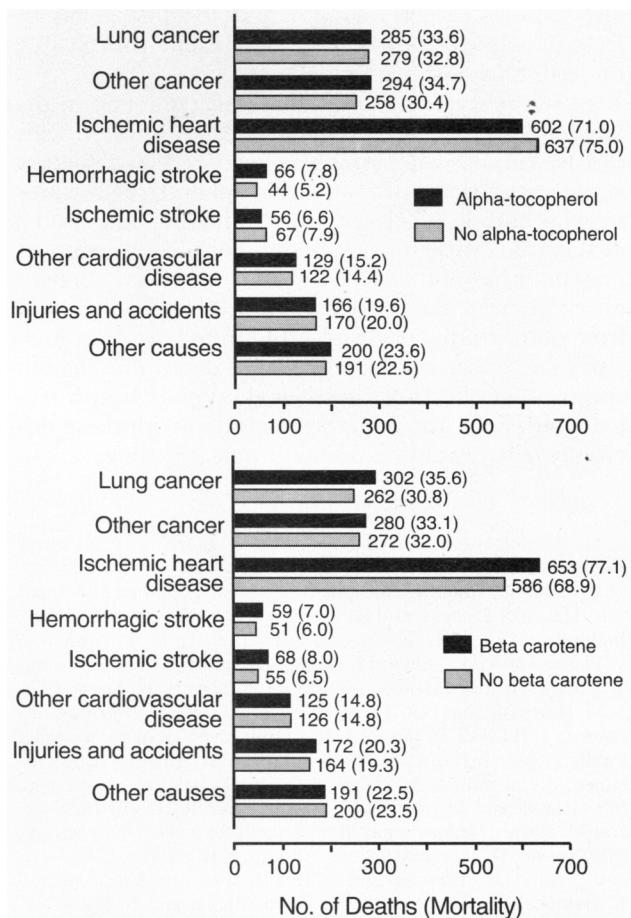


Figure 3. Deaths and Mortality Rates (per 10,000 Person-Years), According to Cause of Death, among Participants Who Received Alpha-Tocopherol Supplements and Those Who Did Not (Upper Panel) and among Participants Who Received Beta Carotene Supplements and Those Who Did Not (Lower Panel). The cause of death was unknown for four participants.

of the participants in this and other intervention studies are needed before conclusions can be drawn about the role of alpha-tocopherol in preventing these cancers.

Our results raise the possibility that supplementation with beta carotene may be harmful in smokers. The higher mortality due to ischemic heart disease and lung cancer among the beta carotene recipients requires more detailed analysis, and information from other studies is also needed. We are aware of no other data at this time, however, that suggest harmful effects of beta carotene, whereas there are data indicating benefit.^{16,23} Furthermore, there are no known or described mechanisms of toxic effects of beta carotene, no data from studies in animals suggesting beta carotene toxicity, and no evidence of serious toxic effects of this substance in humans.²⁴ In the light of all the data available, an adverse effect of beta carotene seems unlikely; in spite of its formal statistical significance, therefore, this finding may well be due to chance.

The higher mortality due to hemorrhagic stroke among the participants receiving alpha-tocopherol also requires careful review. Alpha-tocopherol has effects on platelet function^{25,26} that could conceivably underlie this observation.

In summary, we found no overall reduction in the incidence of lung cancer or in mortality due to this disease among male smokers who received dietary supplementation with alpha-tocopherol, beta carotene, or both in this large trial in Finland. The results of this study raise the possibility that these substances may have harmful as well as beneficial effects. Longer observation of the participants in this trial and data from other studies of people at normal risk^{27,28} or high risk²⁹ for cancer will be required to determine the full spectrum of effects of these agents. Public health recommendations about supplementation with these micronutrients would be premature at this time.

APPENDIX

The participants in the Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group were as follows: *Principal investigators* — O.P. Heinonen and J.K. Huttunen, National Public Health Institute, Helsinki, Finland, and D. Albanes, National Cancer Institute, Bethesda, Md.; *Senior investigators* — J. Haapakoski, J. Palmgren, P. Pietinen, J. Pikkariainen, M. Rautalahti, and J. Virtamo, National Public Health Institute, and B.K. Edwards, P. Greenwald, A.M. Hartman, and P.R. Taylor, National Cancer Institute; *Investigators* — J. Haukka, P. Järvinen, N. Malila, and S. Rapola, National Public Health Institute; *Data management* — P. Jokinen, A. Karjalainen, J. Lauronen, J. Mutikainen, M. Sarjakoski, A. Suorsa, M. Tiainen, and M. Verkasalo, National Public Health Institute, and M. Barrett, Information Management Services, Silver Spring, Md.; *Laboratory measurements* — G. Alftahan, C. Ehnholm, C.G. Gref, and J. Sundvall, National Public Health Institute; *Nutritionists* — E. Haapa, M.L. Ovaskainen, M. Palva-Alhola, and E. Roos, National Public Health Institute; *Cancer Registry* — E. Pukkala and L. Teppo, Finnish Cancer Registry, Helsinki; *Data and Safety Monitoring Committee* — H. Frick (chairman), University of Helsinki, Helsinki, A. Pasternack, University of Tampere, Tampere, Finland, B.W. Brown, Jr., Stanford University, Palo Alto, Calif., and D.L. Demets, University of Wisconsin, Madison; *Collaborating hospitals in Finland* — Coordinators: K. Kokkola, National Public

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SUE ANNE BRENNER, M.D.

Implanted Tumor Growth Is Suppressed and Survival Is Prolonged in Sixty Percent of Food-Restricted Mice

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ABSTRACT To examine the effect of food restriction on immune functions in the tumor-bearing state, mice were divided into a control group (fed 5.0 g diet/d; 71 kJ/d) and a 60% food-restricted group (fed 3.0 g diet/d; 43 kJ/d) at 8-wk of age, and 4 wk later, L1210 tumor cells were inoculated intradermally. In the food-restricted mice, tumor growth was significantly suppressed, and mean survival time after the tumor inoculation was prolonged ($P < 0.05$). The plasma concentrations of two antitumor cytokines, interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α), were greater in the food-restricted group before tumor inoculation ($P < 0.05$). Furthermore, the food-restricted mice had significantly higher plasma levels of IFN- γ and TNF- α after tumor inoculation, although the treatment significantly increased these cytokine levels in both groups. Splenic natural killer cell cytotoxicity was also higher in the tumor-bearing food-restricted mice than in controls ($P < 0.05$). Food-restricted mice have strong antitumor immunity, and as a result, tumor growth is suppressed and survival time is prolonged in these mice. *J. Nutr.* 130: 111–115, 2000.

KEY WORDS: • food restriction • mice • antitumor immunity • interferon- γ • tumor necrosis factor- α
• natural killer cells

Food restriction (FR)² prolongs the mean and maximal life spans of rats, mice and other species (Cheney et al. 1980, Comfort 1963, Ross 1961, Weindruch and Walford 1988). In addition, FR of rodents results in a reduced incidence and delayed onset of tumors and other age-associated diseases (Weindruch 1989, Yu et al. 1982).

FR results in the activation of cellular immunity, altered subsets of T cells (Gartner et al. 1992), enhanced responses of T cells to mitogens and interleukin-2 (Hishinuma et al. 1990), and altered production of antibodies and interleukin-2 (Spear-Hartley and Sherman 1994). It has been proposed that host immune surveillance mechanisms effectively suppress the incidence of tumors in FR animals (Konno et al. 1991). FR also has been shown to greatly reduce white blood cell (WBC) number, particularly the lymphocyte count (Kubo et al. 1984). Although mechanisms by which FR reduces WBC number have not yet been clarified, it has been reported that the incidence of leukemia is lowered in FR mice due to the reduction of leukocytes (Volk et al. 1994). Moreover, strong immune responses and a high resistance to developing spontaneous tumors in FR mice are thought to be closely related to the reduction of lymphocyte number (Weindruch and Walford 1988).

Effects of FR on immune functions in tumor-bearing animals have not yet been well elucidated so we inoculated L1210

tumor cells intradermally into 60% FR mice, and studied host immune functions after the tumor inoculation.

MATERIALS AND METHODS

Animals. Male BDF1 mice (5-wk-old) were obtained from Clea (Osaka, Japan). These mice had free access to water and to a diet reported by Konno et al. (1991), which was composed of 20% casein, 64% potato starch, 5% soybean oil, 5% cellulose powder, 4% salt mixture and 2% vitamin mixture.³ These mice were kept at controlled temperature (23 ± 2°C), humidity (60 ± 10%) and lighting (12-h light and 12-h dark cycle), and housed individually. When 8-wk-old, mice were randomly divided into control and 60% FR groups. Mice in the control group were fed 5.0 g of the diet daily (71 kJ/d), whereas 60% FR mice received 3.0 g (43 kJ/d). All experimental procedures involving laboratory animals were approved by the Animal Care and Use Committee of Osaka Prefecture University.

Inoculation of tumor cells. L1210 leukemia cells (America Type Culture Collection, CCL219), obtained from Riken Cell Bank (Tsukuba, Japan), were maintained in Dulbecco's modified Eagle's medium, supplemented with 5% heat-inactivated fetal calf serum and 10 mmol/L HEPES. At 12 wk of age (4 wk after the initiation of FR),

³ Vitamin mixture contains 46,000 IU vitamin A acetate, 23,300 IU cholecalciferol, 1,200 mg vitamin E acetate, 6 mg vitamin K₃, 59 mg thiamin HCl salt, 59 mg riboflavin, 29 mg vitamin B₆ HCl salt, 0.2 mg vitamin B₁₂, 588 mg vitamin C, 1 mg D-biotin, 2 mg folic acid, 235 mg pantothenic acid Ca salt, 294 mg nicotinic acid, and 1,176 mg inositol in 100 g with a balance with lactose. Mineral mixture contains 0.43 g CaHPO₄ · 2H₂O, 34.31 g KH₂PO₄, 25.06 g NaCl, 0.623 g Fe-citrate (Fe 17%), 4.8764 g MgSO₄, 0.02 g ZnCl₂, 0.121 g MnSO₄ · 4–5H₂O, 0.156 g CuSO₄ · 5H₂O, 0.0005 g KI, 29.29 g CaCO₃, and 0.0025 g (NH₄)₆Mo₇O₂₄ · 4H₂O in 100 g with a balance with cellulose powder.

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² Abbreviations used: FR, food restriction or food-restricted; IFN- γ , interferon- γ ; NK, natural killer; TNF- α , tumor necrosis factor- α ; WBC, white blood cell.

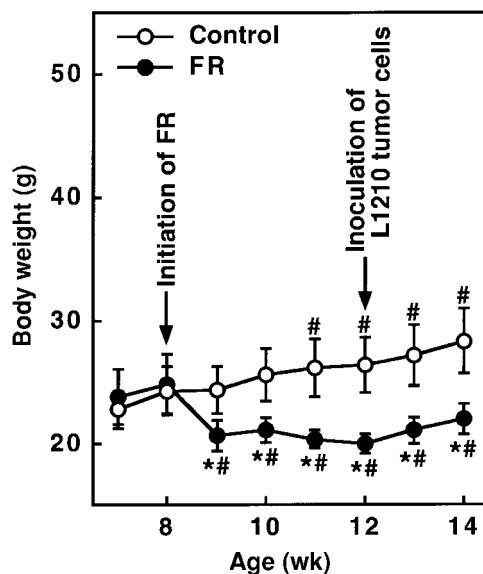


FIGURE 1 Experimental design and body weight change of control and 60% food-restricted (FR) mice. Male mice at 8 wk of age were divided into control (5 g of a diet/d; 71 kJ/d) and 60% FR (3 g of a diet/d; 43 kJ/d) groups, and 4 wk later these mice were inoculated with L1210 tumor cells by intradermal injection. Values are means \pm SD, $n = 7$. *: $P < 0.05$ compared with the control mice at the corresponding time point. #: $P < 0.05$ compared with 8-wk-old mice in each group.

mice in both the FR and control groups were inoculated with L1210 by intradermal injection of $\sim 10^6$ cells. For determination of tumor growth, 2, 3 and 3.5 wk after the inoculation these mice (seven mice in each group at each time point) were anesthetized with ethyl ether, and tumors in these mice were isolated and weighed.

Determination of leukocyte, lymphocyte, neutrophil and monocyte counts. Mice (five mice in each group) were anesthetized with ethyl ether, and blood was collected by cardiac puncture. Blood samples (10% in an EDTA solution) were incubated on ice until measured with an automatic blood corpuscle count apparatus (Sysmex K-1000; Toua Iyuu Electric, Kobe, Japan). For investigation of differential counts, blood smears were prepared on microscope slides, and the slides were air-dried and stained by a modified Giemsa method (Seki et al. 1981). Leukocytes (over 200 cells) on the slides were examined and the percentages of lymphocytes, neutrophils and monocytes were determined.

Determination of plasma cytokine levels. Blood was collected by cardiac puncture, and plasma interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) levels were determined by enzyme-linked immunosorbent assay methods using commercial kits (QuantikineTM M Mouse IFN- γ Immunoassay and QuantikineTM M Mouse TNF- α Immunoassay; R&D Systems, Minneapolis, MN).

Cytotoxicity assay for natural killer (NK) cells. Spleen cells were prepared according to a published method (Weindruch et al. 1983). NK-sensitive YAC-1 cells were used as target cells for the assay of NK cell cytotoxicity. Spleen and target cells were co-cultured in a 96-well microtiter plate at the effector to target ratio of 100:1 for 4 h at 37°C in 5% CO₂, and NK cell cytotoxicity was measured by a lactate dehydrogenase-release method using a commercial kit (Cytotox 96[®] Non-Radioactive Cytotoxicity Assay; Promega, Madison, WI) according to manufacturer's instructions.

Statistical analysis. Statistical analyses were performed with GB-Stat 5.4 (Dynamic Microsystems, Silver Spring, MD). Body weights were compared between the FR and control groups during the experiment by two-way ANOVA for repeated measures, and post-hoc analyses were done by Tukey's method. Mean survival time after the tumor inoculation, and lymphocyte, neutrophil and monocyte counts were compared between the two groups by one-way ANOVA followed by Scheffé post-hoc test. Effects of FR on tumor growth, WBC number and NK cell activity were evaluated by two-way ANOVA, and multiple comparisons were done by Tukey's test. For IFN- γ and

TNF- α data, values were logarithmically transformed to improve normality and to compensate for unequal variance, and were analyzed by two-way ANOVA followed by Tukey's test. All data are shown as means \pm SD, and statistical significance is defined as $P < 0.05$.

RESULTS

When FR was started in BDF1 mice at 8 wk of age, body weight was significantly lowered within 1 wk and at 12 wk of age, the weight in the FR group was about 75% ($P < 0.05$) of that in the control (Fig. 1). The FR-induced body weight change observed in this experiment is consistent with previous reports (Hishinuma et al. 1988, Konno et al. 1991). Tumors appeared at the site of the inoculation in both the FR and control groups by 2 wk after the treatment (at 12 wk), and the tumors progressively grew thereafter. However, the rate of the tumor growth was significantly slower in the FR group, and 3.5 wk (25 d) after the inoculation, tumor weight in the FR mice was $< 30\%$ ($P < 0.05$) of that in controls (Fig. 2A). A significant difference was also seen in survival curves between the FR and control groups (Fig. 2B). Mean survival time after tumor inoculation in the control group was 30.7 d, and all control mice died by 35 d. In contrast, in the FR group, the mean survival time was prolonged to 38.5 d ($P < 0.05$), and the final mouse survived until 54 d.

Although red blood cell number was not different between

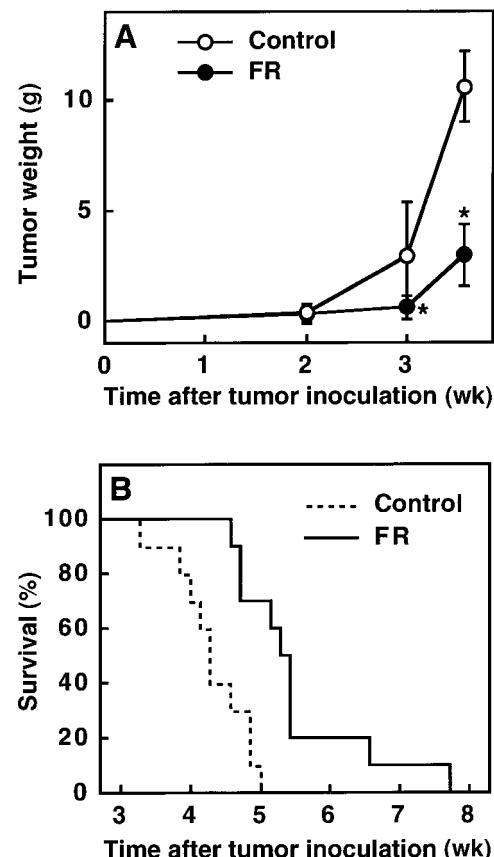


FIGURE 2 Effects of 60% food restriction (FR) of mice on the growth of implanted tumors (A) and tumor-related survival curves (B). *Panel A.* Values are means \pm SD, $n = 9$. *: $P < 0.05$ compared with the control mice at the corresponding time point. *Panel B.* Tumor-related mortality was observed in the FR and control groups after the inoculation of L1210 cells ($n = 10$). The mean survival time of the FR group (38.5 d) is significantly ($P < 0.05$) different from that of the control group (30.7 d).

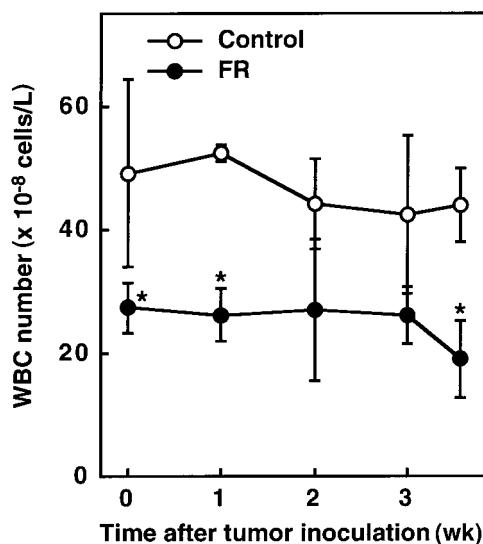


FIGURE 3 White blood cell (WBC) number before and after tumor inoculation in control and 60% food-restricted (FR) mice. Values are means \pm SD, $n = 9$. *: $P < 0.05$ compared with the control mice at the corresponding time point.

the control and FR groups (data not shown), WBC number was 45% lower ($P < 0.05$) in the FR mice (Fig. 3), as reported previously (Kubo et al. 1984). Tumor cell inoculation did not affect WBC number in either group and the number was significantly lower in the FR mice even 3.5 wk after inoculation. Lymphocytes and neutrophils were reduced in the FR group, compared to the controls, in the tumor-bearing state ($P < 0.05$) (Table 1).

Before tumor inoculation, plasma IFN- γ concentration in the FR mice was higher than that in the control ($P < 0.05$). When L1210 tumor cells were implanted, a significant elevation in the plasma IFN- γ level occurred which peaked at 3 wk in both groups (Fig. 4A). However, the FR mice had a higher plasma IFN- γ concentration even after the tumor inoculation, and at the peak, the level in the FR mice was about 3.5-fold greater than that in the controls ($P < 0.05$). The plasma TNF- α concentration also was significantly augmented by FR in the nontumor-bearing mice (Fig. 4B). Furthermore, 3.5 wk after tumor inoculation, plasma TNF- α concentration in the FR mice was also higher (about 2.5-fold greater) than that in the control ($P < 0.05$), although the tumor inoculation significantly increased the TNF- α level even in the control group.

TABLE 1

Lymphocyte, neutrophil and monocyte counts in 60% food-restricted (FR) mice and controls with implanted tumors^{1,2}

	Lymphocytes	Neutrophils	Monocytes
$\times 10^{-8}$ cells/L			
Control mice	19.2 \pm 8.76	22.7 \pm 6.73	1.78 \pm 0.642
FR mice	5.5 \pm 2.51*	11.4 \pm 3.81*	1.48 \pm 0.676

¹ FR and control mice at 12-wk-old were inoculated with L1210 tumor cells, and 3.5 wk later lymphocyte, neutrophil and monocyte counts were determined.

² Values are means \pm SD, $n = 5$. *: $P < 0.05$ compared with the control mice.

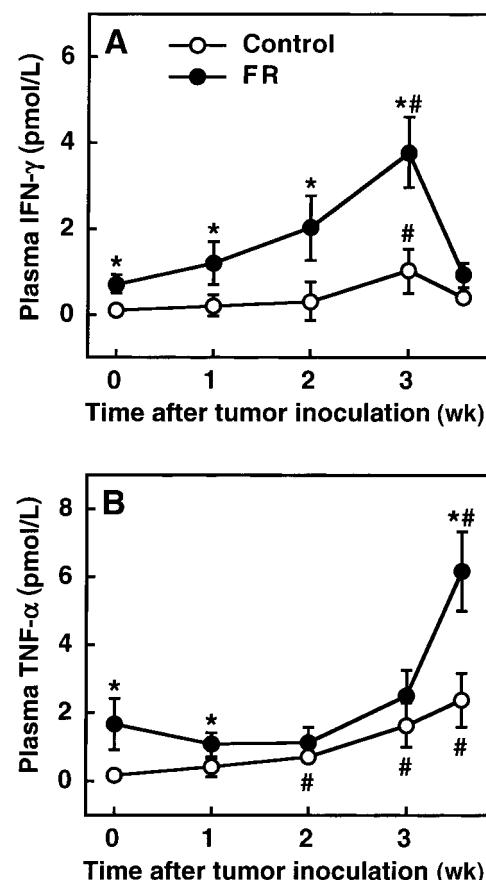


FIGURE 4 Changes in plasma interferon- γ (IFN- γ) (A) and tumor necrosis factor- α (TNF- α) (B) levels after tumor inoculation in food-restricted (FR) mice. FR and control mice at 12-wk-old were inoculated with L1210 tumor cells, and changes in plasma IFN- γ and TNF- α levels were examined. Values are means \pm SD, $n = 5$. *: $P < 0.05$ compared with the control mice at the corresponding time point. #: $P < 0.05$ compared with wk 0.

Before inoculation, there was no significant difference between the FR and control groups in NK cell cytotoxicity (Fig. 5). However, tumor inoculation significantly augmented NK cell cytotoxicity in the FR mice, but not in the control mice. Thus, at 3.5 wk after inoculation, NK cell cytotoxicity was greater in FR mice than in control mice ($P < 0.05$).

DISCUSSION

It has been shown that FR prevents the occurrence of late-life spontaneous tumor development and reduces the incidence of tumors induced by radiation or chemical carcinogens (Higami et al. 1995, Kolaja et al. 1996, Yoshida et al. 1997). In addition, it has been proposed that these effects of FR on the onset of tumors are due to the activation of host immune surveillance mechanisms (Konno et al. 1991, Weintraub et al. 1986). Our present data obtained in mice inoculated with L1210 tumor cells show that the growth of the implanted tumor is significantly suppressed in 60% FR mice, and survival time is longer in these mice (Fig. 2).

In the tumor-bearing state, it has been shown that tumor-primed CD4 $^{+}$ T cells are activated to produce IFN- γ (Yamamoto et al. 1995). IFN- γ possesses an antiproliferative activity against many transformed cell lines in addition to its antiviral and immunoregulatory functions (Abe et al. 1998, Giovarelli et al. 1986, Stark et al. 1998). TNF- α , which was

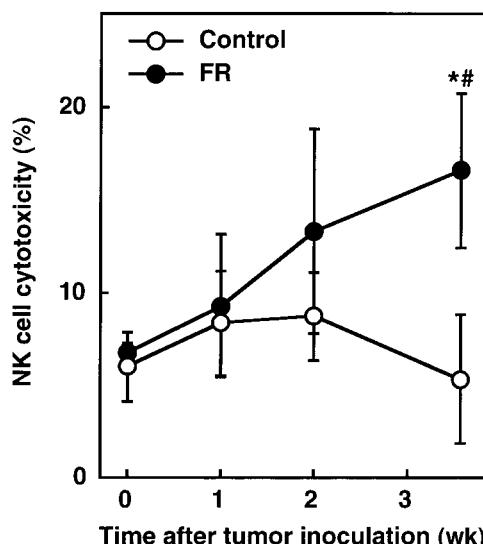


FIGURE 5 Effect of food restriction (FR) on natural killer (NK) cell cytotoxicity after tumor inoculation. FR and control mice at 12-wk-old were inoculated with L1210 tumor cells, and NK cell cytotoxicity on splenocytes was examined after the inoculation. Values are means \pm SD, $n = 4$. *: $P < 0.05$ compared with the control mice at the corresponding time point. #: $P < 0.05$ compared with a value at just before the inoculation (0 wk) in each group.

originally defined by its antitumor activity in vitro and in vivo (Carswell et al. 1975, Feinman et al. 1987, Sugarman et al. 1985), has been reported to be mainly produced by macrophages after stimulation with IFN- γ (Collart et al. 1986, Celada and Maki 1991, Han et al. 1990). L1210 leukemia cells, which were used in this experiment, are insensitive to TNF- α cytotoxicity in vitro (Leu et al. 1991), whereas the IFN- γ receptor is expressed in this cell line (Wietzerbin et al. 1986). It has also been reported that macrophage-mediated cytotoxicity of L1210 cells is augmented by IFN- γ in synergy with interleukin-2 or lipopolysaccharide in vitro, and TNF- α participates in the cytotoxic mechanism to produce nitric oxide by an autocrine mechanism in macrophages (Jiang et al. 1992). Our data (Fig. 4) indicate that in the normal state, IFN- γ and TNF- α levels are significantly greater in the 60% FR group compared to the control. Furthermore, the FR mice have greater abilities to produce IFN- γ and TNF- α even after inoculation of L1210 tumor cells, although this treatment increases cytokine production in both FR and control groups. We contend the activated production of IFN- γ and TNF- α in FR mice is related closely to the effective suppression of tumor growth. However, detailed mechanisms by which IFN- γ and TNF- α production are stimulated by FR are unclear. It has been reported that the CD4 $^+$ T cell subpopulation is augmented in FR mice in both the normal and tumor-bearing states (Gartner et al. 1992, Volk et al. 1994).

NK cells are important antitumor effectors both in vitro and in vivo (Herberman 1985, Ortaldo and Herberman 1984), and high NK cell cytotoxicity has been associated with reductions in tumor development (Reisenfeld et al. 1980). In the 60% FR group, but not in the control group, NK cell cytotoxicity was significantly augmented by the tumor inoculation (Fig. 5), suggesting that NK cells improve antitumor immunity in the FR mice. Since activated NK cells can produce IFN- γ and TNF- α (Perussia 1991), it is thought that NK cells participate in the active production of IFN- γ and TNF- α in the tumor-bearing FR mice.

It has been reported that FR reduces leukocyte number

particularly lymphocytes (Kubo et al. 1984, Weindruch and Walford 1988). In our study, tumor inoculation did not significantly change total WBC number in either the 60% FR or control groups, and lymphocyte number in the FR mice was significantly lower even in the tumor-bearing state. It has been shown that FR induces apoptosis, and immune organs, such as spleen and thymus, are comparatively more sensitive to apoptosis than other organs (Keusch et al. 1983). Thus, the low lymphocyte count in FR mice may be closely related to apoptosis in spleen and thymus. Energy restriction has been suggested to enhance T cell function in aged mice by maintaining apoptosis at levels found in younger mice, thereby removing non- or poorly functioning T cells (Spaulding et al. 1997). It is thus thought that immunologically less effective T cells are eliminated in the FR mice, presumably by apoptosis, even in the tumor-bearing state. Since CD4 $^+$ T cells participate in the production of IFN- γ in tumor-bearing mice (Yamamoto et al. 1995), the elimination of nonfunctional T cells may be one of the important factors in improving antitumor immunity in the FR mice.

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EFFECTS OF A COMBINATION OF BETA CAROTENE AND VITAMIN A ON LUNG CANCER AND CARDIOVASCULAR DISEASE

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Abstract *Background.* Lung cancer and cardiovascular disease are major causes of death in the United States. It has been proposed that carotenoids and retinoids are agents that may prevent these disorders.

Methods. We conducted a multicenter, randomized, double-blind, placebo-controlled primary prevention trial — the Beta-Carotene and Retinol Efficacy Trial — involving a total of 18,314 smokers, former smokers, and workers exposed to asbestos. The effects of a combination of 30 mg of beta carotene per day and 25,000 IU of retinol (vitamin A) in the form of retinyl palmitate per day on the primary end point, the incidence of lung cancer, were compared with those of placebo.

Results. A total of 388 new cases of lung cancer were diagnosed during the 73,135 person-years of follow-up (mean length of follow-up, 4.0 years). The active-treatment group had a relative risk of lung cancer of 1.28 (95 percent confidence interval, 1.04 to 1.57; $P=0.02$), as

LUNG cancer is the leading cause of death from cancer in the United States, accounting for approximately 29 percent of deaths from cancer and 6 percent of all deaths.¹ New approaches are essential to prevent lung cancer in persons who have smoked cigarettes or who have had occupational exposure to asbestos. Twenty-nine percent of men and 25 percent of women who are 45 to 64 years of age currently smoke,² and at least 40 percent of men and 20 percent of women in this age group are former smokers.³ An estimated 4000 to 6000 deaths from lung cancer per year are attributed to exposure to asbestos.^{4,5}

On the basis of epidemiologic observations and laboratory studies, beta carotene and vitamin A have attracted wide interest as agents that may prevent lung cancer.⁶⁻⁹ The Beta-Carotene and Retinol Efficacy Trial (CARET) is one of several recent trials to assess the

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Supported by grants (U01 CA63673, U01 CA63674, U01 CA47989, U01 CA48200, U01 CA48203, U01 CA48196, and U01 CA52596) from the National Cancer Institute.

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compared with the placebo group. There were no statistically significant differences in the risks of other types of cancer. In the active-treatment group, the relative risk of death from any cause was 1.17 (95 percent confidence interval, 1.03 to 1.33); of death from lung cancer, 1.46 (95 percent confidence interval, 1.07 to 2.00); and of death from cardiovascular disease, 1.26 (95 percent confidence interval, 0.99 to 1.61). On the basis of these findings, the randomized trial was stopped 21 months earlier than planned; follow-up will continue for another 5 years.

Conclusions. After an average of four years of supplementation, the combination of beta carotene and vitamin A had no benefit and may have had an adverse effect on the incidence of lung cancer and on the risk of death from lung cancer, cardiovascular disease, and any cause in smokers and workers exposed to asbestos. (N Engl J Med 1996;334:1150-5.)

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chemopreventive efficacy and safety of beta carotene and related agents.¹⁰⁻¹³

This report presents interim efficacy results of the CARET study, which coincided with the announcement of the steering committee's decision on January 11, 1996, to stop the trial's active intervention. Follow-up for additional end points is expected to continue for another five years.

METHODS

Study Design

The study's strategy, design, detailed methods, eligibility, pilot-study findings, and recruitment information have been published elsewhere.^{9,14-16} Briefly, CARET was organized in 1983 and began randomization in Seattle in 1985 in two pilot studies: one enrolled 816 men with substantial occupational exposure to asbestos, who were randomly assigned in a 1:1 ratio to receive either a combination of 15 mg of beta carotene per day and 25,000 IU of retinol per day (active treatment) or placebo; the second enrolled 1029 men and women with extensive histories of cigarette smoking, to receive 30 mg of beta carotene per day, 25,000 IU of retinol per day, both vitamins, or neither vitamin (two-by-two design). The trial was expanded to include additional study centers in 1988 and 1991, and all subjects were randomly assigned in a 1:1 ratio to either active treatment or placebo. The pilot groups receiving active agents were consolidated in 1988 into a single group receiving a standard daily regimen of 30 mg of beta carotene plus 25,000 IU of retinol in the form of retinyl palmitate. Thus, in the pilot study with the cohort of smokers, three subjects were assigned to active treatment for every subject assigned to placebo; therefore, the rates rather than numbers of end points must be compared between active and placebo groups. The design¹⁴ called for active intervention until late 1997 (110,000 person-years), with reporting of results in 1998.

Eligibility, Recruitment, and Randomization

Workers exposed to asbestos were men 45 to 74 years of age in the pilot study and 45 to 69 years of age in the later period of re-

cruitment. To be eligible for the study the subjects had to have first been exposed to asbestos on the job 15 years before randomization, and either have had a chest x-ray film positive for asbestos-related lung disease or have worked in specified high-risk trades — as plumbers and pipe fitters, steamfitters, shipyard boilermakers, non-shipyard boilermakers, shipyard electricians, ship scalers, insulators, plasterboard workers, or sheet-metal workers — for 5 years. The asbestos pilot study had no requirements regarding smoking¹⁵; subsequently, subjects were required to be current smokers or to have smoked within the previous 15 years. For the population of smokers, women and men were recruited from health insurance rolls and managed-care organizations if they were 50 to 69 years of age, had at least 20 pack-years of cigarette smoking, and either were currently smoking or had quit smoking within the previous six years. The participants agreed to limit their supplemental intake of vitamin A to less than 5500 IU per day and to take no supplemental beta carotene. A total of 4060 workers exposed to asbestos and 14,254 heavy smokers (44 percent of whom were women) were randomized. We provided detailed information for informed consent at recruitment and regularly thereafter, including a letter to each participant describing the results of the 1994 Alpha-Tocopherol, Beta Carotene Cancer Prevention Study (ATBC).¹⁰

Active agents and placebos were purchased from Hoffmann-LaRoche and formulated by Tishcon Corporation. Both formulations were given as capsules. Beta carotene beadlets were combined with retinyl palmitate in a single capsule and dispensed in bottles, which were weighed and their contents checked. We assessed the subjects' compliance by weighing the returned bottles to estimate the number of capsules remaining (in 85 percent of the assessments) or by relying on the subjects' own estimates (15 percent). Blood was collected annually from the original pilot participants and every two years from the other participants.

Subjects who stopped receiving study vitamins for any reason other than death were defined as inactive participants and were still followed for end points and counted in the analyses.

Data Collection and Monitoring of Safety and End Points

Each year active participants visited a study center once and were telephoned twice, at four-month intervals. Inactive participants were telephoned semiannually. Over 97 percent of scheduled contacts were completed. As of December 15, 1995, ascertainment of vital status was more than 98 percent complete.

Symptoms and signs and newly diagnosed medical conditions were monitored closely by questionnaire at all contacts and in limited physical examinations during study-center visits; laboratory values for liver function and serum analytes were monitored annually in participants randomized in the pilot studies (for use in adjusting estimates of relative risk with the case-cohort approach).¹⁷ The 13 monitored symptoms were graded according to the CARET symptom-assessment scale.¹⁶ An independent safety and end-points monitoring committee met semiannually to review in blinded fashion data coded according to intervention group. When the results of the ATBC Cancer Prevention trial¹⁰ became available, the committee reviewed the results of the first interim analysis and requested that the blinding be ended. Subsequently, the committee reviewed data unblinded.

Ascertainment and Evaluation of End Points

All initial reports of cancers and deaths from each study center were submitted to the coordinating center and entered into a tracking system. Participants and all study staff members involved in the ascertainment and evaluation of end points and assignment of final diagnoses remained unaware of the participants' treatment assignment throughout the trial. Clinical records and, for tumors involving the lung, pathology specimens were obtained for independent review by the end-points review committee, composed of two oncologists, two internists, and a pathologist. An end point was considered confirmed when the end-point review process was completed. Through December 15, 1995, a total of 2420 end points had been reported: 1446 cancers (in 1353 participants) and 974 deaths. Of the initial reports of

lung cancer for which the end-point-review process was completed, 90 percent were confirmed; most of the remainder were found to be metastases and recurrences.

Statistical Analysis

The primary analysis, based on the intention to treat, was designed to test for differences between treatment groups in the incidence of lung cancer with a weighted log-rank statistic stratified according to the risk group (workers exposed to asbestos or heavy smokers), time of recruitment (pilot study or subsequent period), and study center (six centers).¹⁴ Parameter estimates and the results of statistical tests were similar with and without the weighting; we present here the unweighted results. Estimates of relative risk and confidence intervals were obtained from stratified Cox regression models with the same strata as the log-rank statistics. The cumulative incidence of end points was plotted through 5½ years of follow-up because of the small number of participants beyond that time and involved 354 participants with new cases of lung cancer, 829 deaths, and 67,449 person-years of follow-up. Follow-up for all participants began at randomization.

The prespecified monitoring policy for stopping the trial early because of a benefit or adverse effect of the study vitamins was based on O'Brien-Fleming boundaries¹⁸ applied to the weighted number of confirmed lung-cancer end points, the primary end point. The critical P values were those of 0.0006 or lower for the first interim analysis in 1994 and those of 0.007 or lower for the second interim analysis in 1995. Results are based on active intervention through December 15, 1995, at which time the 18,314 participants had accumulated 73,135 person-years of follow-up (mean, 4.0 years; median, 3.7).

RESULTS

Characteristics of the Participants

The two randomized groups were well matched, with a high-risk profile for lung cancer and cardiovascular disease in both the smokers and the workers exposed to asbestos (Table 1). All smokers were encouraged and assisted, if willing, to stop smoking, and all former smokers were encouraged to maintain that status. Among current smokers, there was a net smoking-cessation rate of 5 percent per year.

Through December 15, 1995, 15 percent of the workers exposed to asbestos who were assigned to active treatment became inactive participants, as compared with 14 percent of those assigned to placebo. The respective values in the group of heavy smokers were 20 percent and 19 percent. Among the active participants, the mean rates of capsule consumption were 93 percent through five years of follow-up, with no significant differences between treatment groups. The percentage of participants who took non-study-related supplemental beta carotene or vitamin A in doses of more than 5500 IU per day was low (2 percent and 1 percent, respectively). After five years of study supplementation, the median serum beta carotene concentration in the active-treatment group was 2100 ng per milliliter, as compared with 170 ng per milliliter in the placebo group; serum retinol levels were about 10 percent higher than those in the placebo group ($P<0.01$). Except for slight skin yellowing in some of those receiving beta carotene (0.3 percent had yellowing of grade 3 or higher on the CARET symptom-assessment scale),¹⁶ there were no differences of clinical importance between groups in any of the 13 monitored symp-

toms and signs, in tests of liver function, or in newly diagnosed conditions.

Incidence of Lung Cancer

The incidence of lung cancer was the primary end point. Through December 15, 1995, 388 participants — 2 percent of the total — were reported to have new cases of lung cancer (5.4 per 1000 person-years). In the case of 286, the end points were confirmed, whereas in the case of 102, further review by the end-points committee was pending. Among the 388, 254 had died. Five participants had two primary lung cancers each. The 388 participants represent 79 percent of the 490 participants projected in our statistical design to have lung cancer by the end of the intervention. The 73,135 person-years of follow-up accrued correspond to 66 percent of the total of 110,000 person-years projected.¹⁴

The active-treatment group had a relative risk of lung cancer of 1.28 (95 percent confidence interval, 1.04 to 1.57; $P=0.02$), as compared with the placebo group (Table 2). This result includes relative risks of 1.40 (95 percent confidence interval, 0.95 to 2.07) for workers exposed to asbestos, 1.42 (95 percent confidence interval, 1.07 to 1.87) for heavy smokers who were smoking at the time of randomization, and 0.80 (95 percent confidence interval, 0.48 to 1.31) for heavy smokers who were no longer smoking at the time of randomization. There was no statistical evidence of heterogeneity of the relative risk among these subgroups. Figure 1 shows the cumulative incidence of lung cancer after randomization; the incidence in the active-treatment and placebo groups was virtually identical for the first 18 months. There was no statistically significant effect of the intervention on

survival after the diagnosis of lung cancer (relative risk of survival after diagnosis of lung cancer in the active-treatment group as compared with the placebo group, 1.05; 95 percent confidence interval, 0.80 to 1.37).

Incidence of Other Cancers

Active treatment had no statistically significant effect on the risk of mesothelioma. There were 23 cases: 14 in the active-treatment group and 9 in the placebo group. The remaining 1030 new cases of cancer (including the 300 prostate cancers, the second most common cancer in this population) were distributed nearly evenly between the two treatment groups.

Mortality Rates

As shown in Table 2 and Figure 2, the mortality rate was 17 percent higher in the active-treatment group than in the placebo group ($P=0.02$). Among the population of heavy smokers, the relative risk was not significantly different between those who were smoking at the time of randomization and those who were no longer smoking at that time (relative risk, 1.15 vs. 1.06).

Analysis according to the cause of death (confirmed causes only; $n=764$) showed that in the active-treatment group, as compared with the placebo group, the relative risk of death from any cause was 1.18 (95 percent confidence interval, 1.02 to 1.37); of death from lung cancer, 1.46 (95 percent confidence interval, 1.07 to 2.00); and of death from cardiovascular causes (codes 390 to 459 and 798 of the *International Classification of Diseases, 9th Revision, Clinical Modification*), 1.26 (95 percent confidence interval, 0.99 to 1.61). As shown in Figure 2, there was no significant difference between treatment

groups in the incidence of death from all causes during the first 24 months. A review of all causes of death revealed no additional statistically significant differences between the treatment groups.

DISCUSSION

CARET was initiated in 1983 to test the hypothesis that beta carotene and vitamin A, through complementary antioxidant and differentiation-promoting actions and possibly through immunologic protective effects, could reduce the incidence of lung cancer in high-risk populations. The trial met high standards for accrual, efficiency, quality assurance, and ascertainment of end points. There have been no side effects attributable to the intervention regimen. The participants have shown a high level of commitment to the trial.

The results of the trial are troubling. There was no support for a

Table 1. Risk Factors among the Participants at Base Line.*

CHARACTERISTIC	WORKERS EXPOSED TO ASBESTOS		HEAVY SMOKERS	
	ACTIVE TREATMENT	PLACEBO	ACTIVE TREATMENT	PLACEBO
No. randomized	2044	2016	7376†	6878†
Age — yr	57±7	57±7	58±5	58±5
Female sex — no. (%)	0	0	3208 (43)	3081 (45)
Race or ethnic group — no. (%)				
White	1805 (88)	1775 (88)	7000 (95)	6487 (94)
Black	152 (7)	153 (8)	103 (1)	122 (2)
Hispanic	36 (2)	43 (2)	101 (1)	95 (1)
Other or unknown	51 (2)	45 (2)	172 (2)	174 (3)
Smoking status — no. (%)				
Never smoked	68 (3)	64 (3)	0	0
Former smoker	1195 (58)	1175 (58)	2473 (34)	2331 (34)
Current smoker	781 (38)	777 (39)	4903 (66)	4547 (66)
Cigarettes smoked/day				
Former smokers	25±12	25±12	28±11	28±11
Current smokers	24±10	25±10	24±9	24±8
Pack-years of smoking‡	43±24	42±24	50±21	49±20
Years since quitting smoking§	10±8	10±8	3±2	3±2

*Plus-minus values are means ± SD. Because of rounding, not all columns total 100 percent.

†The imbalance in the numbers is due to the assignment of 3 pilot participants to active treatment for every 1 assigned to placebo (773 to active treatment vs. 256 to placebo).

‡Only former and current smokers were included.

§Only former smokers were included.

Table 2. Incidence and Estimated Relative Risk of Lung Cancer and Death from All Causes.*

GROUP	NO. OF SUBJECTS		UNREFUTED CASES OF LUNG CANCER/1000 PERSON-YR						DEATHS/1000 PERSON-YR		DEATH FROM ALL CAUSES		
			LUNG CANCER		DEATHS/1000 PERSON-YR			DEATH FROM ALL CAUSES					
	ACTIVE TREATMENT	PLACEBO	ACTIVE TREATMENT	PLACEBO	P VALUE†	RELATIVE RISK‡	95% CI	ACTIVE TREATMENT	PLACEBO	P VALUE†	RELATIVE RISK‡	95% CI	
All subjects	9420	8894	5.92	4.62	0.02	1.28	1.04–1.57	14.45	11.91	0.02	1.17	1.03–1.33	
Workers exposed to asbestos	2044	2016	6.05	4.33	0.08	1.40	0.95–2.07	17.76	14.30	0.04	1.25	1.01–1.56	
Heavy smokers	7376	6878	5.87	4.74	0.09	1.23	0.96–1.56	13.26	10.91	0.14	1.13	0.96–1.32	

*CI denotes confidence interval.

†The P values were calculated by two-sided log-rank tests stratified according to the risk group (workers exposed to asbestos or heavy smokers), time of recruitment (pilot study or subsequent period), and study center (six centers).

‡Estimated relative risk in the active-treatment group as compared with the placebo group. The log-rank test, stratified according to risk group, time of recruitment, and study center, was used to estimate the relative risk.

beneficial effect of beta carotene or vitamin A, in spite of the large advantages inferred from observational epidemiologic comparisons of extreme quintiles or quartiles of dietary intake of fruits and vegetables or of dietary intake or serum levels of beta carotene or vitamin A.^{19,20} With 73,135 person-years of follow-up, the active-treatment group had a 28 percent higher incidence of lung cancer than the placebo group, and the overall mortality rate and the rate of death from cardiovascular causes were higher by 17 percent and 26 percent, respectively.

These results confirm and extend the unexpected results reported for beta carotene in the ATBC Cancer Prevention Study in Finland.¹⁰ We cannot distinguish the effects of beta carotene from those of vitamin A, since the two agents were administered in combination, under the hypothesis that they might have a favorable effect through complementary molecular actions.⁹

The second interim analysis led our safety and endpoints monitoring committee and steering committee to recognize the extremely limited prospect of a favorable overall effect, as well as the possibility of true adverse effects. The decision to stop the intervention was made by the steering committee on January 11, 1996. It is possible that the excess mortality in the active-treatment group may have vanished or become statistically insignificant with completion of the intended intervention period plus several years of follow-up; such reversals of findings have occurred both in the course of a single large, randomized trial^{21,22} and in subsequent randomized trials of the same agent or class of agents.^{23–25} However, it was impossible to ignore the results of the ATBC Cancer Prevention Study¹⁰ in deciding whether to stop the active-intervention phase.

We have no explanation for the possible adverse associations that we have observed to date. There was no evidence of systemic toxicity in any organ from the vitamin A or, except for the expected skin yellowing, the beta carotene. The regimen did not produce clinically important hypertriglyceridemia.²⁶ We considered continuing the trial with the retinyl palmitate alone; however, the need to rerandomize, the extended follow-up required, and the uncertainty about the involvement of the vitamin A made this plan infeasible.

In this trial, beta carotene treatment raised the median serum beta carotene levels to 12 times the baseline levels and the placebo group's median values. Such levels may conceivably be toxic or at least cause serious disequilibrium with other compounds important to redox relations or other cellular mechanisms. Beta carotene has been postulated to have a pro-oxidant effect under certain nonphysiologic conditions.^{27,28} One study reported that the administration of beta carotene drastically lowered vitamin E levels,²⁹ but we³⁰ and three other groups^{10,31,32} have found no such effect. In preliminary analyses of serum beta carotene levels during active treatment, we could find no support for the hypothesis that subjects with the highest serum levels of beta carotene were at greater risk for lung cancer or death from cardiovascular causes, cancer, or any cause.

The results of our study and the ATBC Cancer Prevention Study¹⁰ in populations at high risk for lung cancer and cardiovascular disease and the finding of the Physicians' Health Study¹¹ of no benefit or harm after 12 years of beta carotene treatment clearly do not support

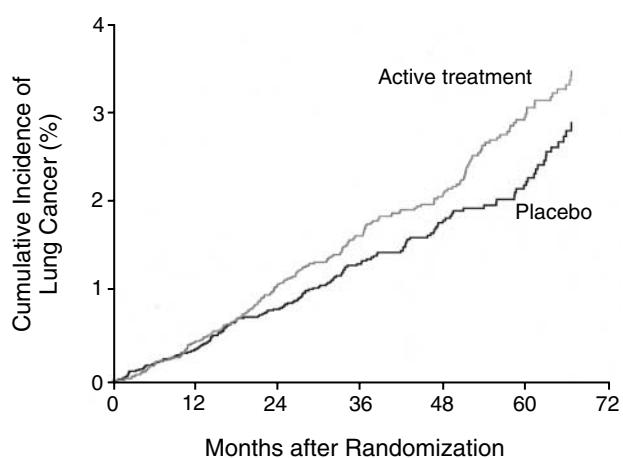


Figure 1. Kaplan-Meier Curves of the Cumulative Incidence of Lung Cancer among Participants Receiving Active Treatment and Those Receiving Placebo.

Data are shown only through 5½ years of follow-up because of the small numbers of participants beyond that time.

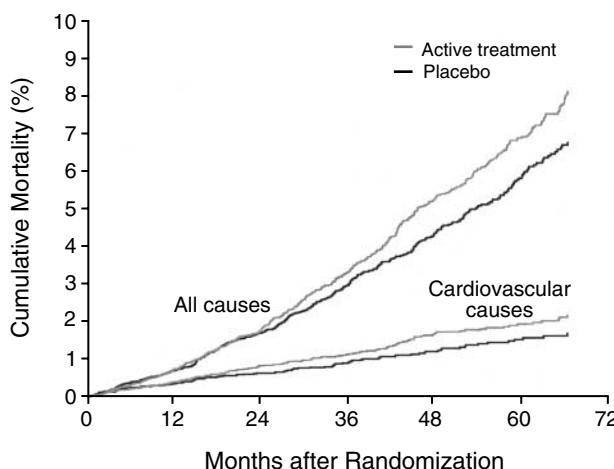


Figure 2. Kaplan-Meier Curves of the Cumulative Incidence of Death from All Causes and Confirmed Cardiovascular Causes among Participants Receiving Active Treatment and Those Receiving Placebo.

Data are shown only through 5½ years of follow-up because of the small numbers of participants beyond that time.

the widely accepted conclusion drawn from observational epidemiologic studies that beta carotene is a primary component responsible for the association of lower risks of cancer and death from cardiovascular causes with high intakes of fruits and vegetables.^{19,33} Such studies typically compare extreme subgroups for such dietary features, ignoring or only crudely adjusting for many other potentially relevant variables, such as the intake of red meat, physical activity, life situations, and other behavior.²⁰ The dietary associations seemed well matched to serum beta carotene measurements; as in other observational analyses, in our study base-line serum beta carotene levels were inversely correlated with the subsequent incidence of lung cancer in both groups. However, randomized prevention trials are needed to test the hypothesis that increased beta carotene intake can be protective.

The results of four large-scale chemoprevention trials of beta carotene and related agents can be summarized. The ATBC Cancer Prevention Study¹⁰ tested daily supplementation with 20 mg of beta carotene and 50 mg of alpha-tocopherol (two-by-two factorial design) in 29,133 male smokers. The Physicians' Health Study¹¹ tested supplementation with 50 mg of beta carotene on alternate days in 22,071 male physicians, 50 percent of whom had never smoked, 39 percent of whom were former smokers, and 11 percent of whom were currently smoking. We tested daily supplementation with a combination of 30 mg of beta carotene and 25,000 IU of retinyl palmitate. Finally, a study conducted in Linxian, China,³⁴ assessed the value of daily supplementation with a combination of 15 mg of beta carotene, 50 µg of selenium, and 30 mg of alpha-tocopherol as compared with three other combinations of vitamins and minerals in a complex factorial design in 29,584 adults presumed

to be vitamin- and mineral-deficient — a very different population from those examined in the other studies. In the ATBC Cancer Prevention Study, 876 new cases of lung cancer were diagnosed, yielding a relative risk of lung cancer of 1.18 among subjects who received beta carotene (with or without alpha-tocopherol), as compared with those who did not. In the Physicians' Health Study, 170 new cases of lung cancer were diagnosed, for a relative risk of lung cancer of 0.93 among men taking beta carotene, as compared with those who received placebo. In our study, there were 388 new cases of lung cancer, yielding a relative risk of such cancer of 1.28 among the subjects who received beta carotene and retinyl palmitate, as compared with those who received placebo. The Linxian study did not report the incidence of lung cancer. Among the subjects who received beta carotene, the relative risk of death from any cause was 1.08 in the ATBC Cancer Prevention Study (3570 deaths), 1.01 in the Physicians' Health Study (1947 deaths), 1.17 in our trial (974 deaths), and 0.91 in the Linxian study (2127 deaths).

Reversing or overcoming lifelong metabolic or exogenous risk factors may require 5 to 10 years or more to account for the latent periods of cancers. Favorable effects may be particularly difficult to achieve in the face of a continuing carcinogenic and atherogenic assault in smokers; alternatively, antioxidants and antiproliferative agents might act on the constituents of cigarette smoke. Long-term follow-up both during and after active treatment with potential chemopreventive agents is essential if we are to have any hope of observing long-term benefits and evaluating long-term risks. During the postintervention follow-up of our study subjects, as end points continue to accrue, we will conduct laboratory analyses and analyze various subgroups, particularly former smokers.

Our findings provide important new information with respect to public policy and public health. When these results are combined with those from the ATBC Cancer Prevention Study¹⁰ and the Physicians' Health Study,¹¹ they make it clear that there can be little enthusiasm about the efficacy or safety of supplemental beta carotene or vitamin A in efforts to reduce the burdens of cancer or heart disease in certain populations. However, we still recommend the dietary intake of fruits and vegetables.

Other agents that prevent lung cancer and coronary heart disease must be identified and subjected to rigorous trials of safety and efficacy. Meanwhile, to reduce the risk of these diseases we must rely primarily on three approaches: smoking cessation, prevention of smoking, and avoidance of occupational and environmental exposure to carcinogenic substances.

We are indebted to the study subjects, staff members, and investigators; to the members of the safety and end-points monitoring committee over the past several years (Anthony Miller, Robert Bruce, Julie Buring, Frank Iber, and O. Dale Williams); and to our colleagues who provided data from the Physicians' Health Study.

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Protease inhibitor suppression of colon and anal gland carcinogenesis induced by dimethylhydrazine

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In the present study, we examined the ability of chymostatin, a highly specific inhibitor of chymotrypsin, to suppress dimethylhydrazine-induced colon carcinogenesis, and the dose-response relationship for an extract of soybeans containing the Bowman-Birk inhibitor (BBI) to suppress dimethylhydrazine-induced colon carcinogenesis, when added to the diet of mice. Our results showed that: (i) diets containing 0.1% BBI reduced the incidence of adenocarcinomas of the colon ~50%, but had no effect on the incidence of squamous cell carcinomas of the anal gland; (ii) the suppressive effect requires protease inhibitor activity, as the autoclaved BBI, in which all protease inhibitory activity has been destroyed, was ineffective at suppressing the incidence of adenocarcinomas; (iii) chymostatin suppressed the incidence of squamous cell carcinomas of the anal gland, but not adenocarcinomas of the colon; and (iv) the growth rates of the animals were the same in each of the experimental groups. Our results indicate that the levels of anticarcinogenic protease inhibitors present in the diets of these animals do not have any adverse effects on the growth or general health of the animals.

Introduction

Cancer of the colon and rectum is the second leading cause of cancer death in US adults (1). In the United States, ~130 000 new cases of colon cancer are reported each year and colon cancer accounts for about 60 000 deaths annually. The incidence and mortality rates of colon cancer have remained unchanged for the last 50 years (1,2). Since colon cancer has been largely refractory to contemporary treatment regimens (2), an alternative approach to controlling this disease is through prevention. Cancer prevention via dietary means may well be the most cost-effective approach towards controlling this disease.

Epidemiological data suggest that nutritional factors play a major role in the etiology of cancer at many different sites (3–7). For example, high dietary levels of legumes have been associated with low cancer rates in general and are inversely correlated with the incidences of breast, colon and prostate cancer (3,6,7). Legumes are known to contain high concentrations of protease inhibitors (8), as well as other agents (such as lectins, etc.) which may modify carcinogenesis. Protease inhibitors have been shown to have anticarcinogenic activity *in vivo* and *in vitro* (e.g. 9–22). We have reported that the soybean-derived Bowman-Birk inhibitor (BBI*) (8,23) suppresses dimethylhydrazine (DMH)-induced colon and liver carcinogenesis in mice, when present in the diet (17,20), 7,12-dimethylbenz[a]anthra-

cene-induced cheek pouch carcinogenesis in hamsters, when topically applied (16), and 3-methylcholanthrene-induced lung tumorigenesis in mice (21). BBI has also been shown to suppress carcinogen-induced malignant transformation *in vitro* (9,22).

In general, inhibitors of chymotrypsin have been found to be the most effective suppressors of transformation *in vitro* (15). For example, nanomolar concentrations of chymostatin, a highly specific inhibitor of chymotrypsin (24), have been shown to inhibit radiation-induced transformation of C3H/10T1/2 cells (14,15). In the current experiments, we have continued our studies examining the ability of the BBI to suppress colon carcinogenesis and have also examined the effect of chymostatin on colon carcinogenesis.

Materials and methods

Chemicals

The BBI was obtained from Central Soya (Fort Wayne, IN) and was prepared as described (17). Chymostatin was obtained from the US/Japan Cooperative Cancer Research Program. 1,2-Dimethylhydrazine dihydrochloride was obtained from Aldrich. Stock solutions of DMH were freshly prepared in 1 mM EDTA and adjusted to pH 6.5 with NaOH before each set of injections.

Preparation of autoclaved BBI (ABBI) and succinylated BBI (SBBI)

ABBI was prepared by autoclaving BBI for 30 min. SBBI was prepared by the method of Smirnoff (25). Briefly, BBI was dissolved in distilled water (5 mg/ml) and brought to pH 9 by the addition of 1 N NaOH. Succinic anhydride was added (in a ratio of 0.5 g succinic anhydride/g BBI), the sample was stirred and maintained at pH 8–9 by the dropwise addition of 1 N NaOH. After 2 h, additional succinic anhydride was added and the reaction was allowed to proceed for an additional 2 h. Next, the pH was adjusted to 3.5 by the addition of concentrated HCl, the sample was centrifuged (10 000 g × 30 min, 4°C), and the pellet was resuspended in distilled water, dialyzed against distilled water (three changes, overnight) and lyophilized to dryness. The modified (succinylated) BBI was assayed for its ability to inhibit chymotrypsin and trypsin; chymotrypsin inhibitory activity was present while >99% of the trypsin inhibitory activity of BBI was lost.

Animals

Eight week old male CD-1 mice were obtained from Charles River Breeding Laboratories (Wilmington, MA), and upon arrival in the laboratory were randomly assigned into treatment groups, housed three mice per cage and placed on the experimental diets. The animals were maintained in a controlled environment animal facility at 25°C with a 12 h light/dark cycle, and received food and water *ad libitum*. The mice were fed a modification of diet no. 101 from Bio Serve (Frenchtown, NJ), a methionine-supplemented diet based on guidelines from the American Institute of Nutrition for a purified diet for rats and mice (26). Animals receiving protease inhibitor supplements also received diet no. 101 with 20% dietary protein, with the protease inhibitor supplement replacing various percentages of the casein component of the diet. All animals were weighed at weekly intervals and received weekly i.p. injections of DMH. The DMH injections began when the mice were 10 weeks old, after a 2 week adaptation to the experimental diets and continued for 20 weeks. The animals were killed by cervical extension. For histopathological analysis, any macroscopically abnormal organs, as well as the colon and pancreas, were removed from each mouse. The pancreas was weighed and examined for grossly observable tumors or other abnormalities. The entire colon was examined and the distance between the anus and any visible tumor was measured. The section of the colon containing the tumor was then excised. In addition to the abnormal sections of the colon, normal appearing areas from each of the three major regions (representing the entire length of the colon) were taken from each mouse for histopathologic analysis. These sections, the tumors, pancreata and any macroscopically abnormal tissues were preserved in 10% buffered formalin and embedded in paraffin; 4–5 µm thick sections were prepared and stained with hematoxylin and eosin. Benign and malignant colon tumors were classified as described (27). Pathological analysis was performed without knowledge of the treatment group to which the animals belonged.

*Abbreviations: BBI, Bowman-Birk inhibitor; DMH, dimethylhydrazine; ABBI, autoclaved BBI; SBBI, succinylated BBI.

Table I. Effects of various protease inhibitor preparations on colon carcinogenesis (adenomatous tumors)

Treatment group ^a	No. and types of tumors		Other pathology ^b	No. of animals with tumors/total no. of animals	
	Benign	Malignant		Benign ^c	Malignant ^d
1. DMH	1 adenoma	12 polypliod adenocarcinomas	2 muc. hyp.	1/19 (5.3%)	12/19 (63%)
2. DMH + ABBI (0.1%)	1 adenoma	10 polypliod adenocarcinomas	1 muc. hyp.	1/16 (6.3%)	10/16 (62%)
3. DMH + BBI (0.1%)	3 adenomas	8 polypliod adenocarcinomas	4 muc. hyp.	3/23 (13%)	8/23 (35%)
4. DMH + BBI (0.01%)	3 adenomas	8 polypliod adenocarcinomas	2 muc. hyp.	3/18 (17%)	8/18 (44%)
5. DMH + BBI (0.0005%)	none	10 polypliod adenocarcinomas	3 dys. hyp.	0/15	10/15 (67%)
6. DMH + SBBI (0.1%)	6 adenomas	10 polypliod adenocarcinomas 1 adenocarcinoma	1 muc. hyp.	5/21 (24%)	11/21 (52%)
7. DMH + chymostatin (0.1%)	2 adenomas	9 polypliod adenocarcinomas	1 muc. hyp.	2/17 (12%)	9/17 (53%)

^aGroups 1, 2, 4, 5 and 7 contained 20 animals/group; groups 3 and 6 contained 23 animals/group.^bOther pathology: muc. hyp., mucosal hyperplasia; dys. hyp., dysplastic hyperplasia.^cStatistical analysis (chi-squared) for benign tumors: groups 1 versus 2–7, $P > 0.05$.^dStatistical analysis (chi-squared) for malignant tumors: groups 1 versus 2, $P > 0.05$; groups 1 versus 3, $P < 0.05$; groups 1 versus 4–7, $P > 0.05$.

Results

The current studies were designed to determine the ability and dose-response relationship of various preparations of BBI to suppress DMH-induced colon carcinogenesis in mice. A second objective of the study was to determine whether chymostatin has the ability to suppress colon carcinogenesis. The mice were given DMH injections while maintained on diets containing: (i) 0.1% (1 part per 1000), 0.01% (0.1 part per 1000) or 0.0005% (0.005 part per 1000) of the BBI extract; (ii) chymostatin, a low mol. wt chymotrypsin inhibitor (24); and (iii) modified preparations containing ABBI (autoclaving destroys all chymotrypsin and trypsin inhibitory activity) or SBBI [this BBI preparation has been treated with succinic anhydride, which specifically succinylates a critical lysine residue (Lys16) in the trypsin inhibitory portion of the BBI molecule. This covalent modification selectively removes the trypsin inhibitory activity, leaving the chymotrypsin inhibitory activity intact (17,25)]. The experimental group receiving ABBI also served as an isocaloric diet control group for the treatment groups receiving the various BBI preparations.

In the first series of experiments, DMH-induced adenocarcinomas in the colon in 63% of the animals; 0.1% BBI in the diet significantly reduced the incidence of these tumors in the DMH-treated animals to 34.8% (Table I). At 0.01% of the diet, BBI produced an insignificant decrease in tumor incidence, while dietary levels of 0.0005% BBI, 0.1% ABBI, 0.1% SBBI or 0.1% chymostatin had no effect on the induction of adenocarcinomas (Table I).

Interestingly, a different pattern of tumor suppression was observed when we compared the incidence of squamous cell carcinomas of the anal gland in animals fed the different diets. The BBI had no effect on the incidence of anal gland tumors in these animals, while a suppressive effect on these tumors was observed with a diet supplemented with chymostatin (Table II). We performed two additional sets of experiments to determine the effects of chymostatin on gastro-intestinal tract tumors. In these studies, DMH injections (15 mg/kg in experiment 1 and 20 mg/kg in experiment 2) were given for 20 weeks; the animals were killed 6 months after the beginning of carcinogen injections. As two different experiments were performed to determine the effect of chymostatin on colon carcinogenesis, we analyzed our data using the likelihood ratio chi-squared test (GLIM Statistical Package, Royal Statistical Society) to rule out the effect of experimental variation. Using this analysis, we found that there

Table II. Effects of various protease inhibitor preparations on anal gland carcinogenesis^a

Treatment group	Animals bearing malignant tumors of the anal gland ^b /total no. of animals in treatment group	Other pathology ^c
1. DMH	7/19 (37%)	5 sq. met.
2. DMH + ABBI (0.1%)	5/16 (31%)	2 sq. met. 1 abscess
3. DMH + BBI (0.1%)	8/23 (35%)	3 sq. met.
4. DMH + BBI (0.01%)	8/18 (44%)	2 dys. hyp.
5. DMH + BBI (0.0005%)	6/15 (40%)	4 sq. met. 1 sq. hyp.
6. DMH + SBBI (0.1%)	11/21 (52%)	1 sq. hyp. 1 hyperkeratosis
7. DMH + chymostatin (0.1%)	3/17 (18%)	carcinoma

^aStatistical analysis (chi-squared): groups 1 versus 2–7, $P > 0.05$.^bAll malignant tumors of the anal gland were squamous cell carcinomas; no benign tumors were observed.^cOther pathology: sq. met., squamous metaplasia; sq. hyp., squamous hyperplasia; dys. hyp., dysplastic hyperplasia. No other tumors were observed.

were no significant differences between the experiments. Further, these studies demonstrate that chymostatin had a significant suppressive effect on DMH-induced squamous cell carcinomas of the anal gland but had no effect on adenomatous tumors of the colon (Table III).

It has been reported that high levels of protease inhibitors in the diet can lead to reduced growth rates, resulting from decreased protein utilization, as well as pathologic changes in the pancreas (28–30). Consequently, the animals were weighed at weekly intervals to assess their rate of growth; no difference in the growth rates for animals in any of the experimental groups was observed. The pancreata from the animals in each group were dissected out and weighed. No difference in the ratio of pancreas wt/body wt was observed between those animals on the control diet and those receiving diets containing protease inhibitor. Further, the protease inhibitor-containing diets had no effect on the gross appearance or histopathological characteristics of the pancreas in these studies.

Table III. Effects of chymostatin on the induction of gastrointestinal tract carcinogenesis (colon and anal gland)

Treatment group	Exp. no. ^a	Total no. of adenomatous colon tumors	Total no. of squamous anal tumors	Fraction of mice with adenomatous tumors (adenomas and adenocarcinomas) of the colon ^b	Fraction of mice bearing anal tumors (squamous cell carcinomas) per experiment ^c	Total ^{c,d}
1. DMH	1	6	6	5/15 (33%)	5/15 (33%)	20/52 (39%)
	2	42	15	22/37 (60%)	15/37 (41%)	
2. DMH + chymostatin (0.1%)	1	5	3	5/15 (33%)	3/15 (20%)	10/51 (20%)
	2	34	7	18/36 (50%)	7/36 (19%)	

^aExperiment 1 contained 15 animals/treatment group; experiment 2 contained 38 animals/treatment group.

^bNo significant difference was observed between the number of colon tumors in the DMH and DMH + chymostatin groups; likelihood ratio chi-squared test: groups 1 versus 2 ($P = 0.48$).

^cA significant difference was observed between the number of anal tumors in the DMH and DMH + chymostatin groups; likelihood ratio chi-squared test: groups 1 versus 2 ($P = 0.03$).

^dIn the DMH treatment group two ear canal tumors were observed; no other tumors were observed besides those of the gastrointestinal tract.

Discussion

In the present study, we have utilized the DMH-induced colon carcinogenesis model to study the inhibition of colon carcinogenesis in mice by dietary protease inhibitors. Our results support our previous findings that dietary intake of BBI will suppress adenomatous tumors of the colon (17,20). BBI was an effective anticarcinogenic agent when present at a concentration of 0.1%, while lower concentrations of the inhibitor were less effective or ineffective at reducing tumor incidence. Consequently, these studies, in addition to previous work (17), suggest that the 0.1% level of the BBI extract is the minimal concentration necessary for a significant reduction in adenomatous tumors of the colon. Lower levels of dietary BBI are still effective at suppressing tumors at other sites; however, at 0.01% of the diet, the BBI extract has been shown to suppress DMH-induced angiosarcomas of the liver (17). It should be noted that the levels of protease inhibitors shown to be anticarcinogenic in our studies are roughly comparable (on a mg/kg basis) to the estimated intake of protease inhibitors by specific human populations consuming relatively high levels of dietary protease inhibitors, such as the Japanese and Seventh Day Adventists (17). These populations have been shown to have lower than normal incidences of breast, colon and prostate cancer (3–7); it is possible that the low cancer incidence in these populations could be, at least in part, attributable to their dietary intake of anticarcinogenic protease inhibitors.

The inability of ABBI to suppress the formation of adenocarcinomas provides strong evidence that protease inhibitory activity is necessary for tumor suppression. We also observed that SBBI had no effect on colon carcinogenesis in these studies. SBBI contains chymotrypsin inhibitory activity but has had the trypsin inhibitory activity of BBI selectively removed. A number of different explanations are possible for our results showing a lack of suppression of carcinogenesis by SBBI. Although SBBI still retains the ability to suppress transformation *in vitro*, it is not known whether it has the same ability as BBI to pass through the stomach in an intact form and enter the intestine in a form capable of interacting with proteases. Even if SBBI does reach the intestine as an active protease inhibitor, it may not be taken up by the colonic epithelial cells as efficiently as BBI and, thus, may not be able to interact with, and inhibit, the critical enzymes in these cells. Additionally, SBBI may be more water soluble than the native inhibitor and, hence, may be more rapidly excreted. Alternatively, the trypsin inhibitory portion of BBI may play an important role in inhibiting adenomatous colonic tumors.

We have observed previously that SBTI (a strong inhibitor of trypsin) does not directly suppress radiation transformation *in vitro*, but will suppress the enhancement of radiation-induced transformation *in vitro* by tumor promoters (12,13). Consequently, while chymotrypsin inhibitors are likely to be the most potent anticarcinogenic agents, trypsin inhibitors, such as SBTI, may also have cancer chemopreventive value by virtue of their ability to inhibit the promotional phase of carcinogenesis. Thus, tumor promotion may play an important role in the development of adenomatous tumors arising in the large bowel.

While BBI suppressed the incidence of adenocarcinomas of the colon, it did not affect the incidence of anal gland squamous cell tumors. In contrast, chymostatin suppressed the incidence of squamous cell tumors in the anal gland, but had no effect on adenocarcinomas of the colon. Several factors could account for these results. (i) The critical target enzymes involved in the malignant transformation of the cells of the colon and anal gland are different. Presumably, BBI would be a more effective inhibitor of the critical target enzymes in the colon while chymostatin is more effective at inhibiting these enzymes in the cells lining the anal gland. (ii) BBI may be absorbed more efficiently by the epithelial cells lining the colon while chymostatin may be more effectively absorbed by the epithelial cells lining the anal gland. While BBI is water soluble, chymostatin is not, and hence its distribution in the gastrointestinal tract contents may not favor uptake by the critical epithelial cells lining the gut. The fact that chymostatin is fat soluble could also explain its selective ability to affect anal gland carcinogenesis. Consequently, water solubility may be an important characteristic of protease inhibitors for the suppression of adenomatous tumors of the colon.

It is somewhat surprising to us that BBI does not affect squamous cell carcinomas of the anal gland, as it has previously been shown to suppress squamous cell carcinomas of the hamster cheek pouch (16). As discussed elsewhere (17), the cells of origin of squamous cell carcinomas are markedly different in the oral and anal gland epithelium, which perhaps accounts for the observed differences in the ability of BBI to affect carcinogenesis at these two sites.

The ability of protease inhibitors to suppress carcinogenesis appears to vary greatly with the dose of carcinogen administered. BBI has previously been shown to prevent completely adenomatous tumors of the gastrointestinal tract when the doses of DMH utilized were such that a considerably lower percentage of the animals developed tumors than observed in the present study (17,20). We observed that BBI was capable of suppressing

the yield of tumors, in terms of tumor-bearing animals, by ~1/2 in this study, which utilized a high carcinogen dose resulting in gastrointestinal tract tumors in most of the DMH-treated animals. Chemopreventive agents appear to act similarly in other organ systems as well, with the degree of suppression of tumorigenicity being inversely correlated with the tumor yield (15).

The results from this study support our previous findings that non-toxic levels of BBI can suppress the development of murine adenomatous colon tumors, which are histopathologically similar to those that occur in the most common form of human colon cancer. Indeed, the colon cancer model utilized in the present studies is considered to be a representative model for the human disease (31). Thus, BBI may have the potential to lower the incidence of colon cancer in the human population.

Acknowledgements

We thank Eric Glasgow, Christine Keller-McGandy and Harrison Weed for technical assistance in these studies. We also thank Dr Jesse Berlin for his help with statistical analysis, Dr Robert McGandy for his help with the histopathological analysis of the data shown in Table III and Dr Walter Troll and the US Japan Cooperative Cancer Research Program for providing the chymostatin used in this study. This research was supported by NIH grants CA 46496 and CA 38246.

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Received on October 12, 1989; revised on March 29, 1990; accepted on April 19, 1990

Soybean Isoflavones Reduce Experimental Metastasis in Mice^{1,2}

Manuscript received 5 November 1998. Initial review complete 11 December 1998. Revision accepted 1 February 1999.

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ABSTRACT We investigated the effect of dietary supplementation with isoflavones on pulmonary metastasis of B16BL6 murine melanoma cells in C57BL/6 mice. Mice were fed a basal AIN-93G diet or the basal diet supplemented with the isoflavones genistein and daidzein at 113 µmol/kg, 225 µmol/kg, 450 µmol/kg, or 900 µmol/kg for 2 wk before and after the intravenous injection of 0.5 × 10⁵ melanoma cells. At necropsy, the number and size of tumors that formed in the lungs were determined. The number of mice that had >15 lung tumors was 17 in the control group, and 16, 15, 13, and 10 in the groups fed isoflavones at 113 µmol/kg, 225 µmol/kg, 450 µmol/kg and 900 µmol/kg, respectively. The latter two were significantly different from the control ($P \leq 0.05$). The median number of tumors in the control group was 67, and those in the isoflavone-supplemented groups were 57, 33, 32, and 17, respectively. The last was significantly different from the control ($P \leq 0.05$). Dietary supplementation with isoflavones at 225 µmol/kg, 450 µmol/kg, and 900 µmol/kg also significantly decreased tumor size (median cross-sectional area and volume) compared to the control values. We conclude that dietary supplementation with isoflavones reduces experimental metastasis of melanoma cells in mice. *J. Nutr.* 129: 1075–1078, 1999.

KEY WORDS: • mice • genistein • daidzein • melanoma
• metastasis

Epidemiologic studies suggest that consumption of foods that are high in soybean-based products is associated with a reduced risk of breast (Lee et al. 1991), prostate (Severson et al. 1989), uterine (Goodman et al. 1997), and gastric cancers (Nagai et al. 1982) in humans. Dietary supplementation with soybean protein isolate (SPI)⁴ (Hawrylewicz et al. 1991) or soybean chips (Barnes et al. 1990) reduces mammary carcinogenesis in female rats. Adding autoclaved raw soybean to the diet of male mice inhibits carcinogenicity of N-nitroso compounds in the liver and urinary

bladder (Mokhtar et al. 1988). These protective effects are associated with soy isoflavones, e.g. genistein and daidzein. Long-term intraperitoneal administration of genistein and daidzein to young rats (Constantinou et al. 1996) and subcutaneous injection of genistein to neonatal rats (Lamartiniere et al. 1995) reduce the development of mammary carcinoma after exposure of the animals to carcinogens. Other studies showed that soy-derived products have no effect on tumorigenesis in some animal models (Reddy et al. 1976).

Soybean is a rich source of dietary isoflavones (Murphy 1982). Isoflavones exist in soybean primarily as conjugated glycosides. Following ingestion, they are hydrolyzed to aglycones by glycosidases produced by intestinal bacteria. The conjugates genistin, daidzin, and glycitein and their aglycones, genistein, daidzein, and glycinein constitute 90–95% of the total soy isoflavones (Murphy 1982). It appears that the unconjugated aglycones are associated with many biological properties of isoflavones that may be responsible for their anticancer activities. These include antiestrogenic activity (Folman and Pope 1966), inhibition of protein tyrosine kinases (Akiyama et al. 1987), regulation of cell cycle progression and apoptosis (Kroemer et al. 1995), and antiangiogenic activity (Fotsis et al. 1993).

Metastasis, the spread of malignant cells from a primary neoplasm to distant organs that results in the development of secondary tumors, is the most devastating aspect of cancer. Advances in surgical techniques and adjuvant therapies have proven useful in the treatment of primary tumors. However, metastasis remains a major cause of poor prognosis and death in cancer patients. We recently reported that dietary supplementation with SPI reduces pulmonary metastasis of murine melanoma cells in mice (Yan et al. 1997). Connolly et al. (1997) reported that soybean chips inhibit metastasis of human mammary carcinoma cells in athymic nude mice. These studies suggest that dietary soybean is useful in preventing the spread of malignant cells.

The objective of the present study was to determine whether isoflavones present in SPI reduce metastasis. To accomplish this, the effect of dietary supplementation with isoflavones genistein and daidzein on pulmonary metastasis of melanoma cells was investigated using an intravenous injection model.

MATERIALS AND METHODS

Animals and diets. The protocol of the present study was reviewed and approved by the Creighton University Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals (National Research Council 1985). Three-week-old male C57BL/6 mice were purchased from Charles River (Wilmington, MA). Mice were housed five per box, in wire-topped plastic boxes, in a pathogen-free room on a 12:12-h light-dark cycle. The temperature in the room was maintained at 25 ± 1°C. Mice were given free access to the diet and deionized water and weighed weekly. Five diets were compared: a basal diet and the basal diet supplemented with genistein and daidzein (Lancaster, Windham, NH) at 113, 225, 450, or 900 µmol/kg, which was equivalent to that provided in the diet containing 2.5, 5, 10 or 20% SPI, respectively (Yan et al. 1997). The concentration of genistein in isoflavone-supplemented diets was 83.3, 166.7, 333.3, and 666.7 µmol/kg, respectively, and the concentration of daidzein was 29.5, 59.1, 118.1, and 236.2 µmol/kg, respectively. Glycitein was omitted from the supplementation because it was not commercially available. Dietary formulations were

¹ Presented in part at Experimental Biology 98, April 21, 1998, San Francisco, CA. [Yan, L., Yee, J. A., Li, D. & McGuire, M. H. (1998) Effect of dietary supplementation of isoflavones on pulmonary metastasis of melanoma cells in mice. FASEB J. 12: A829 (abs.)].

² This work was supported by the State of Nebraska Cancer and Smoking-Related Disease Research Program (Grant No. 98-51) and by Hazel Berve Trust Fund.

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⁴ Abbreviations used: SPI, soybean protein isolate.

TABLE 1

Isoflavone intake and urinary isoflavone excretion in mice fed the control and isoflavone-supplemented diets

Group	Isoflavone Intake ¹ μmol/(mouse · d)	Urinary Excretion ²					Total Excretion
		Genistein	Daidzein	Equol	ODMA ³		
		nmol/(mouse · d)					
Control	0	0	0	0	0	0	0
Isoflavones							
113 μmol/kg	0.3	3.9	14.2	0	2.6	20.7	
225 μmol/kg	0.6	13.3	21.3	0	2.7	37.3	
450 μmol/kg	1.2	22.2	24.6	11.7	4.7	63.3	
900 μmol/kg	2.4	51.1	44.5	27.5	5.2	128.3	

¹ The isoflavone intake was calculated on the basis of dietary isoflavone concentrations and the mean food intake of all mice (2.6 ± 0.6 g/d; $n = 30$).

² Urine samples collected from 6 mice in each group during the 1 wk metabolic study before tumor cell injection were pooled and analyzed for isoflavones.

³ O-desmethylangolensin.

based on the AIN-93G standard diet (Reeves et al. 1993), except that soybean oil was replaced with corn oil. Diet components were purchased from ICN (Costa Mesa, CA). All diets were prepared in our laboratory, and each lot was stored at 4°C for no longer than 3 wk.

Experimental design. Ninety mice were fed the basal diet for 2 d before being assigned to five groups of 18 each. They were then fed the basal diet or one of the isoflavone-supplemented diets. B16BL6 murine melanoma cells (Dr. I. J. Fidler, University of Texas, Houston, TX) were cultured in minimum essential medium with 10% heat-inactivated fetal bovine serum as described previously (Yan et al. 1997). The melanoma cells were collected from monolayer cultures by a brief trypsinization (0.05% trypsin and 0.53 mmol/L EDTA). The viability of the cells was determined with trypan blue, and a single cell suspension was made in serum-free medium. After 2 wk consuming the diets, each mouse was injected via the lateral tail vein with 0.5×10^5 viable cells in 0.2 mL. To avoid possible changes in cell viability, melanoma cells were injected into mice within 30 min after their collection. The order that tumor cells were injected into mice from different dietary groups was randomized. The mice were then fed the diets for another 2 wk. One wk before tumor cell injection, six mice from each group were transferred to metabolic cages, and their food intake was recorded over 7 d. Urine collected from each group ($n = 6$) throughout this week was pooled and analyzed for isoflavones (Xu et al. 1994). Isoflavone intake was calculated on the basis of food intake and the dietary concentration of isoflavones.

At the end of the experiment, mice were anesthetized using ketamine (50 mg/kg body weight) and xylazine (5 mg/kg body weight) and then killed by cervical dislocation. Their lungs were excised and fixed in 10% phosphate-buffered formalin. The number of pulmonary tumors was determined by counting visible black foci by using a dissecting microscope (Yan et al. 1997). The cross-sectional area of tumors in randomly selected fields was measured using a Quantimet 500 image analysis system (Leica Cambridge, Cambridge, UK). Tumor volume was calculated using the mean of the longest and the shortest diameters measured and the assumption that tumors were spherical (Welch et al. 1983).

Statistical analysis. Fisher's exact test (Steel and Torrie 1980) was used to analyze the frequency distribution of the mice that had 1–15 tumors or >15 tumors. Bartlett's test (Bartlett, 1937) for homogeneity of variances revealed that standard deviations for the mean values of the number of tumors, tumor cross-sectional area, and tumor volume differed significantly among the groups ($P \leq 0.05$). Because ANOVA can only be used to compare the means of populations with homogeneous variances, the results were analyzed using the Kruskal-Wallis nonparametric and Dunn's multiple comparison tests (Kruskal and Wallis 1952). The data were analyzed using the statistic program Instat 2.01 for Macintosh. Differences were considered significant at $P \leq 0.05$.

RESULTS

To determine the effect of dietary supplementation with isoflavones on growth, mice were weighed weekly, and food

intake was recorded. The overall body weight of mice at the beginning and at the end of the experiment was 14 ± 1 g and 24 ± 1 g, respectively. There was no difference in body weight among the groups throughout the experiment (data not shown). The mean food intake of all mice ($n = 30$) was 2.6 ± 0.6 g/d. There were no differences in food intakes among the groups (data not shown). The daily isoflavone intakes for each group are shown in Table 1. There were no measurable isoflavones in urine from mice fed the basal diet. The urinary excretion of isoflavones (a sum of genistein, daidzein, and daidzein metabolites equol and O-desmethylangolensin) was increased in a dose-dependant manner in mice fed the diet containing isoflavones at 113, 225, 450 or 900 μmol/kg (Table 1). One mouse from the control group and one from the 900 μmol isoflavones/kg group were excluded from the experiment because their growth was significantly less than all other mice.

Injection of 0.5×10^5 viable melanoma cells into the lateral tail vein resulted in lung metastasis in all the mice fed the control diet (Table 2). Based on the number of lung tumors per mouse, the mice were placed into one of the two categories: 1) 1–15 tumors and 2) >15 tumors. In the control group, all mice had >15 lung tumors (Table 2). By contrast, 89, 83, 72, and 59% of the mice in groups fed the diets containing isoflavones at 113, 225, 450, and 900 μmol/kg, respectively, had >15 tumors. The latter two were significantly different from the control ($P \leq 0.05$). The median number of lung tumors in the control group was 67. The median number of lung tumors reduced in the 900 μmol/kg isoflavone/kg group compared to the control ($P \leq 0.05$). The mean number of lung tumors in mice fed the isoflavone-supplemented diets decreased relative to the control in a dose-dependent manner.

To determine the effect of isoflavones on the growth of metastatic tumors, tumor cross-sectional area and volume were determined. The median cross-sectional area was 0.55 mm^2 , and median volume was 0.21 mm^3 in mice fed the basal diet (Table 3). Dietary supplementation with isoflavones decreased both variables in a dose-dependent manner. The difference in tumor cross-sectional area and volume between the control and the groups supplemented with isoflavones at 225, 450, or 900 μmol/kg was significant ($P \leq 0.01$).

DISCUSSION

We reported that dietary supplementation with SPI reduces experimental metastasis (Yan et al. 1997). Results of the present study demonstrate that dietary supplementation with

TABLE 2

Effect of dietary supplementation of isoflavones on pulmonary metastasis of melanoma cells in mice

Group	n	Mice with lung tumors		Tumors/mouse		
		1–15 Tumors	>15 Tumors ¹	Median ²	Mean ± SEM ³	Range
Control Isoflavones	17	0	17	67	67 ± 8	19–110
113 µmol/kg	18	2	16	57	71 ± 11	4–167
225 µmol/kg	18	3	15	33	50 ± 12	5–201
450 µmol/kg	18	5	13*	32	45 ± 11	3–157
900 µmol/kg	17	7	10**	17*	29 ± 6	4–81

¹ Significantly different from control, *P ≤ 0.05 and **P ≤ 0.01. Data were analyzed using Fisher's exact test.

² Significantly different from the control, *P ≤ 0.05. Data were analyzed using the Kruskal-Wallis nonparametric and Dunn's multiple comparison tests.

³ Because of the heterogenous variances among sample populations, the mean values were not compared by ANOVA.

isoflavones at concentrations equivalent to that provided in the SPI diets decreased the number of lung tumors and the tumor cross-sectional area and volume compared to the controls. The dietary isoflavone content was positively correlated with both the urinary isoflavone concentration and the magnitude of the inhibitory effect on metastasis. Dietary supplementation with isoflavones up to 900 µmol/kg had no adverse effect on the growth of mice during the experimental period. These results indicate that isoflavones effectively reduced pulmonary metastasis of melanoma cells and also retarded the growth of those tumors that developed in the lungs.

The observations from this study provide the first evidence that dietary supplementation with isoflavones reduces experimental metastasis. This is supported by the findings that oral administration of genistein inhibits lung metastasis of melanoma cells intravenously injected into mice (Menon et al. 1998). Interestingly, they found that daidzein was ineffective in reducing metastasis. In the present study, the effect of dietary supplementation with either genistein or daidzein alone was not tested. However, the concentration of genistein in the experimental diets was threefold greater than that of daidzein. Thus, the protective effect of dietary isoflavones on experimental metastasis may be largely attributed to genistein. Whether daidzein is without an effect is difficult to conclude from the currently available data. In the study described by Menon et al. (1998), injection of 1 × 10⁶ melanoma cells into mice led to the development of uncountable massive lung tumors in control and daidzein-treated animals. Thus, they were unable to determine whether or not daidzein was effective in reducing metastasis.

At present, the mechanism whereby dietary soybean or isofla-

vones reduces metastasis remains unknown. The experimental metastasis model employed in the present study measured the extravasation of melanoma cells from the cardiovascular system into the interstitium of the lungs. This requires invasion of the subendothelial basement membrane. Genistein inhibits the invasion of extracellular matrix by BALB/c mammary carcinoma cells *in vitro* (Scholar and Toews 1994). This may be due to an effect of genistein on cell adhesion to the extracellular matrix. Genistein is a potent inhibitor of protein tyrosine kinases (Akiyama et al. 1987). Protein tyrosine kinases phosphorylate tyrosine residues on proteins that participate in signal transduction events, including integrin-mediated cell adhesion (Hynes 1992). It was shown that genistein inhibits epithelial growth factor-stimulated integrin expression by human breast cancer (Narita et al. 1996) and esophageal cancer cells (Sato et al. 1996). Furthermore, genistein inhibits integrin-mediated cell adhesion by lymphoma cells (Weimar et al. 1997) and arterial smooth muscle cells (Hedin et al. 1997). These observations suggest that isoflavones could reduce metastasis by affecting cell adhesion. A second possibility is by reducing protease activity. Genistein inhibits the secretion of urokinase-type plasminogen activator and metalloproteinase by LM3 murine mammary tumor cells (Aguirre Ghiso et al. 1998). These effects were also attributed to the inhibition of protein tyrosine kinases. It should be noted that in these studies genistein was used as a pharmacological tool to study signal transduction events rather than as a dietary component. The high concentrations employed are beyond the level that is achievable in animals consuming a soy-containing diet. Thus, exposure of cultured cells to isoflavones *in vitro* is not comparable to providing animals with soybean- or

TABLE 3

Effect of dietary supplementation of isoflavones on tumor cross-sectional area and volume of metastatic tumors that developed in the lungs of mice

Group	Mice, n	Tumors, n	Tumor cross-sectional area, mm ²		Tumor volume, mm ³	
			Median ¹	Mean ± SEM ²	Median ¹	Mean ± SEM ³
Control Isoflavones	17	139	0.55	0.65 ± 0.03	0.21	0.28 ± 0.02
113 µmol/kg	18	139	0.52	0.61 ± 0.03	0.19	0.25 ± 0.02
225 µmol/kg	18	139	0.36**	0.49 ± 0.03	0.10**	0.19 ± 0.02
450 µmol/kg	18	139	0.32**	0.41 ± 0.03	0.09**	0.15 ± 0.01
900 µmol/kg	17	139	0.16**	0.26 ± 0.03	0.03**	0.08 ± 0.01

¹ Significantly different from the control, **P ≤ 0.01. Data were analyzed using the Kruskal-Wallis nonparametric and Dunn's multiple comparison tests.

² Because of the heterogenous variances among sample populations, the mean values were not compared by ANOVA.

isoflavone-supplemented diets. Therefore, caution should be taken when data from in vitro experiments are used to explain a dietary effect of soybean or isoflavones in animal studies.

The results of the present study demonstrate that tumor cross-sectional area and volume of mice fed the isoflavone diets were significantly reduced compared to those of mice fed the basal diet. A decrease in tumor size could be due to prolonged retention of tumor cells in the circulatory system or an inhibition of malignant cell proliferation after they take up residence in the lungs. Most circulating B16 melanoma cells rapidly die following their intravenous injection (Fidler 1970). Approximately 1% of the cells survive for 24 h, and one tenth of them form tumor colonies in the lungs. Therefore, it is unlikely that retention in the circulation explains the difference in tumor size between the control and the isoflavone-supplemented groups. Rather, it is more likely that this difference is due to the inhibition of mitosis of malignant cells in the lungs. Investigations in our laboratory designed to determine the effect of dietary isoflavones on cell proliferation and angiogenesis during the formation of metastatic tumor will clarify this possibility.

Comparing results of the present study with our previous report on dietary SPI and experimental metastasis (Yan et al. 1997), it appears that SPI is more effective in reducing the number of lung tumors than the isoflavone-equivalent diets. Soybean contains several potential anticancer components other than isoflavones, e. g., protease inhibitors (Kennedy 1993) and saponins (Koratkar and Rao 1997). Although these agents are largely eliminated during the preparation of the SPI, trace amounts may exist. The SPI also contains phytate that has been shown to have a tumor-attenuating action in some animal models (Shamsuddin et al. 1988). Finally, glycinein, which was present in the SPI employed in our previous study (Yan et al. 1997), was not supplemented in the diet in the present study. Thus, these variations could contribute, at least in part, to the differences observed in these two studies.

In summary, results of the present study demonstrate that dietary supplementation with isoflavones reduced experimental metastasis of melanoma cells in mice and also inhibited the growth of metastatic tumors that developed in the lungs. We conclude that isoflavones are responsible, at least in part, for the protective effect of dietary soybean on experimental metastasis of melanoma cells in mice.

ACKNOWLEDGMENTS

The authors thank Vivian W. Huang and Mai-Linh Frascarelli, undergraduate students at Creighton University, for participating in this research project.

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Effect of Selenium and Vitamin E on Risk of Prostate Cancer and Other Cancers

The Selenium and Vitamin E Cancer Prevention Trial (SELECT)

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See also pp 52 and 102.

Context Secondary analyses of 2 randomized controlled trials and supportive epidemiologic and preclinical data indicated the potential of selenium and vitamin E for preventing prostate cancer.

Objective To determine whether selenium, vitamin E, or both could prevent prostate cancer and other diseases with little or no toxicity in relatively healthy men.

Design, Setting, and Participants A randomized, placebo-controlled trial (Selenium and Vitamin E Cancer Prevention Trial [SELECT]) of 35 533 men from 427 participating sites in the United States, Canada, and Puerto Rico randomly assigned to 4 groups (selenium, vitamin E, selenium + vitamin E, and placebo) in a double-blind fashion between August 22, 2001, and June 24, 2004. Baseline eligibility included age 50 years or older (African American men) or 55 years or older (all other men), a serum prostate-specific antigen level of 4 ng/mL or less, and a digital rectal examination not suspicious for prostate cancer.

Interventions Oral selenium (200 µg/d from L-selenomethionine) and matched vitamin E placebo, vitamin E (400 IU/d of all rac- α -tocopherol acetate) and matched selenium placebo, selenium + vitamin E, or placebo + placebo for a planned follow-up of minimum of 7 years and a maximum of 12 years.

Main Outcome Measures Prostate cancer and prespecified secondary outcomes, including lung, colorectal, and overall primary cancer.

Results As of October 23, 2008, median overall follow-up was 5.46 years (range, 4.17-7.33 years). Hazard ratios (99% confidence intervals [CIs]) for prostate cancer were 1.13 (99% CI, 0.95-1.35; n=473) for vitamin E, 1.04 (99% CI, 0.87-1.24; n=432) for selenium, and 1.05 (99% CI, 0.88-1.25; n=437) for selenium + vitamin E vs 1.00 (n=416) for placebo. There were no significant differences (all $P>.15$) in any other prespecified cancer end points. There were statistically nonsignificant increased risks of prostate cancer in the vitamin E group ($P=.06$) and type 2 diabetes mellitus in the selenium group (relative risk, 1.07; 99% CI, 0.94-1.22; $P=.16$) but not in the selenium + vitamin E group.

Conclusion Selenium or vitamin E, alone or in combination at the doses and formulations used, did not prevent prostate cancer in this population of relatively healthy men.

Trial Registration clinicaltrials.gov identifier: NCT00006392

JAMA. 2009;301(1):39-51

www.jama.com

PROSTATE CANCER MORTALITY IN the United States has declined in recent years, but this cancer remains the most common nonskin epithelial malignancy in US men, with 186 320 new cases and 28 660 deaths (the second leading cause

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of cancer death) estimated for 2008.¹ An effective prevention strategy for prostate cancer would have substantial public health benefits, including the potential to reduce the incidence of biologically indolent prostate cancer, which is significantly overdetected by widespread screening with prostate-specific antigen (PSA) and for which most newly diagnosed men still undergo curative-intent therapy involving substantial morbidity despite surgical and other advances.²⁻⁶

Important secondary results of 2 randomized controlled trials, the Nutritional Prevention of Cancer (NPC) study and the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) study, showed prostate cancer risk reductions of 63% for selenized yeast and 32% for α -tocopherol (or vitamin E).⁷⁻¹⁰ In addition, a large-scale randomized controlled trial¹¹ involving several different regimens found that a combination of selenium, vitamin E, and beta carotene reduced overall cancer mortality. These clinical data, supported by epidemiologic and preclinical data,¹²⁻¹⁹ led to the design of the Selenium and Vitamin E Cancer Prevention Trial (SELECT).²⁰

Investigators in the United States and Canada from major cooperative groups of the National Cancer Institute and Department of Veterans Affairs used the Prostate Cancer Prevention Trial (PCPT) accrual infrastructure (200 clinical sites, with 18 882 randomized men) in designing and activating SELECT. We report herein the effects of selenium and vitamin E, alone or in combination, on the risk of prostate cancer and secondary end points in SELECT.

METHODS

Study Design

SELECT is a phase 3 randomized, placebo-controlled trial of selenium (200 $\mu\text{g}/\text{d}$ from L-selenomethionine), vitamin E (400 IU/d of all rac- α -tocopheryl acetate), or both (planned follow-up of minimum of 7 years and maximum of 12 years) for prostate cancer prevention. The major eligibility re-

quirements included age 50 years or older for African American men and 55 years or older for all other men, no prior prostate cancer diagnosis, 4 ng/mL or less of PSA in serum, and a digital rectal examination (DRE) not suspicious for cancer. No current use of anticoagulant therapy other than 175 mg/d or less of acetylsalicylic acid or 81 mg/d or less of acetylsalicylic acid with clopidogrel bisulfate, no history of hemorrhagic stroke, and normal blood pressure were also required because of antiplatelet effects of vitamin E and related findings of the ATBC study.

Participant characteristics were based on self-report, including self-identification of race and ethnicity which were defined by the US Census Bureau. Race and ethnicity data were collected mainly for the generalizability of trial results. All potentially eligible men were required to provide written informed consent before being allowed to participate in the trial. The local institutional review board of each study site approved the study for activation and reviewed its progress annually. The trial was activated in July 2001 and follow-up blinded to the trial results ended on October 23, 2008.

Baseline blood and toenail specimens and a 5-year blood sample were collected for future biological studies. Prostate tissue samples collected during the trial were submitted for confirmation by central pathology review (no samples were collected at baseline). Participants without prostate cancer had clinic visits once every 6 months throughout the trial; with prostate cancer, annually. Adherence and adverse events were monitored every 6 months and a limited physical examination including assessments of blood pressure, weight, and smoking status was conducted annually. Prespecified adverse events known to be associated with vitamin E or selenium were graded according to the National Cancer Institute Common Toxicity Criteria.

Although eligible PSA and DRE results were required at study entry, annual prostate cancer screening with PSA and DRE was not mandatory because the benefits of this screening were un-

der debate when the trial opened and community screening standards were expected to change during the trial. Participants were recommended during annual clinic visits to undergo a PSA test and DRE according to the standard of care at their study sites and the participant's preferences. A formal prerandomization period (28-90 days; no placebo run-in capsules) gave potential participants time to decide if they would agree to stop disallowed over-the-counter supplements of selenium or vitamin E throughout the study and to demonstrate, by returning for randomization, their willingness to adhere to the trial. Other adherence methods included offering each participant a free multivitamin containing no selenium or vitamin E and assessing serum levels of vitamin E and selenium in all participants at a subset of study sites (22 sites representing 7.8% of the trial population). These sites were chosen a priori to be representative of the broad range of sites in the trial.

End Point Assessment

Participants reported prostate cancers to the study site staff. Study staff obtained medical records supporting the diagnosis and abstracted the diagnostic method and clinical stage. Tissue and the corresponding pathology report were sent to the central pathology laboratory for confirmation. Gleason Score was based on central pathology review.

Men were asked at their first 6-month clinic visit to report new events since entering the trial and thereafter to report new events since their last visit. Cardiac-event data were collected in detail from the trial beginning (2001); data on diabetes were added through self-reported glitazone medication use (beginning in 2003) and self-report of diabetes (beginning in 2005) via the following question at each clinic visit: "Does the participant report having diabetes (either his doctor told him he has diabetes or he is taking medication for diabetes)?"

A general question regarding any events considered severe or life-threatening (grade 3 or 4), regardless

of attribution to the study supplements, was also asked. A Social Security Death Index search was conducted in July 2008 for participants who had a last contact date of more than 18 months before the search. Other specifically queried events (known at study inception to be related to either of the study supplements) included alopecia, dermatitis, fatigue, halitosis, nail changes, and nausea.

Statistical Analysis

The primary end point was prostate cancer incidence as determined by routine clinical management. Cancers that were not confirmed centrally were included in the analysis. SELECT was designed as a 4 group trial with 5 prespecified comparisons (selenium vs placebo, vitamin E vs placebo, selenium + vitamin E vs placebo, selenium vs selenium + vitamin E, and vitamin E vs selenium + vitamin E). With a sample size of 32 400 men, using a 1-sided $\alpha=.005$ level (equivalent to a 2-sided $\alpha=.01$ level), there was 96% power to detect a 25% re-

duction in prostate cancer for either of the single agents (vs placebo), 89% power to detect a 25% reduction for selenium + vitamin E (vs an active single agent) and more than 99% power to detect a 44% reduction of selenium + vitamin E (vs placebo).

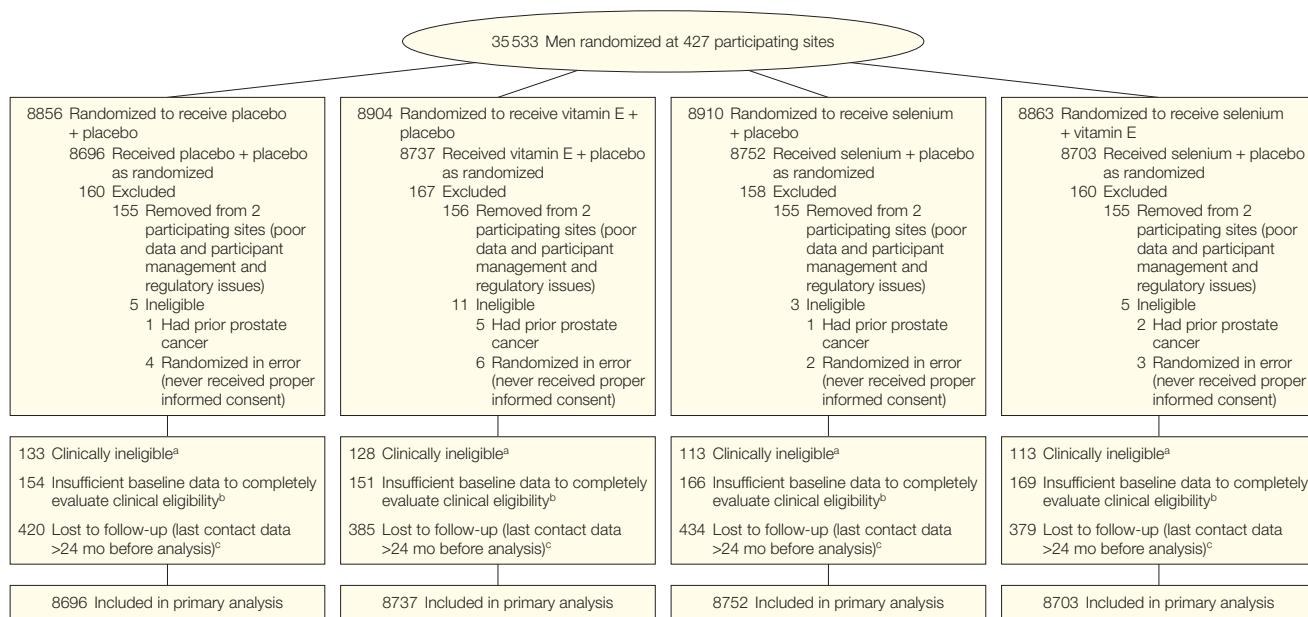
Design assumptions were based on the PCPT, ATBC, and NPC trials. The details of the statistical design have been described elsewhere.²⁰ Important elements included (1) constant accrual over 5 years; (2) prostate cancer incidence in the placebo group based on PCPT for the first 3 years and the 1995 Puget Sound SEER registry afterward; (3) adherence to the study supplements, which was assumed to decrease over the course of the trial with a 5-year rate of 68% and 12-year rate of 51%; (4) a constant 10% drop-in rate, defined as participants receiving placebo who are taking active supplementation off-study; (5) loss to follow-up of 0.5% per year; and (6) deaths estimated from PCPT for

years 1 to 3 and from the 1995 US standard rates of men aged 63 years and all races for year 4 onward. The sample size was calculated to be 32 400 men and the number of prostate cancers expected in the placebo group was 533 over 12 years. Under the assumed conditions, the required median time under observation was estimated to be 8.8 years.

The primary analysis consisted of the 5 prespecified comparisons detailed above. These comparisons allowed for a meaningful analysis of the study results whether an interaction between vitamin E and selenium occurred. Each individual test was conducted at a 1-sided $\alpha=.005$ level (equivalent to a 2-sided $\alpha=.01$ level) using a Bonferroni factor of 5 to preserve an overall 1-sided $\alpha=.0025$ level (equivalent to a 2-sided $\alpha=.05$ level).

An independent data and safety monitoring committee met yearly and reviewed data on safety, adherence, and diagnosis of prostate cancer. In addition to the final analysis, interim analy-

Figure 1. Flow of Participants Included in Analysis by Intervention Group



^aDue to increased blood pressure, high-grade prostatic intraepithelial neoplasia, suspicious digital rectal examination (DRE) or increased prostate-specific antigen (PSA), aspirin dosage, prior cancer less than 5 years before randomization, participation in another clinical trial, or other clinical reason.

^bBlood pressure, PSA, and/or DRE not performed within required time frame (but normal) or other data-related reason.

^cAll data up until the last contact are included; these men also could have been either clinically ineligible or had insufficient baseline data. For time-to-event analyses, these men were censored at their last follow-up.

ses were planned for years 5, 7, 9, 10, and 11 after the first participant was randomized; the percentages of the expected total number of prostate cancer events in the placebo group at each interval were 14%, 35%, 61%, 74%, and 88%, respectively. Each interim analysis resulted in recommendations that could have included modifications to the study, including termination of accrual, modifications to data collection, or early reporting of results. Recommendations were made to the steering committee, which made the final decisions.

The interim analyses tested the null hypothesis at a 1-sided $\alpha=.0005$ level (equivalent to a 2-sided $\alpha=.001$ level)

using the Cox proportional hazards regression model. In addition, the alternative hypothesis of a 25% reduction in prostate cancer incidence was tested at a 1-sided level of $\alpha=.0005$ (equivalent to a 2-sided $\alpha=.001$ level) using an extension of the Cox proportional hazards regression model that allows for testing a relative risk (RR) not equal to 1. The purpose of the second analysis was to allow for the study to stop if it was determined that the expected reduction in prostate cancer would not be observed. The frequencies of the number of cardiovascular events and cases of diabetes were tested with a χ^2 test. For cardiovascular event and diabetes analyses, we did not capture the report of the date

of the event, which thus was not incorporated into the analysis.

Participants were randomized in a randomized block scheme, in which the block was the study site. This ensured a balance of the 4 intervention groups within each study site. All analyses were performed by using an intention-to-treat analysis in which men were classified according to the group to which they were randomized. All men were followed up until death or loss to follow-up. For cancer end points, men were censored at the time of their last follow-up or death. The analysis did not incorporate adjustments for baseline covariates. Data were analyzed by using SAS ver-

Table 1. Baseline Characteristics of Study Participants

Characteristics	No. (%) of Participants			
	Placebo (n = 8696)	Vitamin E (n = 8737)	Selenium (n = 8752)	Selenium + Vitamin E (n = 8703)
Age, y				
Median (interquartile range)	62.6 (58.1-67.8)	62.3 (58.0-67.8)	62.6 (58.2-68.0)	62.4 (58.1-67.8)
50-54	355 (4)	402 (5)	337 (4)	385 (4)
55-64	5078 (58)	5143 (59)	5076 (58)	5052 (58)
65-74	2702 (31)	2641 (30)	2733 (31)	2731 (31)
≥75	561 (6)	551 (6)	606 (7)	535 (6)
Race/ethnicity				
White	6863 (79)	6890 (79)	6942 (79)	6874 (79)
African American	1078 (12)	1107 (13)	1053 (12)	1076 (12)
Hispanic (non-African American)	492 (6)	477 (5)	481 (5)	484 (6)
Hispanic (African American)	76 (1)	103 (1)	86 (1)	95 (1)
Other ^a	187 (2)	160 (2)	190 (2)	174 (2)
Education (highest level)				
≤High school graduate or GED	1993 (23)	1875 (22)	1917 (22)	1898 (22)
Some college/vocational school	2291 (26)	2387 (27)	2327 (27)	2348 (27)
≥College graduate	4317 (50)	4394 (51)	4430 (51)	4372 (50)
Unknown/missing	95 (1)	81 (1)	78 (1)	85 (1)
PSA, ng/mL				
0.1-1.0	4122 (47)	4208 (48)	4218 (48)	4213 (48)
1.1-2.0	2728 (31)	2653 (30)	2661 (30)	2666 (31)
2.1-3.0	1168 (13)	1228 (14)	1211 (14)	1149 (13)
3.1-4.0	666 (8)	634 (7)	652 (7)	659 (8)
>4.0	5 (<1)	3 (<1)	2 (<1)	1 (<1)
Unknown/missing	7 (<1)	11 (<1)	8 (<1)	15 (<1)
Smoking status				
Never	3682 (42)	3752 (43)	3780 (43)	3666 (42)
Current	655 (8)	659 (8)	631 (7)	670 (8)
Former	4208 (48)	4194 (48)	4214 (48)	4242 (49)
Ever (unknown status)	63 (1)	55 (1)	61 (1)	56 (1)
Unknown	88 (1)	77 (1)	66 (1)	69 (1)

Abbreviations: GED, general equivalency diploma; PSA, prostate-specific antigen.

SI conversion: To convert PSA to $\mu\text{g/L}$, multiply by 1.0.

^aOther race/ethnicity include Asian (n=420), Native American (n=99), Pacific Islander (n=39), multiple races (n=34), and unknown (n=119).

sion 9.1 (SAS Institute Inc, Cary, North Carolina).

Supplement Quality Control and Quality Assurance

The Pharmacy Coordinating Center received the study supplements for bottling as finished capsules in shipments containing lots of active capsules along with the appropriate matching placebo. As required by current good manufacturing practice,²¹ each lot of capsules was quarantined upon receipt until testing was performed to ensure that capsules labeled "active" by the

manufacturer contained the appropriate active agent and that capsules labeled as "placebo" did not contain an active agent. In addition, each time the capsules were bottled, production-run-verification testing was performed to ensure that bottles labeled as an active agent or placebo contained the appropriate material. To ensure that the quality of the blind was maintained, capsules received in each subsequent lot were compared with the previous lot and with matching capsules in the current shipment for their characteristics of weight, shape and size,

color and external marking, odor, and comparability of contents of opened capsules. Whether the participant guessed or had an external validation of whether he was getting the active agent or placebo was not assessed.

RESULTS

On September 15, 2008, the independent data and safety monitoring committee met, reviewed data as of August 1, 2008, for the second formal interim analysis, and recommended the discontinuation of study supplements because the alternative hypothesis of no evi-

Table 2. Adherence to Study Supplements by Pill Counts and Bioadherence

Pill Counts ^a	% (Range) ^b			
	Placebo	Vitamin E	Selenium	Selenium + Vitamin E
Selenium/matching placebo				
Year 1 (n=34 708)	85 (76-85)	85 (77-85)	84 (76-84)	85 (77-84)
Year 2 (n=34 163)	81 (72-81)	80 (72-81)	79 (71-80)	80 (72-80)
Year 3 (n=33 616)	76 (68-77)	77 (69-77)	75 (68-76)	76 (69-77)
Year 4 (n=32 976)	69 (65-73)	73 (66-74)	71 (64-72)	72 (65-74)
Year 5 (n=23 419)	69 (63-71)	71 (64-73)	69 (62-70)	70 (64-71)
Vitamin E/matching placebo				
Year 1 (n=34 708)	85 (76-85)	85 (77-85)	85 (76-85)	85 (77-85)
Year 2 (n=34 163)	80 (71-80)	80 (71-80)	79 (70-79)	79 (71-80)
Year 3 (n=33 616)	75 (67-75)	75 (67-76)	74 (67-75)	76 (69-77)
Year 4 (n=32 976)	70 (63-72)	70 (63-72)	69 (62-71)	70 (63-72)
Year 5 (n=23 419)	67 (61-69)	69 (62-71)	67 (61-69)	68 (61-70)
Median (Interquartile Range)				
Bioadherence	Placebo (n = 285)	Vitamin E (n = 290)	Selenium (n = 277)	Selenium + Vitamin E (n = 257)
Serum selenium, µg/L				
Baseline	137.6 (124.7-151.8)	135.9 (122.4-148.4)	135.0 (123.4-145.9)	136.4 (122.9-150.0)
6-mo visit	137.4 (123.3-152.0)	138.4 (124.1-154.0)	223.4 (198.6-251.8)	227.0 (199.4-251.2)
1st annual visit	138.1 (125.2-152.2)	137.7 (124.1-150.4)	232.4 (204.2-261.4)	228.5 (205.5-258.1)
2nd annual visit	132.0 (120.8-143.1)	129.8 (120.1-139.9)	228.0 (206.3-256.9)	220.7 (194.0-249.5)
4th annual visit ^c	140.1 (124.3-150.8)	143.8 (126.2-158.6)	251.6 (218.7-275.0)	253.1 (210.5-283.0)
Cholesterol-adjusted α-tocopherol, µg/mL				
Baseline	12.45 (10.70-14.95)	12.79 (10.69-15.37)	12.58 (10.43-14.75)	12.20 (10.12-15.35)
6-mo visit	11.68 (10.09-13.61)	18.14 (15.21-22.45)	11.62 (10.10-13.44)	17.90 (15.11-20.84)
1st annual visit	11.68 (10.24-13.44)	18.50 (15.08-22.46)	11.69 (10.10-13.03)	18.04 (14.77-22.35)
2nd annual visit	12.13 (10.80-13.72)	18.35 (15.13-22.85)	11.80 (10.57-13.58)	18.44 (15.32-22.89)
4th annual visit ^c	12.09 (9.95-14.41)	16.57 (13.86-22.61)	12.03 (9.57-13.53)	17.87 (14.68-22.31)
Cholesterol-adjusted γ-tocopherol, µg/mL				
Baseline	1.31 (0.83-2.01)	1.43 (0.89-2.21)	1.50 (0.96-2.21)	1.44 (0.96-2.02)
6-mo visit	1.50 (1.07-1.97)	0.78 (0.51-1.12)	1.64 (1.22-2.29)	0.74 (0.48-1.11)
1st annual visit	1.53 (1.09-2.05)	0.75 (0.52-1.16)	1.69 (1.27-2.33)	0.70 (0.48-1.04)
2nd annual visit	1.57 (1.13-2.13)	0.74 (0.49-1.08)	1.76 (1.26-2.43)	0.66 (0.50-1.03)
4th annual visit ^c	1.69 (1.14-2.29)	0.80 (0.50-1.23)	1.90 (1.48-2.70)	0.69 (0.47-1.07)

SI conversions: To convert serum selenium to µmol/L, multiply by 0.0127; α-tocopherol and γ-tocopherol to µmol/L, multiply by 23.22.

^aPercentage of men adherent, defined as taking at least 80% of their study supplements. Denominators decrease over time reflecting the varying amounts of follow-up.

^bThese ranges are estimates including those with missing data and assumes those missing were either all not adherent (low estimate) or all adherent (high estimate).

^cNumbers of participants for 4th annual visit are placebo (n=79), vitamin E (n=78), selenium (n=72), and selenium + vitamin E (n=71).

dence of benefit from either study agent was convincingly demonstrated ($P < .0001$) and there was no possibility of a benefit to the planned degree with

additional follow-up. Study sites were notified to discontinue supplements on October 23, 2008, and the data presented in this article are current as of this date.

Participants

A total of 35 533 men were accrued and randomly assigned at 427 participating sites in the United States, Canada,

Table 3. Clinically Diagnosed Prostate Cancers

	No. (%) of Participants			
	Placebo (n = 8696)	Vitamin E (n = 8737)	Selenium (n = 8752)	Selenium + Vitamin E (n = 8703)
Total No. of prostate cancers diagnosed by study site	416	473	432	437
Method of diagnoses				
Prostate biopsy	404 (97)	458 (97)	419 (97)	420 (96)
Other/unknown	12 (3)	15 (3)	13 (3)	17 (4)
No. of total prostate biopsies	1020	1011	982	997
PSA tests ^a				
Year 1	6708 (83)	6876 (84)	6807 (84)	6838 (84)
Year 2	6641 (86)	6652 (85)	6635 (85)	6673 (86)
Year 3	6284 (85)	6334 (85)	6376 (85)	6349 (85)
Year 4	6043 (85)	6087 (84)	6065 (85)	6045 (84)
Year 5	4265 (84)	4246 (84)	4271 (84)	4257 (84)
DRE tests ^a				
Year 1	5766 (72)	5936 (73)	5870 (72)	5833 (72)
Year 2	5567 (72)	5563 (72)	5561 (72)	5551 (72)
Year 3	5180 (70)	5188 (70)	5198 (70)	5190 (70)
Year 4	4862 (69)	4823 (67)	4878 (69)	4878 (68)
Year 5	3420 (68)	3418 (68)	3397 (68)	3425 (68)
Reason for biopsy (positive biopsies)				
Increased PSA	259 (64)	324 (71)	296 (71)	263 (63)
PSA prompting biopsy, median (IQR), ng/mL	4.60 (4.00-5.50)	4.60 (3.99-5.60)	4.83 (4.05-5.70)	4.70 (4.00-5.60)
PSA velocity	12 (3)	10 (2)	13 (3)	16 (4)
Abnormal DRE	66 (16)	58 (13)	46 (11)	56 (13)
Increased PSA/PSA velocity + abnormal DRE	55 (14)	49 (11)	56 (13)	72 (17)
Other	8 (2)	13 (3)	12 (3)	17 (4)
T stage				
T1a-c	278 (70)	343 (75)	301 (73)	286 (69)
T2a-b	122 (30)	114 (25)	108 (26)	128 (31)
T3a-b	0 (0)	2 (0)	5 (1)	3 (1)
TX/not staged	16	14	18	20
N stage				
N0	109 (100)	127 (100)	125 (99)	117 (100)
N1	0 (0)	0 (0)	1 (1)	0 (0)
NX/not staged	307	346	306	320
M stage				
M0	124 (100)	134 (99)	122 (96)	119 (98)
M1a-b	0 (0)	2 (1)	5 (4)	2 (2)
MX/not staged	292	337	305	316
Gleason score ^b				
No. graded by central laboratory	365	396	361	365
2-6	240 (66)	249 (63)	217 (60)	220 (60)
7 (grade 3 + grade 4)	80 (22)	97 (24)	105 (29)	91 (25)
7 (grade 4 + grade 3)	21 (6)	27 (7)	19 (5)	24 (7)
8-10	24 (7)	23 (6)	20 (6)	30 (8)

Abbreviations: DRE, digital rectal examination; IQR, interquartile range; PSA, prostate-specific antigen.

SI conversion: To convert PSA to $\mu\text{g/L}$, multiply by 1.0.

^aPercentages are based on alive participants who are prostate cancer-free and for whom the form was submitted.

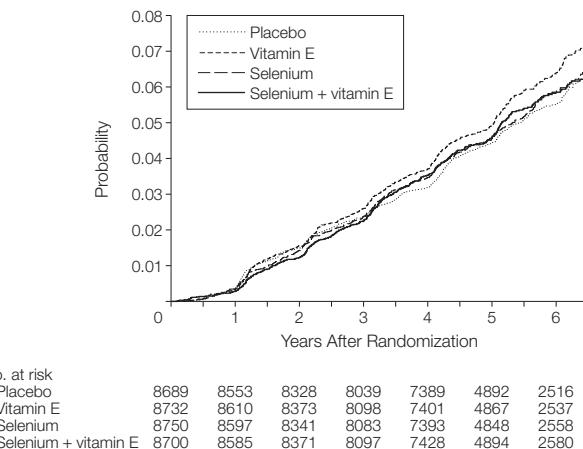
^bGleason score was based on central pathology review. The Gleason grade ranges from 1 to 5, with 5 having the worst prognosis. The Gleason score ranges from 2 to 10, with 10 having the worst prognosis.

and Puerto Rico between August 22, 2001, and June 24, 2004. **FIGURE 1** shows the SELECT randomization scheme including participants who were excluded from analyses; all 621 participants at 2 study sites were removed from the analysis because of severe problems that were detected early on including poor data and participant management and regulatory issues. These participants differed substantially from the rest of the SELECT population in being from sites in the south of the United States, 99% African American, younger (median age 57 years), and of a lower education level (67% had <high school education), and in having lower PSA levels (57% had <1.0 ng/mL) and a higher prevalence of current smokers (33%). An additional 9 participants were removed because they were found to have had prostate cancer at randomization and 15 were removed because their informed consent was never received. More men were accrued (35 533 in 3 years) than initially planned (32 400 in 5 years) mainly because of a faster-than-expected accrual rate and the administrative time it takes to close down accrual.

The baseline characteristics of SELECT participants by each of the 4 groups (placebo, vitamin E, selenium, and selenium + vitamin E) are shown in **TABLE 1**. All potentially important risk factors were well balanced among the groups. A total of 2.6% of SELECT men were former PCPT men randomized to finasteride; during the trial, 4.8% of the non-PCPT participants reported use of finasteride at 5 mg ($n=1602$) or 1 mg ($n=86$).

The median overall follow-up was 5.46 years (range, 4.17-7.33 years). The percentages of participants with a recent last-contact date were more than 88% within 7 months and 92% within 13 months of the SELECT data analysis. Loss to follow-up, defined as having a last contact date of more than 24 months before analysis, involved 5.1% of participants, which was higher than had been estimated for the trial design (3.5% at 7 years after trial activation).

Figure 2. Cumulative Incidence of Prostate Cancer Detected Each Year by Intervention Group



Compared with placebo, there was a statistically nonsignificant increase in prostate cancer in the vitamin E group ($P=.06$) and not in the selenium + vitamin E group ($P=.52$) or the selenium group ($P=.62$).

Adherence to both study agents as determined by pill count was similar across all study groups, and averaged 83% at year 1 and 65% at year 5. Adherence to at least 1 of the 2 agents was 87% at year 1 and 72% at year 5 (the design-estimated adherence rates were 90% at year 1 and 68% at year 5). Bioadherence was measured in a subset of participants by serum levels of selenium and cholesterol-adjusted α -tocopherol and γ -tocopherol (which is suppressed by α -tocopherol) and showed a good separation in agent serum levels between the groups (**TABLE 2**). The drop-in rate was assessed by a direct question to the participants about taking either of the supplements. Positive responses were 3.1% or less for vitamin E and 1.8% or less for selenium in each year (below the design drop-in estimate of 10%). Prostate tissue samples were sent to the central pathology laboratory for confirmation in 86% of cases. The central laboratory agreed with the clinical site's prostate cancer diagnosis in 99% of these cases.

Prostate Cancer

There were no statistically significant differences in the rates of prostate cancer between the 4 groups (placebo, 416 cases [5-year rate of 4.43%]; selenium, 432

cases [4.56%]; vitamin E, 473 cases [4.93%]; selenium + vitamin E, 437 cases [4.56%]) (**TABLE 3** and **FIGURE 2**). Compared with placebo, the hazard ratios (HRs) for prostate cancer were 1.13 (99% confidence interval [CI], 0.95-1.35; 95% CI, 0.99-1.29; $P=.06$) in the vitamin E-alone group, 1.05 (99% CI, 0.88-1.25; 95% CI, 0.91-1.20; $P=.52$) in the selenium + vitamin E group, and 1.04 (99% CI, 0.87-1.24; 95% CI, 0.90-1.18; $P=.62$) in the selenium-alone group. The data and safety monitoring committee had some concern over the statistically nonsignificant increase in prostate cancer in the vitamin E-alone group ($P=.09$ per interim data of August 1, 2008) and over a nonsignificant increase in diabetes mellitus associated with selenium ($P=.08$ per interim data of August 1, 2008).

The majority of prostate cancers diagnosed during the trial were early-stage and low-grade, and cancer stage and grade were similar across all groups (**Table 3**). The percentage of patients who had an annual PSA examination and DRE was similarly high and the biopsy rate was similar across all groups, indicating that the prostate cancer findings were not due to screening-associated detection bias. More than 95% of prostate cancers were diag-

nosed by biopsy, the triggers for which (based on PSA and other factors) are shown in Table 3 and were similar across all groups. The number of prostate cancers in the placebo cohort was higher than what was estimated at study inception. This was due to the faster than expected accrual, the larger than expected sample size, and higher baseline PSA levels than anticipated.

Secondary Outcomes

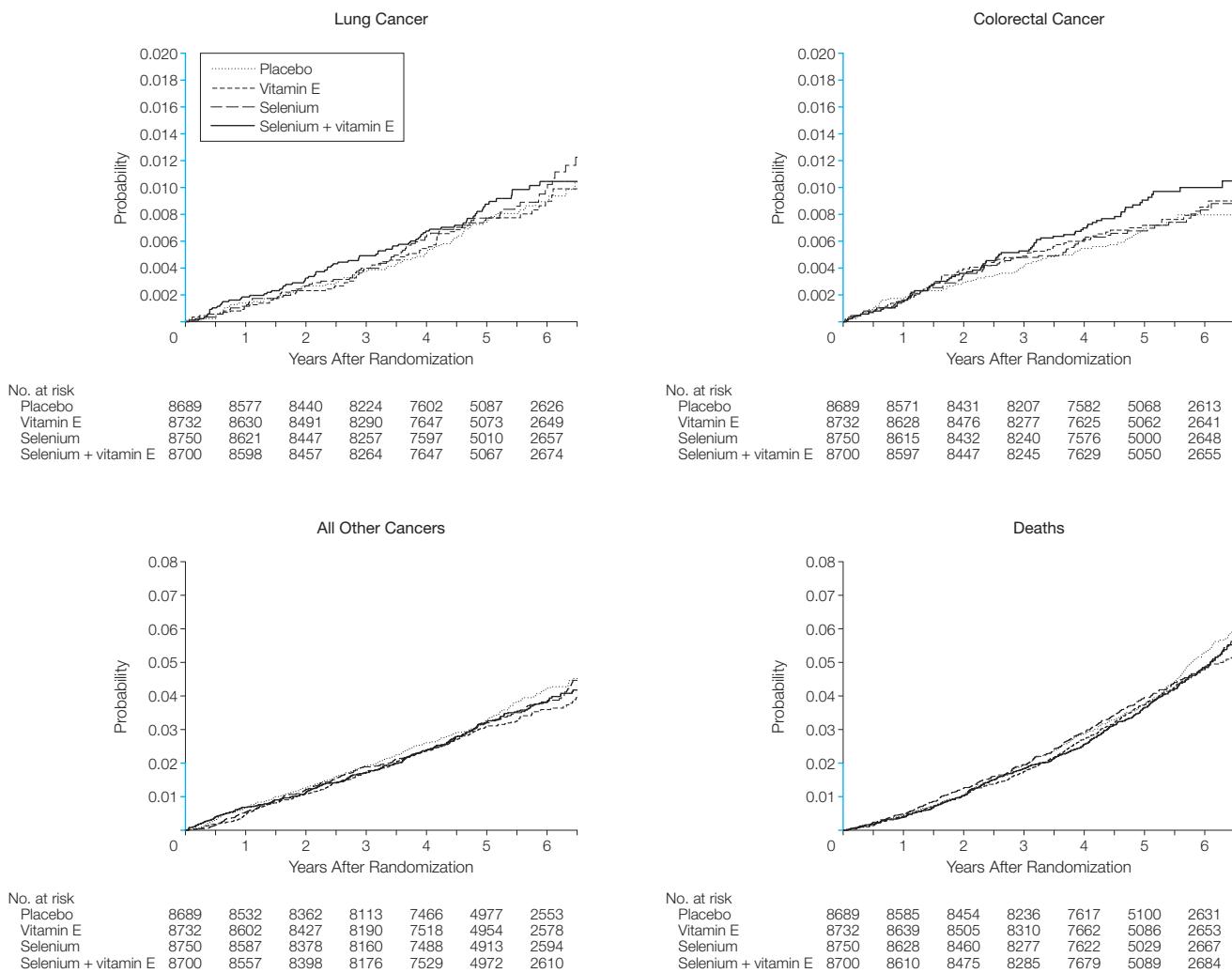
There were no significant differences (all $P > .15$) in any prespecified secondary cancer end points (FIGURE 3 and TABLE 4). At 5 years, the cumulative

death rate in the placebo group was 38 deaths per 1000 participants (95% CI, 34 deaths per 1000 participants to 42 deaths per 1000 participants); the estimated rate at trial inception was 48 deaths per 1000 participants. The numbers of deaths from any cause were similar across the 4 groups (382 in placebo group, 358 in vitamin E group, 378 in selenium group, and 359 in selenium + vitamin E group).

The study agents had no significant effects on the overall incidence of cardiovascular events (Table 4). A statistically nonsignificant increase in type 2 diabetes mellitus (diagnosed after ran-

domization) occurred in the selenium-alone group vs placebo group ($n=724$; 10.0%; 99% CI, 9.1%-11.0%; vs $n=669$; 9.3%; 99% CI, 8.5%-10.2%, respectively; RR, 1.07; 99% CI, 0.94-1.22; $P=.16$). The number (percentage) of cases of diabetes mellitus was 700 (9.7%; 99% CI, 8.8%-10.6%) in the vitamin E group and 660 (9.1%; 99% CI, 8.2%-10.0%) in the selenium + vitamin E group (P values of these data compared with placebo were 0.47 for vitamin E and 0.61 for selenium + vitamin E). Data on known, clinically less significant adverse effects of the study agents (alopecia, dermatitis, halitosis,

Figure 3. Cumulative Incidence of Lung Cancer, Colorectal Cancer, All Other Primary Cancers, and Deaths by Intervention Group



There were no significant differences in any prespecified secondary cancer or death end points (all $P > .15$). The blue portions of the y-axes indicate 0 to 0.02 cancer probability.

nail changes, fatigue, and nausea) are shown in TABLE 5. The only statistically significant differences ($P < .01$) were for selenium vs placebo for alopecia and grades 1 to 2 dermatitis.

COMMENT

In SELECT, neither 200 µg of selenomethionine or 400 IU of synthetic DL α-tocopherol, given orally alone or combined for a median of 5.5 years had significant effects on the primary or secondary end points. A statistically nonsignificant increased incidence of prostate cancer ($P = .06$) was observed in the vitamin E group but not in the selenium + vitamin E group. The trial supplements were dis-

continued early (in year 7 of the overall 12-year study) in accordance with a unanimous recommendation of the data and safety monitoring committee stating that, based on the evidence to date from the 7-year planned interim analyses, there was no evidence of a benefit from either study agent and no possibility of a benefit to the planned degree with additional follow-up. Sensitivity analyses suggested that the prespecified 25% risk reduction was extremely unlikely to be reached for either agent even with additional exposure.

The statistical assumptions made in SELECT involving accrual rate, study supplement adherence and drop-in rates, prostate cancer incidence, death

rate, and loss to follow-up were largely met and gave the trial significant power to detect the estimated preventive effects. Furthermore, the large sample size, inclusion of a substantial proportion of non-white men, and equal distribution of known risk factors across all trial groups make the conclusions drawn from SELECT especially robust and generalizable.

Why were selenium and vitamin E ineffective in preventing prostate cancer in SELECT despite strong secondary evidence suggesting efficacy?^{7,8} Considering selenium first, the secondary reduction in prostate cancer incidence in the NPC study could have been subject to

Table 4. Secondary Outcomes Including Diagnosis of Other Primary Cancers, Diabetes, Cardiovascular Events, and Deaths^a

	Placebo (n = 8696)		Vitamin E (n = 8737)		Selenium (n = 8752)		Selenium + Vitamin E (n = 8703)	
	No. of Men	HR (99% CI)	No. of Men	HR (99% CI)	No. of Men	HR (99% CI)	No. of Men	HR (99% CI)
Any cancer (including prostate) ^b	824	1 [Reference]	856	1.03 (0.91-1.17)	837	1.01 (0.89-1.15)	846	1.02 (0.90-1.16)
Lung	67	1 [Reference]	67	1.00 (0.64-1.55)	75	1.12 (0.73-1.72)	78	1.16 (0.76-1.78)
Colorectal	60	1 [Reference]	66	1.09 (0.69-1.73)	63	1.05 (0.66-1.67)	77	1.28 (0.82-2.00)
Other primary cancer ^c	306	1 [Reference]	274	0.89 (0.72-1.10)	292	0.95 (0.77-1.17)	290	0.94 (0.76-1.16)
	No. of Men	RR (99% CI)	No. of Men	RR (99% CI)	No. of Men	RR (99% CI)	No. of Men	RR (99% CI)
Diabetes ^d	669	1 [Reference]	700	1.04 (0.91-1.18)	724	1.07 (0.94-1.22)	660	0.97 (0.85-1.11)
Cardiovascular events								
Any (including death)	1050	1 [Reference]	1034	0.98 (0.88-1.09)	1080	1.02 (0.92-1.13)	1041	0.99 (0.89-1.10)
Nonfatal strokes								
Hemorrhagic	11	1 [Reference]	7	0.63 (0.18-2.20)	11	0.99 (0.33-2.98)	12	1.09 (0.37-3.19)
Ischemic	56	1 [Reference]	49	0.87 (0.53-1.44)	51	0.90 (0.55-1.49)	67	1.20 (0.75-1.90)
Not specified ^e	25	1 [Reference]	14	0.56 (0.24-1.32)	11	0.44 (0.17-1.11)	20	0.80 (0.37-1.73)
Other nonfatal (worst grade) ^f								
Grade 3	626	1 [Reference]	642	1.02 (0.89-1.17)	685	1.09 (0.95-1.25)	624	1.00 (0.87-1.15)
Grade 4	190	1 [Reference]	203	1.06 (0.82-1.38)	193	1.01 (0.78-1.31)	201	1.06 (0.82-1.37)
	No. of Men	HR (99% CI)	No. of Men	HR (99% CI)	No. of Men	HR (99% CI)	No. of Men	HR (99% CI)
Deaths	382	1 [Reference]	358	0.93 (0.77-1.13)	378	0.99 (0.82-1.19)	359	0.94 (0.77-1.13)
Cancer	125	1 [Reference]	106	0.84 (0.60-1.18)	128	1.02 (0.74-1.41)	117	0.93 (0.67-1.30)
Prostate	0	1 [Reference]	0	NA	1	NA	0	NA
Lung	41	1 [Reference]	38	0.92 (0.52-1.65)	45	1.10 (0.63-1.91)	39	0.95 (0.53-1.69)
Colorectal	10	1 [Reference]	13	1.30 (0.44-3.83)	10	1.00 (0.32-3.16)	15	1.49 (0.52-4.28)
Other primary cancer ^c	74	1 [Reference]	55	0.74 (0.47-1.17)	72	0.97 (0.63-1.49)	63	0.85 (0.55-1.32)
Cardiovascular	142	1 [Reference]	119	0.84 (0.61-1.15)	129	0.91 (0.66-1.24)	117	0.82 (0.60-1.13)
Hemorrhagic stroke	8	1 [Reference]	9	1.12 (0.32-3.92)	9	1.12 (0.32-3.93)	12	1.49 (0.46-4.84)
Other cardiovascular	134	1 [Reference]	110	0.82 (0.59-1.14)	120	0.89 (0.65-1.24)	105	0.78 (0.56-1.09)
Other deaths	115	1 [Reference]	133	1.15 (0.83-1.60)	121	1.05 (0.75-1.47)	125	1.08 (0.78-1.51)

Abbreviations: CI, confidence interval; HR, hazard ratio; NA, not applicable; RR, relative risk.

^aThe HRs and RRs given for vitamin E, selenium, and selenium + vitamin E groups are compared with the placebo group.

^bNo. of participants that had more than 1 cancer for each group are placebo (n=25), vitamin E (n=24), selenium (n=25), and selenium + vitamin E (n=36).

^cExcluding basal cell and squamous cell skin cancers.

^dBased on self-report or reported use of diabetes medications of the glitazone class; excludes prevalent cases at randomization.

^eNot specified as to whether an ischemic or hemorrhagic stroke.

^fAccording to National Cancer Institute Common Toxicity Criteria.

limitations inherent in secondary analyses, such as chance findings due to multiple testing, especially because the overall NPC sample size was relatively small (1312 men and women vs 29 133 men in the ATBC study). Second, the formulation (high-selenium yeast) given in the NPC trial may have been more active than the *l*-selenomethionine given in SELECT (both trials gave an equivalent selenium dose). In designing SELECT, we carefully evaluated the choice of *l*-selenomethionine vs high-selenium yeast (and other formulations),²⁰ and our rationale for selecting *l*-selenomethionine included the following considerations: selenomethionine was the major component of apparently active high-selenium yeast; evidence indicated substantial batch-to-batch variations in specific organoselenium compounds in samples of NPC yeast, making it unlikely that we could duplicate the selenium yeast formulation used in the NPC study; potential genotoxicity of highly active inorganic selenium compounds, such as selenite, made them potentially unsuitable for long-term prevention; lowering (vs selenomethionine) of overall body selenium stores with selenite, which is neither absorbed nor retained well; practical and safety concerns over newer selenium compounds, such as mono-methylated forms (eg, lacking availabil-

ity, investigational new drug certification, and clinical data); and in vitro data indicating that selenomethionine was effective in suppressing malignant and not normal prostate cells.¹⁵

Despite this careful rationale, it is impossible to know now whether selenized yeast would have been more active than *l*-selenomethionine was in SELECT. Finally, the NPC trial was conducted in men chosen for deficient levels of selenium, finding that selenium was most preventive in the men with the lowest baseline selenium levels⁹; SELECT men generally were replete in selenium at baseline, with median serum selenium levels of 135 ng/mL vs 113 ng/mL in NPC. The NPC cutpoint for the lowest 2 tertiles was 121.6 units; 78% of SELECT men were above this level. The NPC trial found a nonsignificant increase in overall cancer rate in its highest tertile (HR, 1.20; 95% CI, 0.77-1.86).²²

There are potential reasons why vitamin E did not prevent prostate cancer in SELECT. First, the high dose (400 IU/d) of the α -tocopherol form of vitamin E in SELECT may have been less effective than a lower dose such as the 8-fold lower 50 mg/d (roughly equivalent to 50 IU/d) that produced the earlier positive secondary findings in the ATBC study.⁷ (The vitamin E formulation, synthetic *all rac*- α -tocopherol acetate, was the same in

SELECT and the ATBC study.) A secondary analysis of the HOPE trial²³ found that a relatively high dose of natural vitamin E did not reduce prostate cancer incidence. Achieving higher plasma or tissue levels of α -tocopherol within the physiological range, such as through a 50-mg/d supplement, may have some prostate cancer (or other) preventive effect such as cell proliferation or tumor growth inhibition.²⁴ Furthermore, high pharmacological doses of α -tocopherol may have an adverse effect on cytochrome p450 enzyme and other regulatory mechanisms²⁵ that a lower dose would not have. It is also possible (but not certain) that the known effect of α -tocopherol in suppressing potentially beneficial plasma γ -tocopherol levels would have been less with the lower than higher dose of α -tocopherol.²⁰ Nevertheless, men taking vitamin E with the highest baseline (and thus total) serum vitamin E levels in the ATBC study had the highest reduction in prostate and lung cancer,²⁶ which supported our choice of the higher dose. A higher dose also was associated with potential benefits such as reductions in aging-related Alzheimer disease and macular degeneration.

Second, several studies have suggested that vitamin E is more protective against prostate cancer in smokers, and

Table 5. Adverse Events Known to Be Associated With the Study Supplements^a

Adverse Event	Placebo (n = 8696)		Vitamin E (n = 8737)		Selenium (n = 8752)		Selenium + Vitamin E (n = 8703)	
	No. of Men	RR (99% CI)	No. of Men	RR (99% CI)	No. of Men	RR (99% CI)	No. of Men	RR (99% CI)
Alopecia	206	1 [Reference]	220	1.06 (0.83-1.36)	265	1.28 (1.01-1.62) ^b	238	1.15 (0.91-1.47)
Dermatitis								
Grades 1-2	516	1 [Reference]	591	1.14 (0.98-1.32)	605	1.17 (1.00-1.35) ^b	554	1.07 (0.92-1.25)
Grades 3-4	8	1 [Reference]	12	1.49 (0.46-4.83)	14	1.74 (0.56-5.44)	16	2.00 (0.66-6.09)
Halitosis	427	1 [Reference]	493	1.15 (0.97-1.36)	503	1.17 (0.99-1.38)	531	1.24 (1.06-1.46)
Nail changes	1035	1 [Reference]	1041	1.00 (0.90-1.11)	1087	1.04 (0.94-1.16)	1075	1.04 (0.93-1.15)
Fatigue								
Grades 1-2	586	1 [Reference]	604	1.03 (0.89-1.19)	645	1.09 (0.95-1.26)	612	1.04 (0.90-1.20)
Grades 3-4	24	1 [Reference]	29	1.20 (0.59-2.45)	21	0.87 (0.40-1.88)	20	0.83 (0.38-1.81)
Nausea								
Grades 1-2	203	1 [Reference]	191	0.94 (0.72-1.21)	244	1.19 (0.94-1.52)	202	0.99 (0.77-1.28)
Grade 3	9	1 [Reference]	3	0.33 (0.06-1.85)	9	0.99 (0.30-3.34)	8	0.89 (0.25-3.10)

Abbreviations: CI, confidence interval; RR, relative risk.

^aThe RRs given for vitamin E, selenium, and selenium + vitamin E groups are compared with the placebo group. Maximum grade experienced by a participant are given. Alopecia, halitosis, and nail changes were only defined for grades 1 and 2. National Cancer Institute Common Toxicity Criteria were used for alopecia, nail changes, fatigue, and nausea. Halitosis and dermatitis were defined in the study protocol. Generally, grade 1=mild, grade 2=moderate, grade 3=severe, and grade 4=life-threatening.

^b $P < .01$.

less than 60% of SELECT men were current or former smokers (whereas all men in the ATBC study were smokers). For example, observational analyses in a trial-based cohort of the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO),²⁷ a trial of screening vs standard health care routines, showed a 71% reduction in the incidence of advanced prostate cancer associated with supplemental vitamin E use in current and recent smokers. A subgroup analysis of current and former smokers in SELECT, however, did not show a smoking-related benefit (placebo, 4.6% [223/4863] vs vitamin E alone, 4.8% [232/4853]). As with selenium in the NPC study, vitamin E effects on prostate cancer incidence in the ATBC study could have been due to chance findings in secondary analyses.

Selenium was not associated with significant effects on cardiovascular events, lung cancer, other cancers, or overall mortality in SELECT. One safety concern with selenium is a potential association with increased risk for type 2 diabetes mellitus, for which there are mixed data from prior studies.^{28,29} A recent analysis of the NPC study population showed a significant increase in type 2 diabetes mellitus (by self-report and medical records), largely limited to the top tertile of plasma selenium levels at baseline.³⁰

In SELECT, a nonsignificant increase in risk (RR, 1.07; $P=.16$) of diabetes mellitus compared with placebo was observed in the selenium group but not in the selenium + vitamin E group (RR, 0.97; $P=.62$). Concerns about the safety of vitamin E supplementation arose during SELECT. One meta-analysis³¹ found that vitamin E at doses of at least 400 IU/d increased all-cause mortality, and another study³² found evidence that vitamin E supplementation, alone or in combination with other antioxidants, may increase mortality. Neither study is directly relevant to the doses and population studied in SELECT; many studies included in these meta-analyses were in patients with serious disease, and the finding of increased mortality was driven by studies using doses

far higher than 400 IU/d. In more relevant, placebo-controlled trials completed in healthy men and women, there were no associations of vitamin E supplementation with increased risks of either cardiovascular disease or overall mortality.³³ SELECT results support the safety of vitamin E at 400 IU/d in healthy men, because there were no increases in either cardiovascular disease or total mortality in the vitamin E groups.

The 35 533 randomized men of SELECT were needed because of the robust statistical design accommodating 4 study groups with 5 primary comparisons; this large trial population made SELECT the largest cancer chemoprevention trial ever conducted to our knowledge. African American men have among the highest prostate cancer risks in the world, and SELECT had the highest participation of African American men (13%) of any large-scale cancer chemoprevention trial to date.

The statistical rigor of the trial was matched by the rigor of its implementation. Features of this implementation included the SELECT Workbench, a secure Web site administered by the SELECT statistical center and used by study-site staff and investigators. The SELECT Workbench was used to access participant and site-specific reports, the study protocol, and a detailed study manual and to submit data using Web-based forms. Form submission included detailed edit checks and a tracking system to identify all expected forms. Training and monitoring consisted of semi-annual workshops, quality assurance audits at least once every 3 years, and mentoring by trained statistical center staff and experienced clinical research associates. SELECT also maintained a public Web site initially designed to recruit participants and later used to promote participant adherence and to keep SELECT in the public's eye.²⁰

Potential limitations of SELECT include that it did not test different formulations or doses of selenium and vitamin E and that it did not definitively assess results in subgroups of men who may have responded differently than did the overall population. Because of ac-

tive annual screening (eg, PSA in 85%; Table 3) and early detection (eg, 99.4% stage T1 or T2; Table 3), SELECT could not assess effects in reducing advanced or fatal prostate cancer, which recent data suggest may be a potential benefit of vitamin E and selenium.^{18,27,34-36} SELECT also could not assess intervention effects in a population deficient in vitamin E, selenium, or both since our trial population was well-nourished at baseline, or in current smokers since they represented only 7.5% of the SELECT population, a substantial difference from the ATBC study in predominantly heavy smokers.

Cancer chemoprevention is an important approach for reducing cancer burden.³⁷ Several randomized controlled trials have demonstrated significant cancer or premalignancy risk reductions in the breast, colon-rectum, prostate, and stomach.³⁸⁻⁴⁴ Prostate cancer is a particularly attractive target for chemoprevention because of its clinical ubiquity, substantial treatment-associated morbidity, and step-wise molecular pathogenesis. In the large-scale PCPT, which was reported 2 years after SELECT was activated, finasteride produced a 25% relative reduction in the 7-year period prevalence of prostate cancer (vs placebo),⁴³ and recent data suggest that finasteride reduces the risk of clinically significant disease and may not induce high-grade cancers despite initial concerns to the contrary.⁴⁵⁻⁴⁹

CONCLUSION

In conclusion, SELECT has definitively demonstrated that selenium, vitamin E, or selenium + vitamin E (at the tested doses and formulations) did not prevent prostate cancer in the generally healthy, heterogeneous population of men in SELECT. These data underscore the prudence that is needed in considering recommendations to use agents for the prevention or control of disease in the absence of convincing clinical trial results. These findings also compel the medical research community to continue the search for new, effective agents for prostate cancer prevention.

Published Online: December 9, 2008 (doi:10.1001/jama.2008.864).

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Author Contributions: Drs Lippman and Klein had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Both contributed equally to the study.

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Obtained funding: Lippman, Coltman.

Administrative, technical, or material support: Lippman, Klein, P. Goodman, Lucia, Thompson, Ford,

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Financial Disclosures: Dr Lucia reported serving as a consultant for GlaxoSmithKline and Veridex, and being a member of the Advisory Board for GenProbe. Dr Thompson reported serving as a consultant for Veridex and Mission Pharmacal (with fees paid to University of Texas Health Sciences Center, San Antonio). Dr Gaziano reported receiving investigator-initiated research funding from VeroScience, Amgen, and BASF Corporation, and research support in the form of study agents and packaging from BASF Corporation, Wyeth Pharmaceuticals, and DSM Nutritional Products Inc (formerly Roche Vitamins); serving as a consultant or receiving honoraria from Bayer AG and Pfizer; and serving as an expert witness for Merck. Dr Meyskens reported being a co-founder of Cancer Prevention Pharmaceuticals. No other authors reported financial disclosures.

Funding/Support: This work was supported in part by Public Health Service Cooperative Agreement grant CA37429 awarded by the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, and in part by the National Center for Complementary and Alternative Medicine (National Institutes of Health). Study agents and packaging were provided by Perrigo Company (Allegan, Michigan), Sabinsa Corporation (Piscataway, New Jersey), Tishcon Corporation (Westbury, New York), and DSM Nutritional Products Inc (Parsippany, New Jersey).

Role of the Sponsor: The National Cancer Institute was involved in the design and conduct of the study, in the analysis and interpretation of the data, and in the preparation, review, and approval of the manuscript.

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Disclaimer: Dr Gaziano, a contributing editor for *JAMA*, was not involved in the editorial review or decision to publish this article.

Additional Contributions: We thank the 35 533 men and many principal investigators and clinical research associates at our 427 clinical sites, whose participation in SELECT has written an important chapter in the history of cancer prevention. We also thank the many personnel of the Southwest Oncology Group (the coordinating group of this Intergroup trial), whose tireless efforts allowed SELECT to successfully complete the test of its primary hypotheses. No compensation was received.

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Baseline Characteristics and the Effect of Selenium Supplementation on Cancer Incidence in a Randomized Clinical Trial: A Summary Report of the Nutritional Prevention of Cancer Trial¹

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Abstract

The Nutritional Prevention of Cancer Trial was a randomized, clinical trial designed to evaluate the efficacy of selenium as selenized yeast (200 µg daily) in preventing the recurrence of nonmelanoma skin cancer among 1312 residents of the Eastern United States. Original secondary analyses through December 31, 1993 showed striking inverse associations between treatment and the incidence of total [hazard ratio (HR) = 0.61, 95% confidence interval (CI) = 0.46–0.82], lung, prostate, and colorectal cancer and total cancer mortality. This report presents results through February 1, 1996, the end of blinded treatment. Effect modification by baseline characteristics is also evaluated. The effects of treatment overall and within subgroups of baseline age, gender, smoking status, and plasma selenium were examined using incidence rate ratios and Cox proportional hazards models. Selenium supplementation reduced total (HR = 0.75, 95% CI = 0.58–0.97) and prostate (HR = 0.48, 95% CI = 0.28–0.80) cancer incidence but was not significantly associated with lung (HR = 0.74, 95% CI = 0.44–1.24) and colorectal (HR = 0.46, 95% CI = 0.21–1.02) cancer incidence. The effects of treatment on other site-specific cancers are also described. The protective effect of selenium was confined to males (HR = 0.67, 95% CI = 0.50–0.89) and was most pronounced in former smokers. Participants with

baseline plasma selenium concentrations in the lowest two tertiles (<121.6 ng/ml) experienced reductions in total cancer incidence, whereas those in the highest tertile showed an elevated incidence (HR = 1.20, 95% CI = 0.77–1.86). The Nutritional Prevention of Cancer trial continues to show a protective effect of selenium on cancer incidence, although not all site-specific cancers exhibited a reduction in incidence. This treatment effect was restricted to males and to those with lower baseline plasma selenium concentrations.

Introduction

The NPC⁴ Trial (1) contributed substantially to the evidence supporting selenium as a chemopreventive agent. These results, which have been cited in the medical literature over 400 times in the last 5 years, have also received considerable public attention. The study was originally designed to test the efficacy of selenium supplementation in preventing NMSC recurrence in men and women with a history of two or more BCCs or one SCC of the skin. The hypothesis for this trial was supported by Clark's observation that populations in the southeastern United States, a region with soil selenium concentrations lower than those of the rest of the country, showed elevated NMSC rates (2). Thus, Clark and colleagues initiated a randomized clinical trial of selenium supplementation for preventing the recurrence of NMSC in this high-risk population. The original trial results failed to confirm that selenium supplementation prevented NMSC recurrence. Indeed, the incidence of new BCCs was increased by a nonsignificant 10% among selenium-supplemented individuals, whereas the incidence of new SCCs was increased by an again nonsignificant 14%.

Nevertheless, early in the intervention, an unexpected deficit of other cancer and mortality endpoints among selenium-supplemented participants became apparent, so that in 1993, endpoints for the trial were expanded to include lung, prostate, and colorectal cancer, as well as total cancer incidence and total cancer mortality. In 1994, the Safety Monitoring and Advisory Committee recommended the trial be unblinded and results published. The National Cancer Institute audited the study in May 1995, and, with the National Cancer Institute's approval, the blinded phase of patient treatment and follow-up ended in February 1996. At this time, all participants were informed of their treatment status, given the opportunity to take selenium supplements, and reconsented to participate in the Open-Label Phase of this trial.

Received 11/2/01; revised 4/1/02; accepted 4/23/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported in part by National Cancer Center Grant RO1 CA49764 from the NIH.

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⁴ The abbreviations used are: NPC, Nutritional Prevention of Cancer; HR, hazard ratio; CI, confidence interval; NMSC, nonmelanoma skin cancer; BCC, basal cell carcinoma; SCC, squamous cell carcinoma; BMI, body mass index; PY, person-year(s); RR, relative risk; PHS, Physicians' Health Study.

The apparent effects of selenium supplementation on cancer incidence through the end of 1993 (representing an average of 6.4 years of subject follow-up) were striking. The original report indicated that selenium supplementation led to a marginally statistically significant decrease in (a) lung cancer incidence ($HR = 0.56$, 95% CI = 0.0.31–1.01, $P = 0.05$), (b) statistically significant decreases in prostate cancer incidence ($HR = 0.35$, 95% CI = 0.18–0.65, $P = 0.001$), (c) colorectal cancer incidence ($HR = 0.39$, 95% CI = 0.17–0.90, $P = 0.03$), (d) total cancer incidence ($HR = 0.61$, 95% CI = 0.46–0.82, $P < 0.001$), and (e) total cancer mortality ($HR = 0.48$, 95% CI = 0.31–0.76, $P = 0.001$).

The current report adds considerably to the statistical precision of this trial by extending the previously reported results (September 15, 1983 to December 31, 1993) through the entire blinded phase of the trial (September 15, 1983 to February 1, 1996). With total cancer incidence as the primary end point, mean subject follow-up time was enhanced by 1 year to an average of 7.4 years. Overall, selenium supplementation continued to reduce the incidence of total cancer and prostate, colorectal, and lung cancers, although the reduction in incidence of the latter two cancers was not statistically significant. Not all site-specific cancers presented in this report exhibited a reduction in risk with selenium supplementation. In addition, this analysis describes the effect of selenium supplementation on total cancer incidence within subgroups defined by key baseline characteristics including age, gender, smoking status, and plasma selenium. The protective effect of selenium supplementation on total cancer incidence was most prominent in males and those with lower baseline plasma selenium concentrations. Although the examination of treatment effects in subgroup analyses is fraught with potential limitations, and the modest sample size limits statistical power and interpretation, subgroup analyses in this important dataset provide an opportunity to evaluate trends in the data, which may further our understanding of the effectiveness of selenium as a chemopreventive agent.

Materials and Methods

The protocol for the NPC study is described in the original report by Clark *et al.* (1). Briefly, this study was a randomized, double-blind, placebo-controlled trial conducted among 1312 participants living in the Eastern United States. Participants had a history of two or more BCCs or one SCC of the skin, with one of these occurring within the year prior to randomization. Participants had a life expectancy of at least 5 years and had no internal malignancies treated within the previous 5 years. Exclusion criteria included a history of significant liver or kidney disorders. Although recruitment was gender neutral, approximately three-quarters of the participants were male.

This study was conducted in dermatology clinics in seven cities located in low-selenium areas of the United States, including Augusta, Georgia; Macon, Georgia; Columbia, South Carolina; Miami, Florida; Wilson, North Carolina; Greenville, North Carolina; and Newington, Connecticut. Recruitment began on September 15, 1983 and continued each year through 1991. Participants were randomized in a double-blinded fashion to the experimental treatment or an identical placebo. Experimental participants were treated with 200 μ g of selenium supplied in a 0.5-g high-selenium baker's yeast tablet provided by Nutrition 21 (La Jolla, CA) through 1995 and by Cypress Systems (Fresno, CA) thereafter. The selenium content of each batch of pills was determined in the laboratories of Dr. G. F. Combs, Jr. and of Dr. I. S. Palmer (South Dakota State University, Brookings, SD) using the diaminonaphtha-

lene-fluorometric procedure after nitric-perchloric acid digestion (3). Plasma selenium concentration was determined in the laboratory of Dr. G. F. Combs, Jr. by automated electrothermal atomic absorption spectrophotometry (Perkin-Elmer 3030; Perkin-Elmer Corp., Norwalk, CT) equipped with an electrodeless discharge lamp and automatic Zeeman-effect background correction. Quality control included multiple aliquots of human plasma as external control samples. A coefficient of variation of <7% (for duplicate analyses) was the criterion for acceptance (4).

At the baseline interview, sociodemographic and behavioral variables including education (number of years of schooling, 0–18), occupation (classified according to NIH standards), numbers of years on farm, use of vitamin supplements, use of sunscreen, cancer screening information, number of alcoholic drinks/day, smoking status (current, former, never), number of cigarettes smoked/day, and years of smoking were collected from the participants. In addition, a thorough medical and medication history was obtained at baseline and updated at each biannual follow-up visit. Patient medical records from each clinic were reviewed periodically to ascertain information from both study and nonstudy visits to ensure the completeness and accuracy of the information. For participants who became inactive, annual contact was attempted using the National Death Index and ChoicePoint (formerly Equifax Inc.) to determine vital status and identify diagnoses of new illnesses. In the event of reports of new illnesses or medical procedures, research nurses requested medical, surgical, and pathology records from physicians in hospitals for documentation. Searches for additional cases of cancer were also performed at each state tumor registry in which a clinic site was located. An oncologist or appropriate medical specialist reviewed every cancer record and confirmed the diagnosis. A nosologist coded the death certificates. Review and coding of all records occurred in a blinded manner.

At the end of the blinded period of treatment on February 2, 1996, 35.9% of participants were still on treatment, 16.6% were off treatment but still having routine dermatological examinations, 22.1% of participants were censored for dermatological endpoints but not other endpoints, and 24.8% had died. After a total of 9301 PY of follow-up, no participants were lost to vital follow-up, and only seven subjects (three in the selenium group and four in the placebo group) declined to provide additional illness information. Participant-reported compliance indicated that 79.3% of participants (80.3% in the placebo group and 78.4% in the selenium group) missed taking a pill less than twice a month.

Sixty-two participants (including two cancer cases in each treatment group) whose initial blood draws were drawn >4 days after the randomization date were excluded from the analysis. Thus, all statistical analyses were based on data from those 1250 participants with initial blood draws within 4 days of randomization. Results obtained from the total cohort of 1312 participants and the subsample of 1250 participants with valid baseline selenium values (621 participants in the selenium group and 629 participants in the placebo group) showed no significant differences when continuous (age, BMI, and plasma selenium concentrations) and categorical (gender and smoking status) baseline variables were compared using *t* tests and χ^2 tests, respectively. In addition, no significant differences in incidence data from the total cohort and subsample of the NPC participants were detected.

Within the subsample of 1250 NPC participants, *t* tests and χ^2 tests were conducted to determine any differences in the distribution of these baseline variables between treatment groups. PY of follow-up were calculated among the subsample

Table 1 Baseline characteristics of participants by treatment group

Characteristic	Selenium	Placebo
Participants randomized (no.)	621	629
Age (yrs) (mean \pm SD)	63.4 \pm 10.2	63.0 \pm 9.9
Gender (% male)	74	75
BMI (kg/m^2) (mean \pm SD)	25.6 \pm 3.9	25.5 \pm 4.1
Smoking status (%)		
Never	34	30
Former	39	40
Current	27	30
Plasma selenium (ng/ml)		
Mean \pm SD	114.4 \pm 22.6	114.0 \pm 21.5
33 rd centile	105.6	104.8
50 th centile	113.6	113.2
66 th centile	122.4	121.2

of 1250 subjects. For subjects without cancer, PY were computed using the date of randomization as the start date, and the earlier of February 1, 1996 or the date of death as the closing date. PY of follow-up for cancer cases were calculated through the date of the first category-specific, postrandomization primary cancer diagnosis (excluding NMSC) documented in pathology, surgery, or medical reports. Participants with multiple cancers at different sites were counted only once in the analysis of total cancer incidence and once in each site-specific analysis in which an incident cancer was diagnosed.

Total cancer incidence data between treatment groups were analyzed statistically through the comparison of Nelson-Aalen (5) cumulative hazard function estimates calculated at different time points of the trial and the two-sided log-rank test. RRs, calculated using the ratio of the incidence density for the treatment groups, and the corresponding 95% CIs for site-specific and total cancer incidence and total cancer mortality were calculated. *P*s were derived from log-rank tests. Supporting analyses included the calculation of HRs and 95% CIs using the Cox proportional hazards model, which allowed adjustment for age at baseline (continuous variable), gender, and smoking status (never, former, current) as covariates when appropriate. Throughout this report, results of both the incidence rate ratio and Cox proportional hazards models will be displayed in the tables, although only the latter will be presented in the text. Among the 1250 participants with baseline blood draws within 4 days of randomization, the effect of selenium supplementation on total cancer incidence was assessed within subgroups determined by baseline characteristics. Effect modification by median age (65 years), gender, and smoking status (never, former, current) at randomization was tested using the Mantel-Haenszel test for heterogeneity in the unadjusted models. The statistical significance of the interaction between each baseline characteristic and treatment group, adjusted for other important baseline variables, was tested in a Cox proportional hazards model that included this interaction and the corresponding main effect terms, in addition to the variables for the adjustment.

The statistical association between total cancer incidence and concentrations of baseline plasma selenium was also determined. Based on the distribution among the 1250 participants with valid values, baseline plasma selenium concentrations were divided by the median ($\leq 113.4 \text{ ng}/\text{ml}$ and $> 113.4 \text{ ng}/\text{ml}$) and by tertiles ($\leq 105.2 \text{ ng}/\text{ml}$, $105.3\text{--}121.6 \text{ ng}/\text{ml}$, and $> 121.6 \text{ ng}/\text{ml}$). The effect of selenium supplementation on total cancer incidence was assessed within these subgroups of baseline plasma selenium with the same techniques used for the analyses within subgroups of baseline age, gender, and smoking status.

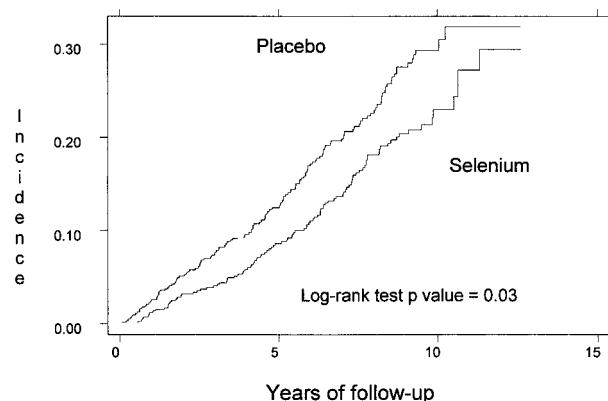


Fig. 1. Cumulative incidence of total cancer in the NPC Trial by treatment group.

HRs, 95% CIs, and tests of statistical significance adjusted for age, gender, and smoking status at baseline were calculated to determine the association between baseline plasma selenium concentrations and the subsequent development of total cancer, according to treatment group. To confirm the consistency of this association, three different measures of baseline plasma selenium were used: (a) as a continuous variable (each unit = 10 ng/ml); (b) by the median value; and (c) by tertiles. The subgroups below the median or in the first tertile of baseline plasma selenium were used as the referent groups in their respective models. Tests for trends in the effects of baseline plasma selenium across tertiles were conducted using the tertile number as a continuous term in Cox proportional hazards models.

All techniques were implemented using STATA 6.0 (6).

Results

Selected baseline characteristics of participants, by treatment group, are displayed in Table 1. The treatment groups were well balanced for all evaluated baseline characteristics. At randomization, the mean age was 63.4 years among participants randomized to selenium and 63.0 years among those randomized to placebo. The mean BMI (kg/m^2) and proportions of current, never, and former smokers at baseline did not vary appreciably across treatment groups. The mean baseline plasma selenium concentrations were 114.4 and 114.0 ng/ml for selenium- and placebo-supplemented individuals, respectively. The distributions of baseline plasma selenium by median and tertile were almost identical across treatment groups.

At unblinding (February 1, 1996), the trial had 9301 PY of follow-up (4694 and 4607 years for the selenium and placebo groups, respectively). Throughout this period, 242 cases of incident cancer were diagnosed. Of these, 105 occurred in the selenium-supplemented group, and 137 occurred in the placebo-supplemented group. Total cancer cumulative incidence curves over time since randomization are shown in Fig. 1. Cumulative incidence was lower among those receiving selenium than among those receiving placebo, throughout the entire trial. At the end of the study, the selenium group showed a significantly lower incidence (25%) of total cancer (HR = 0.75, 95% CI = 0.58–0.97, *P* = 0.03) than the placebo group (Table 2). Table 2 also shows the RR and HR (HR = 0.61, 95% CI = 0.46–0.82, *P* < 0.001) estimates for total cancer from the 1983–1993 analysis published in 1996 (1). The overall effect of

Table 2 Total cancer incidence by treatment group and follow-up period

Follow-up period	Cases		Unadjusted ^a			Adjusted ^b		
	Se	Placebo	RR	95% CI	P	HR	95% CI	P
1983 to Feb. 1, 1996	105	137	0.75	0.58–0.98	0.03	0.75	0.58–0.97	0.03
1983 to Dec. 31, 1993	77	119	0.63	0.47–0.85	0.001	0.61	0.46–0.82	<0.001

^a RR and 95% CI were derived from incidence rate ratios, Ps were derived from log-rank tests.

^b 95% CI and Ps were derived from the Cox proportional hazards model adjusted for age (continuous), gender, and smoking (never, former, current) at randomization.

Table 3 Site-specific cancer incidence by treatment group (through February 1, 1996)

Cancer	Cases		Unadjusted ^a			Adjusted ^b		
	Se	Placebo	RR	95% CI	P	HR	95% CI	P
All sites	105	137	0.75	0.58–0.98	0.03	0.75	0.58–0.97	0.03
Prostate	22	42	0.51	0.29–0.87	0.009	0.48	0.28–0.80	0.005
Lung	25	35	0.70	0.40–1.21	0.18	0.74	0.44–1.24	0.26
Colorectal	9	19	0.46	0.19–1.08	0.055	0.46	0.21–1.02	0.057
Other carcinomas	6	9	0.66	0.19–2.07	0.44	0.67	0.24–1.88	0.44
Other noncarcinomas	3	5	0.59	0.09–3.04	0.50	0.59	0.14–2.47	0.47
Esophageal	2	5	0.39	0.04–2.41	0.28	0.40	0.08–2.07	0.28
Melanoma	11	9	1.21	0.46–3.30	0.68	1.18	0.49–2.85	0.71
Bladder	10	8	1.24	0.44–3.61	0.66	1.28	0.50–3.25	0.60
Breast	11	6	1.82	0.62–6.01	0.24	1.89	0.69–5.14	0.21
Head and neck	9	7	1.27	0.42–4.01	0.65	1.27	0.47–3.42	0.63
Lymphoma and leukemia	8	6	1.32	0.40–4.61	0.62	1.25	0.43–3.61	0.68
Cancer mortality, all sites	40	66	0.59	0.39–0.89	0.008	0.59	0.39–0.87	0.008

^a RR and 95% CI were derived from incidence rate ratios, and Ps were derived from log-rank tests.

^b 95% CI and Ps were derived from the Cox proportional hazards model adjusted for age (continuous), gender, and smoking (never, former, current) at randomization.

selenium is diminished slightly by the inclusion of 25 months of additional follow-up.

Analyses by cancer site are displayed in Table 3. The most frequent site-specific cancer in this cohort was prostate cancer ($n = 64$), closely followed by lung cancer ($n = 60$) and then by colorectal cancer ($n = 28$). Prostate cancer incidence was significantly reduced by selenium supplementation (HR = 0.48, 95% CI = 0.28–0.80, $P = 0.005$); lung cancer incidence showed a nonsignificant 26% reduction (HR = 0.74, 95% CI = 0.44–1.24, $P = 0.26$), and colorectal cancer incidence exhibited a marginally significant reduction of 54% (HR = 0.46, 95% CI = 0.21–1.02, $P = 0.057$). Selenium-supplemented individuals experienced nonsignificant reductions in incidence for other carcinomas (thyroid, pancreatic, gastric, renal, endometrial, mesothelioma, and unknown primary), other noncarcinomas (glioblastoma, Kaposi's sarcoma, astrocytoma, histiocytoma, liposarcoma, leiomyosarcoma, and sarcoma), and cancer of the esophagus. Conversely, participants supplemented with selenium showed nonsignificantly increased incidence of five of the other specific cancers, including melanoma, bladder cancer, breast cancer, head and neck cancer, lymphoma, and leukemia, compared with those supplemented with placebo (Table 3).

Table 3 also shows total cancer mortality by treatment group. One hundred and six cancer deaths occurred throughout the trial. Of these, 40 were among selenium-supplemented individuals, and 66 were among placebo-supplemented participants (HR = 0.59, 95% CI = 0.39–0.87, $P = 0.008$).

The effects of selenium supplementation on total cancer incidence within subgroups defined by baseline cancer risk factors are shown in Table 4. There was no evidence that the effect of selenium supplementation was related to age at baseline. The adjusted treatment effects for males and females were 0.67 (95% CI = 0.50–0.89, $P = 0.005$) and 1.20 (95% CI =

0.66–2.20, $P = 0.55$), respectively. Thus, any protective treatment effect in the study was confined to males. Multivariate adjustment for age and smoking status did not alter the treatment effects within either gender subgroup. Ps for heterogeneity and interaction were not statistically significant.

Table 4 also presents subgroup analysis by baseline cigarette smoking status. Selenium supplementation decreased unadjusted total cancer incidence, although not significantly so, for each category of smoking status (never, former, and current smokers). Similar treatment effects were observed in never smokers (HR = 0.81, 95% CI 0.47–1.41, $P = 0.46$) and current smokers (HR = 0.86, 95% CI 0.56–1.31, $P = 0.47$). Former smokers experienced a statistically significant treatment benefit (HR = 0.66, 95% CI = 0.44–0.97, $p = 0.04$). Nevertheless, Ps for heterogeneity and interaction were not statistically significant.

The report by Clark *et al.* (7), which described a more extensive analysis of incident prostate cancer in the 1983–1993 dataset, indicated that the effect of selenium supplementation was strongest among participants with the lowest baseline plasma selenium concentrations (RR = 0.08, $P = 0.002$ for individuals with baseline plasma selenium concentrations <106.4 ng/ml). We investigated the association between selenium supplementation and the incidence of total cancer across strata of baseline plasma selenium (Table 5). A statistically significant inverse association between selenium supplementation and total cancer incidence was apparent in participants below the median baseline selenium (HR = 0.62, 95% CI = 0.43–0.90, $P = 0.01$), whereas those above the median value at baseline experienced a nonsignificant reduction in incidence (HR = 0.91, 95% CI = 0.63–1.30, $P = 0.60$). However, a significant interaction between treatment group and baseline plasma selenium divided by the median concentration was not apparent (P for interaction = 0.14).

Table 5 shows that selenium supplementation led to a

Table 4 Total cancer incidence by treatment group and subgroups defined by baseline characteristics

	Cases		Unadjusted ^a				Adjusted ^b			
	Se	Placebo	RR	95% CI	P	P, M-H	HR	95% CI	P	P, int ^c
Age (yrs)										
≤65	46	64	0.75	0.50–1.11	0.13	0.95	0.76	0.52–1.12 ^d	0.17	0.98
>65	59	73	0.74	0.51–1.05	0.08		0.75	0.54–1.07 ^d	0.11	
Gender										
Female	23	20	1.14	0.60–2.20	0.66	0.13	1.20	0.66–2.20 ^e	0.55	0.14
Male	82	117	0.68	0.51–0.92	0.008		0.67	0.50–0.89 ^e	0.005	
Smoking status										
Never	25	26	0.85	0.47–1.53	0.57	0.65	0.81	0.47–1.41 ^f	0.46	0.76
Former	42	61	0.67	0.44–1.01	0.05		0.66	0.44–0.97 ^f	0.04	
Current	38	50	0.86	0.55–1.33	0.47		0.86	0.56–1.31 ^f	0.47	

^a RR and 95% CI were derived from incidence rate ratios; Ps were derived from log-rank (P) test and Mantel-Haenszel (P, M-H) test for heterogeneity.

^b HR, 95% CI, and Ps from the Cox proportional hazards model: ^d, adjusted for gender and smoking status (never, former, current) at baseline; ^e, adjusted for age (continuous) and smoking status (never, former, current) at baseline; and ^f, adjusted for age (continuous) and gender at baseline.

^c Ps for treatment group characteristic interaction is for the (treatment group × factor) cross-product term in separate Cox proportional hazards models.

Table 5 Total cancer incidence by treatment group and baseline plasma selenium

Baseline plasma Se	Cases		Incidence ^a		Unadjusted ^b				Adjusted ^c			
	Se	Placebo	Se	Placebo	RR	95% CI	P	P, M-H	HR	95% CI	P	P, int ^d
By median												
≤113.4 (ng/ml)	46	73	1.93	3.12	0.62	0.42–0.91	0.01	0.15	0.62	0.43–0.90	0.01	0.14
>113.4 (ng/ml)	59	64	2.13	2.82	0.90	0.62–1.31	0.57		0.91	0.63–1.30	0.60	
By tertile												
≤105.2 (ng/ml)	27	54	1.71	3.44	0.50	0.30–0.80	0.002	0.02	0.51	0.32–0.81	0.005	0.007
105.3–121.6	34	46	2.13	3.03	0.70	0.44–1.12	0.12		0.70	0.44–1.09	0.11	
>121.6 (ng/ml)	44	37	2.91	2.44	1.19	0.75–1.90	0.43		1.20	0.77–1.86	0.43	

^a Annual cumulative incidence per 100 PY.

^b RR and 95% CI were derived from incidence rate ratios; Ps were derived from log-rank (P) test and Mantel-Haenszel (P, M-H) test for heterogeneity.

^c HR, 95% CI, and P values from the Cox proportional hazards models adjusted for age (continuous), gender, and smoking status (never, former, current) at baseline.

^d Ps for treatment group characteristic interaction is for the (treatment group × factor) cross-product term in separate Cox proportional hazards models.

Table 6 Total cancer incidence according to baseline plasma selenium, by treatment group

Baseline plasma Se	Se ^a				Placebo ^a			
	HR	95% CI	P	P, trend ^b	HR	95% CI	P	P, trend ^b
Continuous								
Per 10 ng/ml	1.12	1.03–1.22	0.005		0.97	0.90–1.05	0.49	
By median								
≤113.4 ng/ml	1.00				1.00			
>113.4 ng/ml	1.45	0.98–2.15	0.06		0.95	0.68–1.34	0.79	
By tertile								
≤105.2 ng/ml	1.00				1.00			
105.2–121.6 ng/ml	1.29	0.78–2.15	0.32		0.88	0.59–1.31	0.52	
>121.6 ng/ml	1.88	1.15–3.05	0.01	0.01	0.76	0.50–1.16	0.20	0.20

^a HR, 95% CI, and P values from the Cox proportional hazards models adjusted for age (continuous), gender, and smoking status (never, former, current) at baseline.

^b Ps for trend across tertiles were conducted using the tertile variable as a continuous term.

significant 49% reduction in incidence among those in the lowest tertile of baseline plasma selenium (HR = 0.51, 95% CI = 0.32–0.81, $P = 0.005$) and to a nonsignificant 30% reduction in incidence among those in the second tertile (HR = 0.70, 95% CI = 0.44–1.09, $P = 0.11$). For those in the third tertile, selenium supplementation was associated with a nonsignificant 20% increase in incidence (HR = 1.20, 95% CI = 0.77–1.86, $P = 0.43$). A significant interaction between treatment group and tertile of baseline plasma selenium was evident (P for interaction = 0.007).

As a means of exploring the nature of this interaction, we present the HRs for total cancer according to baseline selenium

status within treatment group. Table 6 presents these HRs, 95% CIs, and tests of statistical significance, relating baseline plasma selenium concentrations to the subsequent development of total cancer. HRs were calculated using three different exposure measures of baseline plasma selenium: (a) as a continuous variable (each unit = 10 ng/ml); (b) by the median value; and (c) by tertiles.

A strong positive association between baseline plasma selenium and the incidence of total cancer is seen within the selenium group for the continuous, dichotomous, and trichotomous analyses of baseline selenium concentrations. When baseline plasma selenium is treated as a continuous variable,

selenium supplementation increased total cancer incidence by 12% ($HR = 0.12$, 95% CI = 1.03–1.22, $P = 0.005$) for every unit (where 1 unit = 10 ng/ml) increase in baseline plasma selenium concentration. When selenium is treated as a dichotomous variable, the comparison of total cancers above the median with those below the median yielded a HR of 1.45 (95% CI = 0.98–2.15, $P = 0.06$). Using the first baseline selenium tertile as the referent group among selenium-supplemented subjects, the HR was 1.29 (95% CI = 0.78–2.15, $P = 0.32$) in the second tertile and 1.88 (95% CI = 1.15–3.05, $P = 0.01$) in the third tertile of baseline plasma selenium. The trend for this association was statistically significant ($P = 0.01$). Thus, the trichotomous analysis revealed that among selenium-supplemented participants, those in the third tertile experienced an almost 2-fold, statistically significant elevation of incidence compared with participants in the first tertile.

The association of baseline plasma selenium and total cancer in the placebo group, albeit weak, was in the protective direction, with individuals of higher status showing a lower incidence of total cancer (Table 6). There was a nonsignificant decrease in incidence of total cancer with increasing baseline selenium in increments of 10 ng/ml ($HR = 0.97$, 95% CI = 0.90–1.05, $P = 0.49$). The decrease in incidence was also nonsignificant when comparing the effects of baseline selenium above the median, as opposed to below the median ($HR = 0.95$, 95% CI = 0.68–1.34, $P = 0.79$). This nonsignificant reduction in total cancer incidence is again apparent in the comparison of tertiles of baseline plasma selenium. Using the first tertile as the referent group, the HR was 0.88 (95% CI = 0.59–1.31, $P = 0.52$) in the second and 0.76 (95% CI = 0.50–1.16, $P = 0.20$) in the third tertile. The P for trend in this trichotomous analysis was 0.20.

Discussion

The NPC Trial is the only double-blind, placebo-controlled, randomized trial to date to have tested the effect of selenium supplementation on cancer incidence in a Western population. The original secondary analyses of the NPC data showed a highly significant inverse association of selenium supplementation with the incidence of total cancer through December 31, 1993, over a mean period of 6.4 years of follow-up (1). In this report, we describe analyses of the effect of selenium supplementation on total cancer incidence through the end of randomized, blinded treatment (February 1, 1996). This extended follow-up attenuated the protective effect of selenium supplementation on total cancer incidence, although selenium supplementation continued to reduce the incidence of total cancer over a mean follow-up of more than 7 years. A significant inverse association with the most common cancer, prostate cancer, was observed. For the next most common sites, lung and colorectal cancers, respectively, inverse but nonsignificant associations with selenium supplementation and incidence were determined. These results are consistent with the majority of epidemiological studies that support the efficacy of selenium as a chemopreventive agent against all cancers (8–16) and prostate (17–19), lung (12, 20–24), and colorectal cancers (25–28). However, not all epidemiological trials consistently support a protective association between selenium and cancer (26, 29–41).

Of the remaining eight cancer sites evaluated in this report, nonsignificant reductions in incidence were apparent in three categories: (a) other carcinomas; (b) other noncarcinomas; and (c) esophageal cancer. Results from epidemiological trials on these three cancer categories have been inconsistent (41–45).

Conversely, nonsignificant increases in incidence were

evident in five cancer types, including melanoma, bladder cancer, breast cancer, head and neck cancer, and lymphoma and leukemia. These results, although nonsignificant and based on small case numbers, may indicate potential increased risk with selenium supplementation. Previous reports on the effects of selenium on melanoma (24, 46–49), bladder cancer (33, 48, 50), head and neck cancer (51–53), and lymphoma and leukemia (24, 37, 41, 54) in epidemiological trials have been varied. The evidence associating selenium status and breast cancer is conflicting. Analogous to that observed in the NPC Trial, several prospective studies have shown nonsignificant positive associations between serum (24) and toenail (55, 56) selenium status and breast cancer. However, the lack of an association between serum (57), toenail (58), and four indicators (59) of selenium status and breast cancer risk has been suggested by several case-control studies. Similarly, several prospective trials have shown equivocal associations between serum (37, 60) and toenail (61) selenium status and breast cancer risk. Many case-control studies have suggested a protective effect of higher selenium status (26, 62, 63) in postmenopausal but not premenopausal women (64). In addition, inverse trends between breast cancer and selenium concentrations in serum (29), toenails (65), and drinking water (41) have been suggested in prospective trials, although results were nonsignificant. Moreover, significant inverse associations between breast cancer risk and serum (66–68) and hair (23) selenium have been documented.

Methodological issues, primarily the difficulty of assessing long-term selenium exposure, may explain some of the inconsistencies in the association of selenium and cancer reported from epidemiological trials (69). In addition, treatment and disease may alter selenium status and thus may lead to temporal ambiguity and misclassification of selenium status. Nevertheless, a meta-analysis of cohort studies comparing associations of serum selenium, retinol, β -carotene, and vitamin E with cancer suggests that selenium has a remarkably consistent protective effect (70).

Furthermore, evidence for the chemopreventive efficacy of selenium has been consistently represented in laboratory trials, although the exact mechanism(s) of its activity is unclear. The overwhelming majority of *in vivo* studies in rodents have shown that various forms of selenium inhibit carcinogen-induced covalent DNA adduct formation and retard oxidative damage to DNA, lipids, and proteins at multiple organ sites (71). *In vitro* and *in vivo* systems have shown that tumor cell growth, cell proliferation, and cell cycle biomarkers; apoptosis; p53 expression; cyclooxygenase 2 expression; DNA, RNA, and protein synthesis; the activation of transcriptional factors activator protein 1 and nuclear factor κ B; the activities of protein kinase C and protein kinase A, thymidine kinase, c-Jun-NH₂-kinase, and DNA cytosine methyltransferase; and 8-isoprostanate formation are modified by various forms of selenium treatment (71).

Effect Modification. Several potential effect modifiers for the effect of selenium supplementation on total cancer were presented in this analysis, including age at baseline, gender, and baseline smoking and plasma selenium statuses. Our data suggest that gender and baseline plasma selenium status predict the effect of selenium supplementation on total cancer.

Gender. The high proportion of males in the trial reflects the higher age-adjusted incidence of NMSC among men living in the United States (for BCC, 247/100,000 PY for males and 150/100,000 PY for females; for SCC, 65/100,000 and 24/100,000 PY for males and females, respectively) (72). The difference in the participation of women also reflects the reli-

ance on three Veterans Administration hospitals for subject recruitment, where there was an overwhelming preponderance of males among the potentially eligible patients. Whereas the recruitment of patients was gender blind, it is likely that the lower number of females in the trial and their lower incidence of cancer limited the power to investigate the interaction between gender and treatment. Thus, the apparent interaction between gender and treatment was not statistically significant, and the overall protective treatment effect was detectable only among males. Moreover, although the effect in men appeared to be concentrated in those whose baseline plasma selenium concentrations were below the median or in the lowest tertiles, baseline plasma selenium status did not modify the treatment effect among women (data not shown).

This discrepancy in the effect of selenium supplementation on cancer protection by gender has also been noted elsewhere. A case-control study of total cancer mortality nested within a prospective trial of 10,532 persons in the Netherlands showed that among males, the mean serum selenium for cases was significantly less than that for controls and that the adjusted risk of cancer mortality for the lowest quintile of serum selenium ($<100.8 \text{ ng/ml}$) was more than twice that of men with higher concentrations (RR = 2.7, 90% CI = 1.2–6.2; Ref. 30). In females, however, selenium concentrations were similar among cases and controls, with no evidence of increased cancer mortality associated with low serum selenium (30). Similar results were observed in a longitudinal study of 39,268 men and women participating in the Finnish Social Insurance Institution's Mobile Clinic Health Examination Survey (29).

Effect modification by gender may be attributed in part to gender differences in selenium metabolism. In a European study, females excreted significantly higher amounts of selenium per kilogram of body weight compared with males (73). Furthermore, whole-body residence time and body load adjusted for body weight have been estimated to be greater in males than females (74). Patterson *et al.* (74) speculated that these gender differences might reflect hormonal differences and the strong affinity of the testes for selenium. Thus, future chemoprevention trials may need to consider dose adjustments for gender (74) or whether large numbers of women should be included in the study samples. Clearly, we need to further evaluate how gender may modify the effect of selenium on cancer outcomes.

Smoking. After adjustment for age and gender, selenium supplementation was associated with a statistically significant reduction in total cancer incidence among former smokers and with nonsignificant reductions among never and current smokers. This is consistent with the proposal that former smokers are an ideal target population for chemoprevention trials (75, 76). The α -Tocopherol β -Carotene Cancer Prevention Study Group (77) documented a nonsignificant reduction in lung cancer incidence with α -tocopherol and increased lung cancer incidence with β -carotene in heavy current smokers. Cumulative and continuing exposure to tobacco smoke may have overwhelmed the effect of chemopreventive agents usually associated with early initiation and promotion stages of carcinogenesis (78).

Baseline Plasma Selenium. Clark *et al.* (1) reported that selenium supplementation had the greatest effect on prostate cancer prevention in men from this trial with the lowest baseline plasma selenium status. In the current analyses, the protective effect of treatment on total cancer incidence was likewise confined to participants in the lowest tertile of baseline plasma selenium. Moreover, a formal interaction between base-

line plasma selenium by tertile and treatment was detected in this analysis. Modification of the association between treatment and cancer incidence by baseline status of the supplemented nutrient is strikingly similar to the treatment effects for prostate cancer observed in the PHS (79, 80). Within the placebo group of the PHS, those in the lowest *versus* the highest quartile of baseline plasma β -carotene experienced a marginally significant increased risk of prostate cancer (RR = 1.45, 95% CI = 0.98–2.15), with a marginally significant *P* for trend over plasma quartiles. However, men in the lowest quartile randomly assigned to β -carotene supplementation had a significant reduction in prostate cancer risk (RR = 0.68, 95% CI = 0.46–0.99) compared with those assigned to placebo. Supplementation of those in the highest baseline quartile was associated with a nonsignificant increase in risk (RR = 1.33, 95% CI = 0.91–1.96). Thus, β -carotene supplementation in the PHS reduced the risk of prostate cancer only among those individuals with low baseline plasma β -carotene levels.

In the current analysis of the NPC Trial, attempts to glean information on the nature of the effect modification of selenium treatment by baseline plasma selenium reveal a complex and confusing pattern, one that is not entirely consistent with our understanding of selenium as protective against cancer. Indeed, these results clearly indicate the lack of a protective effect among participants whose baseline plasma selenium concentrations were in the upper tertile. It is noteworthy that this group of participants was selected on the basis of residency in an area in which the selenium intake was likely to be lower than in other regions of the United States. Thus, these results provide little support for the use of 200 μg selenium/day to protect against cancer among average-risk individuals with plasma concentrations at or above the United States estimated average of 123 ng/ml (mean \pm SD serum selenium in 16,693 subjects obtained from the Third National Health and Nutrition Examination Survey (NHANES III) was $123 \pm 17 \text{ ng/ml}$ (81).

In addition, among selenium-supplemented individuals, we observe a striking association; those with higher baseline concentrations experienced an elevated incidence of cancer. A pattern of modestly decreased incidence among placebo participants coupled with no risk gradient among treated participants seems somewhat plausible; however, the pattern we observed was clearly unpredicted and unsettling. It is critical that this effect be further evaluated in carefully controlled mechanistic studies.

There are several limitations in this study. First, as mentioned earlier, total and site-specific cancers (excluding NMSC) were not primary endpoints of the NPC Trial (82). Nevertheless, the ascertainment of these endpoints did not change throughout the entirety of the trial through February 1, 1996. Second, this trial possessed differential statistical power to detect an overall treatment effect in males *versus* females, as well as for gender-specific cancers. An adequately powered hypothesis-driven biomarker study should thus be conducted in females, before women are included in large-scale chemoprevention trials with selenium. A third limitation is the inherent difficulty in the assessment of selenium status. Due to variations in levels of the element between foodstuffs and the uncertainty about the availability for absorption of the different forms of the element, simple measures of dietary selenium intake indicative of the general status of a population are not sufficient for determining selenium status in individuals (83). It is therefore necessary to measure biochemical concentrations of selenium in the body and not simply gross intake (83). Ideally, the combination of two or more indices of selenium status would ensure a more accurate assessment. However, because

98.5% of the NPC participants had plasma selenium concentrations greater than the 70–90 ng/ml required to maximize plasma selenoproteins (84, 85), plasma selenium was the only index used to measure selenium status in this trial. Although plasma selenium concentrations reflect long-term selenium status in populations with relatively constant selenium intakes, it seems prudent that future selenium chemoprevention trials include multiple sequential plasma measures before randomization to more accurately define an individual's baseline plasma selenium status.

Finally, although the incidence estimates were adjusted for potential confounders such as age, gender, and smoking status, the lack of detailed information on unmeasured risk factors such as family history of cancer, physical activity level, biochemical status of other nutrients, and dietary intake data from foods and alcohol is a possible limitation. Randomization should minimize the likelihood of confounding by these factors. However, it is noteworthy that, during 1994–1995, plasma selenium concentrations measured in 1134 British people ages 65 years and over were strongly and directly correlated with plasma zinc, cholesterol, vitamin C, several carotenoids, and α -tocopherol, independent of age (86). Thus, future investigations of this cohort will consider the impact of other antioxidants, including the tocopherols and carotenoids, on cancer incidence and the extent to which they may modulate the effect of selenium supplementation. In addition, we plan to conduct genotype analyses on the study cohort to determine the effect of selenium supplementation on the activity of the inducible enzyme thiolmethyltransferase, which is critical in the methylation of selenium compounds; the prevalence of genetic polymorphisms of key genes in selenium and carcinogen metabolism, including thiolmethyltransferase, classical glutathione peroxidase (GSHPx-1; Refs. 87 and 88), and the recently identified M_r 15,000 selenoprotein (89); and the effect of these polymorphisms on cancer incidence and response to selenium supplementation.

In conclusion, this summary analysis of the NPC Trial, which includes data from the entire period of blinded treatment, continues to provide support for the efficacy of selenium supplementation in reducing total cancer incidence, total cancer mortality, and the incidence of prostate cancer and, to a lesser extent, lung and colorectal cancer. Not all site-specific cancers exhibited a reduction in incidence, although small case numbers limit the precision of our data. Indeed, the incidences of several site-specific cancers, including breast cancer, were nonsignificantly increased with an average of 7.4 years of follow-up. The protective effect of selenium supplementation on total cancer incidence was most prominent in males and in those with lower baseline plasma selenium concentrations. Those participants with baseline plasma selenium concentrations above the United States average showed a nonsignificant elevated incidence of total cancer. Our data suggest that effect modification of the association between selenium supplementation and cancer incidence (and/or biomarkers of carcinogenesis) by baseline plasma selenium status should be monitored and assessed in ongoing and future selenium prevention trials including those underway at the University of Arizona (90) and the recently initiated Selenium and Vitamin E Cancer Prevention Trial (91).

Future research studies should evaluate selenium metabolism and the effect of supplementation on genetic biomarkers of cancer risk in males and females to identify disparities that may account for the apparent differences in the chemopreventive effects of selenium between genders. Future studies should also assess the effect of selenium supplementation on these biomarkers in populations of varying selenium status both

within the United States and around the world to help elucidate the mechanism by which selenium status mediates the chemopreventive activity of this element.

Acknowledgments

We thank all participants for their adherence and commitment throughout the duration of this study and acknowledge the collaborating dermatologists for their cooperation in facilitating the trial. We are grateful to Patricia Wilkins for her meticulous data collection and study management, Edward Wittke for his critical role in computer hardware, software and database management, and Phyllis Click, R.N., and Victoria Brummet, R.N., for their tireless efforts in reviewing medical documentation. We also wish to recognize the members of the Data Safety and Monitoring Board: Dr. Tim Byers, Dr. Harvey Cohen, Dr. Stephen George, and Dr. Robert Greenberg.

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Baseline Characteristics and the Effect of Selenium Supplementation on Cancer Incidence in a Randomized Clinical Trial: A Summary Report of the Nutritional Prevention of Cancer Trial

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Cancer Epidemiol Biomarkers Prev 2002;11:630-639.

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Dietary glycemic index, glycemic load and ovarian cancer risk: a case-control study in Italy

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Received 3 April 2002; Revised 3 July 2002; accepted 18 July 2002

Background: Dietary carbohydrates vary in their ability to raise blood glucose and insulin levels, which, in turn, influence levels of sex hormones and insulin-like growth factors. We analyzed the effect of type and amount of carbohydrates on ovarian cancer risk, using the glycemic index (GI) and the glycemic load (GL) measurement in a large case-control study conducted in Italy.

Materials and methods: Cases included 1031 women with incident, histologically confirmed epithelial ovarian cancer, from four Italian regions. Controls included 2411 women admitted to the same hospital networks for acute, non-neoplastic conditions. Average daily GI and GL were calculated from a validated food frequency questionnaire. Odds ratios (OR) and the corresponding 95% confidence intervals (CI) were computed using multiple logistic regression.

Results: Ovarian cancer was directly associated with dietary GI (OR for highest versus lowest quartile = 1.7, 95% CI 1.3–2.1) and GL (OR = 1.7, 95% CI 1.3–2.1). The associations were observed in pre- and post-menopausal women, and they remained consistent across strata of major covariates identified.

Conclusions: This study supports the hypothesis of a direct association between GI and GL and ovarian cancer risk and, consequently, of a possible role of hyperinsulinemia/insulin resistance in ovarian cancer development.

Key words: carbohydrate, case-control study, glycemic load, ovarian cancer

Introduction

Ovarian cancer is directly related to nulliparity, and inversely related to oral contraceptive use, but little is known of its potential dietary correlates [1]. It has been suggested that diet may have a potential influence on ovarian carcinogenesis, and several case-control studies have reported a beneficial effect on the risk of ovarian cancer of a diet rich in vegetables [2, 3]. A few case-control studies showed that women with cancer of the ovary reported more frequent meat consumption [4, 5], and others suggested that a diet rich in eggs may also increase the risk of ovarian cancer [2, 6]. Fish, on the other hand, seemed to exert a protective effect [5, 6].

With reference to specific nutrients, descriptive epidemiology and ecological studies have reported positive relationships between fat, protein and total calory intake, and ovarian cancer

risk [7]. Data from analytical, mainly case-control studies supported the hypothesis of a possible increased risk in relation to various types of fat [2, 6]. Carbohydrates have also been shown to increase the risk of epithelial ovarian cancer [8].

Different carbohydrates, however, affect blood glucose and insulin levels to varying degrees depending on the nature of the carbohydrate and the type and extent of food processing [9]. On this basis they have been ranked using the glycemic index (GI) and glycemic load (GL). Foods with high GI tend to increase glucose and insulin levels to a greater extent than low GI foods [9]. In turn, it has been proposed that insulin may be directly or indirectly involved in the carcinogenic process by modulating hormonal levels such as sex hormones and insulin-like growth factors (IGF) [10]. High-GL diets have been directly associated with risk of various Western chronic conditions, including diabetes [11], coronary heart disease [12], colorectal [13] and breast [14] cancer, and high insulin levels may be one of the mechanisms of action of risk factors shared by these diseases [15].

We thus evaluated the possible differential effects of carbohydrate-rich foods on epithelial ovarian cancer risk by means of the GI and GL measurements in a large case-control study.

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Materials and methods

A multicenter case-control study of ovarian cancer was conducted between January 1992 and September 1999 in four Italian regions: Greater Milan, the provinces of Pordenone, Padua and Gorizia (north-eastern Italy); the province of Latina (central Italy); and the urban area of Naples (southern Italy).

Cases included women with incident, histologically confirmed epithelial ovarian cancer diagnosed within 1 year prior to interview and with no previous diagnosis of cancer. Overall, 1031 women aged 18–79 years (median age 56 years) were included. Controls included patients with no history of cancer who were admitted to hospitals serving the same areas as those where cases had been identified. Eligible diagnoses were acute, non-neoplastic, non-gynecological conditions, unrelated to hormonal or digestive tract diseases, or associated with long-term modifications of diet. They included 2411 women, aged 17–79 years (median age 57 years), belonging to the following diagnostic categories: trauma, mostly fractures and sprains (26%); other orthopedic disorders, such as low back pain and disc disorders (28%); acute surgical conditions (15%); and other illnesses, such as eye, ear, nose, skin and dental conditions (31%). Cases were frequency matched to controls according to quinquennium of age and area of residence. Approximately 4% of cases and controls approached for interview during their hospital stay refused to participate.

The same structured questionnaire and coding manual were used in each center, and all interviewers were centrally trained and routinely supervised. The checking of data for consistency and reliability was also conducted centrally. The questionnaire included information on sociodemographic characteristics, such as education and occupation, lifelong smoking habits, physical activity at various ages, anthropometric measures, a problem-oriented personal medical history, family history of selected cancers, menstrual and reproductive history, history of use of oral contraceptives, hormone replacement treatment, and female hormone-containing drugs for other indications. Dietary habits were investigated through an interviewer-administered food frequency questionnaire (FFQ) that included 78 items. This questionnaire was used to assess the subjects' habitual diet during the 2 years prior to cancer diagnosis or hospital admission (for controls), and included questions on the average weekly frequency of consumption of foods or food groups, as well as complex recipes. Satisfactory reproducibility [16] and validity [17] of the FFQ have been reported. Details on methodology used have been described elsewhere [13, 14]. To compute energy and nutrient intake, an Italian food-composition database was used. For each food, we expressed GI as a percentage of the glycemic response elicited using 'white bread' as a standard food. We then calculated daily average GI by summing the products of the carbohydrate content per serving for each food or recipe, multiplied by the average number of servings of that food per week, multiplied by its GI, all divided by the total amount of available carbohydrate weekly intake. This represents the 'quality of the carbohydrates', namely slow versus fast absorbable carbohydrates. A score for the daily average GL was computed as the GI, but without dividing by the total amount of carbohydrates. For these calculations we used the carbohydrate content of 50 foods or recipes, since 28 foods or recipes, chiefly cheese, meat and fish-based, contained a negligible amount of carbohydrate. With respect to GI values, we chiefly used international tables. In order to take into account Italian cooking habits (e.g. pasta 'al dente'), Italian sources were used for a few local recipes. Food items for which a GI had not been determined were assigned the GI of the nearest comparable food (e.g. tangerines were assigned the same GI as oranges).

Odds ratios (ORs) and the corresponding 95% confidence intervals (CI) for quartiles of GI and GL intake were computed using unconditional multiple logistic regression models [18]. The regression equations included terms for quinquennia of age, study center, years of education, occupational physical activity, history of diabetes, oral contraceptive use, parity, menopausal status,

number of daily meals, intakes of fiber, alcohol and total energy intake. Adjustment for energy was made using the residuals method. The modifying effect of various covariates was evaluated comparing the differences between the $-2 \log$ likelihood of the model with and without interaction terms, and referring it to the chi square distribution with degrees of freedom equal to the number of interaction terms minus one.

Results

Table 1 gives the distribution of ovarian cancer cases and control subjects according to age, education, menopausal status and other potential confounding factors. Cases were better educated than controls, had a lower parity, frequently reported a family history of ovarian and/or breast cancer, and a lower occupational physical activity.

GI was positively correlated with GL (Pearson correlation coefficient, $r = 0.53$), intake of bread ($r = 0.59$), cereals ($r = 0.56$), cakes and sweets ($r = 0.33$), sugar ($r = 0.26$), available carbohydrates ($r = 0.37$), cereal fibre ($r = 0.42$), and negatively correlated with fruit ($r = -0.19$) and vegetables ($r = -0.11$). Correlations of GI with other dietary and non-dietary factors were weak (i.e. $|r| < 0.10$).

Table 2 shows the ORs of epithelial ovarian cancer according to the quartiles of GI and GL, and total carbohydrate intake by menopausal status. Dietary GI and GL were directly associated with ovarian cancer risk, and the ORs, for the highest versus the lowest quartile, were 1.7 (95% CI 1.3–2.1) and 1.7 (95% CI 1.3–2.1), respectively. However, ORs by quartile of GI and GL did not show linear trends, but were already elevated in the second quartile and tended to plateau thereafter. Associations, particularly for GI, were appreciably stronger in postmenopausal compared with premenopausal women, although no significant heterogeneity emerged. Total carbohydrate intake was also associated with ovarian cancer (OR = 1.8, in the highest quartile, 95% CI 1.3–2.4) in postmenopausal women (Table 2).

Table 3 shows the relationship between GI and epithelial ovarian cancer in different strata of known or suspected risk factors for ovarian cancer. No substantial effect modification was apparent in strata of: family history of ovarian or breast cancer; oral contraceptive use; and parity.

The relationship between GI and epithelial ovarian cancer risk was also analyzed in separate strata of history of diabetes, body mass index (BMI), BMI increase from age 30, waist to hip (W/H) ratio, occupational physical activity and alcohol intake (Table 4). There was no consistent pattern of risk among diabetic subjects or in different strata of BMI, BMI increase from age 30, and W/H ratio. There was, however, a significant modifying effect of alcohol, with no consistent association with GI in alcohol abstainers. The association with GI was stronger in women reporting higher physical activity.

Although risk factors, including dietary factors, may differ in their relationship to specific histological subtypes of ovarian cancer [19], no relevant difference emerged when we replicated the analyses for GI and GL in invasive serous ovarian cancer only. Other histological subtypes represented <10% of cases in our data set.

Table 1. Distribution of 1031 cases of epithelial ovarian cancer and 2411 controls^a, according to age and selected variables (Italy, 1992–99)

Characteristic	Cases		Controls		χ^2 ^b (P value)
	n	%	n	%	
Age groups (years)					
<45	183	17.8	443	18.4	
45–54	287	27.8	615	25.5	
55–64	325	31.5	724	30.0	
≥65	236	22.9	629	26.1	
Education (years)					
<7	570	55.6	1417	59.4	
7–11	227	22.2	620	26.0	38.90
≥12	22	22.2	349	14.6	(<0.01)
Menopausal status					
Premenopausal	346	33.6	803	33.4	0.02
Postmenopausal	683	66.4	1603	66.6	(0.89)
Parity (number of births)					
Nulliparae	184	17.8	381	15.8	
1–2	572	55.5	1268	52.6	48.20
≥3	275	26.7	762	31.6	(<0.01)
Oral contraceptive use					
Never	921	89.3	2142	88.8	0.18
Ever	110	10.7	269	11.2	(0.67)
Diabetes history					
No	986	95.6	2324	96.4	0.06
Yes	45	4.4	87	3.6	(0.81)
Family history of breast or ovarian cancer ^c					
No	902	87.5	2291	95.0	55.95
Yes	129	12.5	120	5.0	(0.01)
Occupational physical activity					
Low	331	33.2	677	28.9	
Medium	492	49.3	1237	52.9	22.75
High	175	17.5	426	18.2	(<0.01)
Alcohol intake (drinks per week)					
Abstainers	288	27.9	833	34.5	
1–6	261	25.3	542	22.5	
7–13	226	21.9	421	17.5	0.31
≥14	256	24.9	615	25.5	(0.58)
Meal frequency					
1 per day	40	3.9	83	3.5	0.47
2 per day or more	991	96.1	2325	96.5	(0.48)
Fibre intake (g/day)					
<17.5	218	21.1	647	26.8	
17.5–22.2	257	24.9	611	25.3	
22.2–27.1	280	27.2	568	23.6	6.71
≥27.1	276	26.8	585	24.3	(0.01)

^aSome figures do not add up to the total as some values are missing.

^bCochran–Mantel–Haenzel χ^2 adjusted for center and age.

^cIn immediate relatives.

Table 2. Odds ratios (ORs) and 95% confidence intervals (CIs)^a of epithelial ovarian cancer by quartile of energy-adjusted glycemic index, glycemic load and total carbohydrate intake (Italy, 1992–99)

Cases:controls ^b	Quartile, OR (95% CI)				χ^2 (trend) (<i>P</i> value)
	1 ^c	2	3	4	
Glycemic index					
Upper limit ^d	70.8	74.4	77.7	—	
Premenopausal	346:803	1	1.33 (0.88–2.00)	1.42 (0.95–2.14)	1.36 (0.90–2.05)
Postmenopausal	683:1603	1	1.83 (1.36–2.47)	2.10 (1.57–2.82)	1.84 (1.37–2.48)
All cases	1031:2411	1	1.61 (1.27–2.04)	1.80 (1.43–2.27)	1.65 (1.30–2.09)
Glycemic load					
Upper limit ^d	147	185	234	—	
Premenopausal	346:803	1	1.49 (0.99–2.25)	1.68 (1.09–2.57)	1.39 (0.92–2.10)
Postmenopausal	683:1603	1	1.37 (1.02–1.84)	1.49 (1.11–2.00)	1.83 (1.36–2.46)
All cases	1031:2411	1	1.40 (1.11–1.78)	1.54 (1.22–1.96)	1.65 (1.30–2.09)
Total carbohydrate intake (g)					
Upper limit ^d	7.57	9.44	11.55	—	
Premenopausal	346:803	1	1.31 (0.86–1.98)	1.33 (0.86–2.06)	1.39 (0.90–2.15)
Postmenopausal	683:1603	1	1.49 (1.11–1.99)	1.55 (1.14–2.10)	1.75 (1.28–2.39)
All cases	1031:2411	1	1.44 (1.13–1.82)	1.48 (1.16–1.90)	1.62 (1.27–2.08)

^aAdjusted for age, study center, years of education, occupational physical activity, meal frequency, alcohol consumption, fibre and energy intake, history of diabetes, oral contraceptive use, parity and menopausal status (when appropriate).

^bSome figures do not add up to total because of some missing value.

^cReference category.

^dIn overall population of cases and controls.

Table 3. Odds ratios (ORs) and 95% confidence intervals (CIs)^a of epithelial ovarian cancer by strata of selected variables and quartile of energy-adjusted glycemic index (Italy, 1992–99)

Cases:controls ^b	Quartile, OR (95% CI)				χ^2 (<i>P</i> value)
	1 ^c	2	3	4	
Upper limit^d					
	70.8	74.4	77.7	—	
Family history of breast and/or ovarian cancer^e					
Yes	129:120	1	2.05 (0.85–4.96)	1.59 (0.72–3.53)	1.45 (0.61–3.44)
No	902:2291	1	1.64 (1.27–2.11)	1.84 (1.44–2.37)	1.70 (1.32–2.19)
Oral contraceptive use					
Yes	110:269	1	2.25 (1.06–4.76)	2.02 (0.98–4.18)	1.53 (0.71–3.28)
No	921:2142	1	1.52 (1.18–1.96)	1.77 (1.38–2.28)	1.61 (1.25–2.07)
Parity (no. of births)					
Nulliparae	184:381	1	2.30 (1.26–4.20)	2.01 (1.10–3.66)	1.39 (0.77–2.51)
1–2	572:1268	1	1.51 (1.10–2.07)	1.75 (1.27–2.40)	1.62 (1.16–2.25)
≥3	275:762	1	1.95 (1.20–3.18)	2.39 (1.51–3.81)	2.26 (1.42–3.59)

^aAdjusted for age, study center, years of education, occupational physical activity, meal frequency, alcohol consumption, fibre and energy intake, history of diabetes, oral contraceptive use, parity and menopausal status (when appropriate).

^bSome figures do not add up to the total as some values are missing.

^cReference category.

^dIn the overall population of cases and controls.

^eIn immediate relatives.

Table 4. Odds ratios (ORs) and 95% confidence intervals (CIs)^a of epithelial ovarian cancer by strata of selected variables and quartile of energy-adjusted glycemic index (Italy, 1992–99)

Cases:controls ^b	Quartile, OR (95% CI)				χ^2 (trend) (<i>P</i> value)
	1 ^c	2	3	4	
Upper limit ^d	70.8	74.4	77.7	—	
Diabetes					
Yes	45:87	1	0.75 (0.16–3.47)	1.66 (0.37–7.55)	1.04 (0.22–5.05)
No	986:2324	1	1.69 (1.32–2.15)	1.88 (1.48–2.39)	1.69 (1.33–2.16)
Body Mass Index (BMI)					
<25	549:1266	1	1.63 (1.16–2.28)	1.84 (1.32–2.54)	1.48 (1.06–2.07)
≥25	472:1128	1	1.66 (1.18–2.35)	1.71 (1.20–2.43)	1.79 (1.26–2.54)
BMI increase from age 30 years ^e					
≤0	228:467	1	1.57 (0.96–2.57)	1.79 (1.11–2.87)	1.74 (1.07–2.83)
>0 to 4	465:1001	1	1.73 (1.20–2.50)	1.75 (1.22–2.52)	1.65 (1.14–2.37)
>4	233:716	1	1.61 (0.99–2.62)	2.09 (1.28–3.40)	1.67 (1.01–2.75)
Waist to hip ratio					
<0.83	319:922	1	1.62 (1.08–2.44)	1.95 (1.31–2.90)	1.43 (0.93–2.19)
≥0.83	407:925	1	1.82 (1.23–2.68)	1.80 (1.23–2.63)	1.95 (1.33–2.84)
Occupational physical activity					
Low	331:677	1	1.58 (1.01–2.48)	1.62 (1.05–2.49)	1.27 (0.82–1.97)
Medium	492:1237	1	1.55 (1.11–2.18)	1.68 (1.20–2.36)	1.56 (1.11–2.21)
High	175:426	1	1.95 (1.03–3.68)	3.16 (1.72–5.81)	3.05 (1.64–5.67)
Alcohol intake (drinks per week)					
Abstainers	288:833	1	1.56 (1.01–2.40)	1.32 (0.86–2.03)	0.98 (0.63–1.51)
1–6	261:542	1	1.78 (1.09–2.93)	2.54 (1.55–4.15)	2.22 (1.35–3.63)
7–13	226:421	1	1.34 (0.75–2.37)	2.29 (1.29–4.07)	2.15 (1.20–3.84)
≥14	256:615	1	1.89 (1.15–3.11)	1.96 (1.21–3.16)	2.24 (1.37–3.66)

^aAdjusted for age, study centre, years of education, occupational physical activity, meal frequency, alcohol consumption, fibre and energy intake, history of diabetes, oral contraceptive use, parity and menopausal status (when appropriate).

^bSome figures do not add up to the total as some values are missing.

^cReference category.

^dIn the overall population of cases and controls.

^eFor subjects aged 35 years or more.

Discussion

The present study shows direct associations between dietary GI and GL and epithelial ovarian cancer risk. An elevated risk was found in the second quartile of GI and GL, but did not show a further increase in the third and fourth (highest) quartile. These associations were consistent across different strata of known or potential risk factors for ovarian cancer. However, the relationship between GI and epithelial ovarian cancer in our study was somewhat stronger in post- compared with premenopausal women, and in women without a family history and in parae.

Diets with high GI or GL are associated with a high consumption of refined carbohydrates, which are quickly absorbed and are capable of elevating blood glucose and insulin level to a greater

extent than slowly absorbed ones, such as pulses and whole grains, which are low GI foods [20]. The main sources of carbohydrates in the Italian population are bread, a high GI food, and pasta, a medium-low GI food, representing 20.5% and 13.4% of total carbohydrate intake, respectively [13].

High insulin levels have been suggested as a potential unifying mechanism for the risk of several Western chronic diseases related to high intakes of energy, fat, refined carbohydrates, and low physical activity and obesity [15]. Central obesity (i.e. high W/H ratio) was associated with ovarian cancer risk in this study [21]. Diabetes, which is characterized by high insulin levels in its early stages, was considered as a possible correlate of ovarian cancer risk. However, in line with other studies [22, 23] a history of diabetes was not found to consistently affect ovarian cancer

risk in the present study. This could, however, have resulted from the small absolute number of diabetic subjects (~4%).

Insulin is a growth factor for cancer cells, and it has been shown to act as a cancer promoter in *in vitro* and in animal studies [15, 24]. Insulin also has affinity for IGF receptors, particularly the IGF-1 receptor, which has strong mitogenic effects on normal and neoplastic cells, including ovarian carcinoma cell lines, where it has been found at higher levels than in non-malignant cells [25]. Epidemiological evidence suggests a promoting effect of hyperinsulinemia [15] and of IGF-1 in carcinogenesis [25].

Insulin and IGF-1 are also powerful negative regulators of sex hormone-binding globulin (SHBG) synthesis *in vitro*, and they may stimulate ovarian cancer proliferation through a hormonal pathway [26]. An interaction between insulin, IGFs and sex hormones has also been suggested for breast cancer [27].

As in most case-control studies, recall and selection biases are possible [18]. However, awareness about any dietary hypotheses, and particularly those related to GI and GL, for ovarian cancer was limited in the Italian population when the study was conducted. While it is conceivable that dietary habits of hospital controls may have differed from those of the general population, great attention was paid in this study to minimize bias by excluding control subjects admitted for conditions that might have been associated with special dietary habits. Of greater concern is the early weight loss often occurring in ovarian cancer patients, which may have led cases to increase their energy and, hence, carbohydrate intake [21]. We had, however, information on weight loss during the year prior to cancer diagnosis or interview. Stratification and adjustment for weight or recent weight loss did not modify the association with GI and GL. Interviewing all subjects in a hospital setting may have allowed greater comparability of dietary history between cases and controls [28], and adjustment for total energy intake should have controlled for potential dietary over- and under-reporting. Furthermore, participation among eligible patients was practically complete and the catchment areas for cases and controls were highly comparable.

GI estimates have some limitations. Some GI estimates have been derived from small samples and their variability is unclear [13]. Statistics on the average dietary GI and GL in the general Italian population are not available, but intakes of bread and pasta in the present study were similar to those reported in the Italian population [29]. In addition, it would be important to confirm the association between GI, GL and ovarian cancer in different populations, since the genotype for insulin resistance may vary between ethnic groups [30].

The major strength of this study is its uniquely large dataset, which allowed reasonably precise risk estimates. Other strengths include consistency of findings, when major categories of controls were used separately, and its reliance on a validated food frequency questionnaire [16, 17].

In conclusion, this study found associations between dietary GI, GL and ovarian cancer risk, thus supporting a possible role of insulin and insulin-related factors in ovarian carcinogenesis. Similar associations were observed for colorectal [13] and breast [14] cancer, indicating the potential role of these factors on several common neoplasms.

Acknowledgements

This work was supported by the contribution of the Italian Association for Research on Cancer, and the Italian League Against Cancer, Milan, Italy. The authors wish to thank Mrs Luigina Mei for editorial assistance.

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ARTICLES

Nutrition Intervention Trials in Linxian, China: Supplementation With Specific Vitamin/Mineral Combinations, Cancer Incidence, and Disease- Specific Mortality in the General Population

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Background: Epidemiologic evidence indicates that diets high in fruits and vegetables are associated with a reduced risk of several cancers, including cancers of the esophagus and stomach. Vitamins and minerals in these foods may contribute to the reduced cancer risk. The people of Linxian County, China, have one of the world's highest rates of esophageal/gastric cardia cancer and a persistently low intake of several micronutrients. **Purpose:** We sought to determine if dietary supplementation with specific vitamins and minerals can lower mortality from or incidence of cancer as well as mortality from other diseases in Linxian. **Methods:** Individuals of ages 40-69 were recruited in 1985 from four Linxian communes. Mortality and cancer incidence during March 1986-May 1991 were ascertained for 29584 adults who received daily vitamin and mineral supplementation throughout this period. The subjects were randomly assigned to intervention groups according to a one-half replicate of a 2⁴ factorial experimental design. This design enabled testing for the effects of four combinations of nutrients: (A) retinol and zinc; (B) riboflavin and niacin; (C) vitamin C and molybdenum; and (D) beta carotene, vitamin E, and selenium. Doses ranged from one to two times U.S. Recommended Daily Allowances. **Results:** A total of 2127 deaths occurred among trial participants during the intervention period. Cancer was the leading cause of death, with 32% of all deaths due to esophageal or stomach cancer, followed by cerebrovascular disease (25%). Significantly ($P = .03$) lower total mortality (relative risk [RR] = 0.91; 95% confidence interval [CI] = 0.84-0.99) occurred among those receiving supplementation with beta carotene, vitamin E, and

selenium. The reduction was mainly due to lower cancer rates (RR = 0.87; 95% CI = 0.75-1.00), especially stomach cancer (RR = 0.79; 95% CI = 0.64-0.99), with the reduced risk beginning to arise about 1-2 years after the start of supplementation with these vitamins and minerals. No significant effects on mortality rates from all causes were found for supplementation with retinol and zinc, riboflavin and niacin, or vitamin C and molybdenum. Patterns of cancer incidence, on the basis of 1298 cases, generally resembled those for cancer mortality. **Conclusions:** The findings indicate that vitamin and mineral supplementation of the diet of Linxian adults, particularly with the combination of beta carotene, vitamin E, and selenium, may effect a reduction in cancer risk in this population. **Implications:** The results on their own are not definitive, but the promising findings should stimulate further research to clarify the potential benefits of micronutrient supplements. [J Natl Cancer Inst 85:1483-1492, 1993]

Linxian, a rural county in Henan Province of north-central China, has one of the world's highest rates of esophageal cancer (1). Mortality rates from this cancer exceed the Chinese national average by 10-fold and the American average for Whites by 100-fold (2). The tumors arise not only as squamous cell carcinomas in the esophagus, but also beyond the esophageal-gastric junction as adenocarcinomas in the cardia region of the stomach. Historically, esophageal and gastric cardia cancers have been considered as a single

*See "Notes" section following "References."

clinical entity for incidence and mortality rate calculations in Linxian (1).

Reasons for the clustering of esophageal-gastric cardia cancer in Linxian are unknown; case-control studies (3,4) have failed to detect strong dietary or other risk factors. A number of investigations (5) conducted in other areas of the world have found that consumption of fresh vegetables and fruits is associated with a reduced risk of both esophageal and stomach cancers. The particular constituents of vegetables and fruits responsible for the protective effect have not been determined, but inverse trends have been shown between cancer risk and indices of intake of several micronutrients found in these foods, especially vitamin C and beta carotene (6). In experimental animals also, deficiencies of certain nutrients may enhance chemical carcinogenesis, while nutrient supplementation may inhibit tumor formation (7).

Food availability and variety in Linxian have historically been limited. Although completion of a massive agricultural irrigation system in 1965 resulted in increased production of several foods, diets typically have remained low in intake of fresh fruits and meat and other animal products. The major staples are corn, millet, sweet potatoes, and wheat. In surveys (8-11) of Linxian residents during the 1970s and early 1980s, blood levels of various micronutrients, including retinol, beta carotene, riboflavin, vitamin C, and vitamin E were consistently low by Western standards, although overt clinical deficiencies were not common.

Because of its extraordinarily high rates of epithelial cancers (i.e., esophageal and gastric) and subclinical deficiencies of several micronutrients among the population, Linxian was selected as the site for two randomized intervention trials to test whether supplementation with multiple vitamins and minerals might reduce the rates of cancer. One trial involved approximately 3300 participants with esophageal dysplasia and is the subject of another article in this issue of the Journal (12). In this presentation, we describe results from a larger trial involving nearly 30000 residents from the high-risk Linxian general population. We present tests of the initial effectiveness of four specific combinations of vitamins and minerals in reducing cancer incidence and mortality as well as mortality from other diseases during the course of the intervention.

Subjects and Methods

Participants in the trial were recruited in 1985 from four communes in Linxian. Residents aged 40-69 years without debilitating diseases or prior esophageal or stomach cancer who were willing to take part in a multiyear, daily pill-taking regimen were sought for enrollment. These individuals were given a brief physical examination, had a 10-mL blood sample collected and stored, and were interviewed regarding aspects of their medical, family, dietary, and tobacco and alcohol consumption histories.

The participants were randomly assigned to receive one of eight vitamin/mineral supplement combinations in the form of individual oral tablets. The treatment combinations were randomly assigned within blocks defined by commune (four communes), sex, and age (by single years). The eight intervention groups were derived from a one-half replicate of a 2⁴ factorial design (13). The factorial design allowed us to assess four factors (i.e., nutrient combinations) in a single experiment. The four factors, which we designate by the letters A, B, C, and D, are defined in Table 1. Doses of each nutrient ranged from one to two times U.S. Recommended Daily Allowances (RDAs).

Table 1. Types and daily doses of micronutrients by treatment factor

Factor	Micronutrients	Dose per day
A	Retinol (as palmitate)	5000 IU
	Zinc (as zinc oxide)	22.5 mg
B	Riboflavin	3.2 mg
	Niacin	40 mg
C	Ascorbic acid	120 mg
	Molybdenum (as molybdenum yeast complex)	30 µg
D	Beta carotene	15 mg
	Selenium (as selenium yeast)	50 µg
	Alpha-tocopherol	30 mg

While a separate evaluation of each of the nine, and perhaps additional, nutrients listed in Table 1 would have been desirable, a 2⁹ or higher factorial experiment was impractical. The feasible options were to delete certain nutrients or to combine them into a smaller number of groups. We chose the latter approach, combining zinc, which enhances the delivery of retinol to tissues, and retinol (Factor A); the B vitamins riboflavin and niacin (Factor B); vitamin C and molybdenum, which are thought to inhibit the formation of carcinogenic nitrosamines and nitrosamine-induced esophageal carcinogenesis, respectively (Factor C); and the fat-soluble antioxidants beta carotene, vitamin E, and selenium (Factor D). A fractional factorial design was selected because it permitted testing of the main effects of four factors at less cost and complexity than a full 2⁴ factorial design. The eight intervention groups in this fractional design were defined by the following combinations of supplements: AB, AC, AD, BC, BD, CD, ABCD, or placebo. Thus, persons in group AB, e.g., received retinol, zinc, riboflavin, and niacin, while those in group ABCD received all nine vitamins and minerals and those in the placebo group received none. This choice of groups resulted in half the participants receiving each of the four factor nutrient combinations. For example, half received factor A (AB, AC, AD, ABCD) and half did not (BC, BD, CD, placebo), and the subjects that received versus those that did not receive factor A were balanced with respect to receipt of all other nutrients.

The eight vitamin/mineral combinations were packaged in coded bottles containing a 1-month supply and were distributed monthly by approximately 200 village doctors beginning in March 1986 and continuing through May 1991. Compliance was assessed in two ways: by counting unused pills and by assaying nutrient levels in blood collected from approximately 120 individuals randomly selected without replacement every 3 months during the course of the trials.

Mortality among trial participants was ascertained via follow-up by village doctors. Diagnoses of cancer were ascertained through local commune and county hospitals and supplemented by a study medical team that provided clinical and diagnostic services, including endoscopy, for participants with symptoms suggestive of esophageal or stomach cancer. Diagnostic materials (e.g., x rays, cytology, biopsy, and surgical specimens) for 85% of the cancer cases were reviewed by a panel of senior Chinese and American experts in gastroenterology, radiology, cytology, and pathology. Reviews were conducted in parallel by senior Chinese diagnosticians for the remaining cancer cases and for deaths due to causes other than cancer.

Statistical analyses focused on estimating the effects of supplementation with each of the four vitamin/mineral factors upon 5½-year (March 1986-May 1991) total mortality and cancer mortality rates. Incidence and mortality rates were calculated for esophageal, gastric cardia, other stomach, and other cancers. In addition, we calculated rates of cerebrovascular disease mortality and other causes of death. Proportional hazards regression analyses (14) were employed to estimate relative risks (RRs) of mortality and cancer incidence and corresponding 95% confidence intervals (CIs) for the four main effects after adjustment for matching variables. Additional adjustment for baseline data on cigarette smoking and parental history of cancer, two risk factors for esophageal/stomach cancer in this population, resulted in essentially no change and is not presented. Regressions were also run with only the use of events and person-years occurring 12 or more months after the intervention began, a procedure that allowed for a latency period before a treatment effect might occur. Tests for pairwise interactions between factors were able to be calculated, but with the fractional design, only three of the six two-way interactions could be evaluated. Furthermore, because the interaction between any two of the four factors, say A and B, is mathematically equivalent to the interaction between the remaining two factors (i.e., C and D), interactive effects for one pair cannot be distinguished from the other. Thus, these tests were not

pursued. In addition to calculating 5½-year rates, we plotted cumulative mortality and incidence by calendar quarter throughout the study period. All *P* values associated with comparisons of those receiving versus those not receiving a particular vitamin/mineral factor are nominal and based on two-sided tests, even though one-sided tests would have been appropriate because of the a priori hypotheses of beneficial effects of the supplements.

Results

Of the approximately 50000 potentially eligible participants, 16% refused to participate, 12% were out of the area, 3% were too sick, and 8% did not join the trial for other reasons. In addition, 1.4% were excluded due to self-reported cancer at screening or death or diagnosis of cancer prior to the start of intervention. After these exclusions, the study population consisted of 29584 randomly assigned participants. Characteristics of these individuals are given in Table 2. The various treatment groups were well balanced with regard to sex, age, smoking, alcohol consumption, and diet and familial cancer history.

Compliance assessed by monthly pill counts and biochemical measures was excellent throughout the study. The overall pill disappearance rate was 93% for all participants, with no difference by treatment group (range, 92%-93%) and little change during the trial (range, 92%-93% in year 1;

91%-92% in year 5). For 86% of all participants, pill disappearance exceeded 90% (range, 85%-87% across treatment groups), while just 5% were poor compliers (i.e., <50% pill disappearance) (range, 5%-6% across treatment groups). Biochemical assessments during the intervention showed significantly higher nutrient blood levels for individuals who received supplementation compared with those who did not; the proportional increase was greatest for beta carotene (Table 3). In contrast, there were no significant differences in baseline levels, except for lower ascorbate levels in those who received vitamin C and molybdenum.

A total of 2127 deaths (7.2% of the study participants) occurred during the period March 1986-May 1991. The percentages of deaths were higher among men (9.3%) than women (5.5%) and rose with age at start of follow-up (2.3% age <50, 7.3% age 50-59, and 17.3% age ≥60). Cancer was the leading cause of death, accounting for 37% of all deaths. Of the 792 cancer deaths, 87% (or 32% of all deaths) were attributed to cancers of the esophagus (360 deaths) or stomach (331 deaths; 253 from gastric cardia and 78 from other stomach cancers). There were 101 deaths from other cancers—32 from lung cancer, 28 from liver cancer, and fewer than 10 from any other specific malignancy. Cerebrovascular disease accounted for 523 (25%) of the deaths, while the remaining 812 (38%) were caused by a variety of conditions, none of which accounted for more than 9% of all deaths.

Table 4 presents numbers of deaths and the death rates among the eight intervention groups by cause of death, and these data serve as the basis for subsequent calculations. In Table 5, RRs for mortality from cancer, cerebrovascular disease, and other diseases associated with factors A (retinol, zinc), B (riboflavin, niacin), C (vitamin C, molybdenum),

Table 2. General population trial participant characteristics

Participant characteristics	All participants	Range for individual treatment groups
No. of participants*	29584	3677-3709
Age at start of intervention, y		
<50	42%	42%
50-59	35%	34%-35%
≥60	23%	23%-24%
Sex		
Male	45%	44%-45%
Female	55%	55%-56%
Education		
None	40%	39%-41%
Any	60%	59%-61%
Tobacco (ever smoke cigarettes regularly >6 mo)		
No	70%	70%-71%
Yes	30%	29%-30%
Alcohol (any use past 12 mo)		
No	77%	76%-77%
Yes	23%	23%-24%
Pickled vegetable (any use past 12 mo in winter or spring)		
No	91%	90%-92%
Yes	9%	8%-10%
Moldy food (any use past 12 mo)		
No	82%	81%-83%
Yes	18%	17%-19%
Family history of esophageal or stomach cancer		
No	68%	68%-69%
Yes	32%	31%-32%

*Data missing for variables other than age and sex on 104-109 participants, depending on the characteristic.

Table 3. Compliance assessed biochemically over the 5-year intervention

Factor	Biochemical assessment						
	Baseline*			During intervention			
	No.	Mean	SD	No.	Mean	SD	<i>P</i> †
<i>Retinol (μg/dL, plasma)</i>							
A	47	35.7	8.8	479	54.0	16.0	.0001
No A	60	35.5	13.1	419	43.0	14.9	
<i>Riboflavin (EGR activation coefficient)‡</i>							
B	56	1.73	0.34	747	1.19	0.25	.0001
No B	51	1.78	0.40	745	1.44	0.31	
<i>Ascorbic acid (mg/dL, plasma)</i>							
C	49	0.15	0.13	730	0.81	0.47	.0001
No C	49	0.25	0.29§	740	0.54	0.41	
<i>Beta carotene (μg/dL, plasma)</i>							
D	47	5.9	5.2	443	85.5	78.5	.0001
No D	60	6.8	5.8	455	12.0	15.0	

* Baseline nutritional assessment conducted in May 1985; values adjusted for season.

† *P* values are for *t* tests of factor versus no factor during intervention.

‡ EGR = erythrocyte glutathione reductase. Lower EGR activation coefficient indicates higher riboflavin status.

§ *P* value for C versus no C at baseline = .03.

Table 4. Numbers and rates of death by major disease category according to intervention group

Intervention group	Person-years of observation	Cause of death							
		Cancer		Cerebrovascular		Other		Total	
		No. of deaths	Deaths per 1000 person-years	No. of deaths	Deaths per 1000 person-years	No. of deaths	Deaths per 1000 person-years	No. of deaths	Deaths per 1000 person-years
Placebo	18626	107	5.7	77	4.1	96	5.2	280	15.0
AB	18736	94	5.0	66	3.5	105	5.6	265	14.1
AC	18701	121	6.5	71	3.8	104	5.6	296	15.8
AD	18745	81	4.3	55	2.9	114	6.1	250	13.3
BC	18686	101	5.4	60	3.2	107	5.7	268	14.3
BD	18729	103	5.5	58	3.1	102	5.4	263	14.0
CD	18758	90	4.8	67	3.6	92	4.9	249	13.2
ABCD	18792	95	5.1	69	3.7	92	4.9	256	13.6
Total	149773	792	5.3	523	3.5	812	5.4	2127	14.2

Table 5. RRs and 95% CIs of death by cause according to vitamin/mineral factor

Cause of death	n	Factor*							
		A		B		C		D	
	n	RR	95% CI	RR	95% CI	RR	95% CI	RR	95% CI
Total	2127	1.00	0.92-1.09	0.97	0.89-1.06	1.01	0.93-1.10	0.91	0.84-0.99
Cancer	792	0.97	0.85-1.12	0.98	0.85-1.13	1.06	0.92-1.21	0.87	0.75-1.00
Esophageal	360	0.93	0.76-1.15	0.90	0.73-1.11	1.05	0.85-1.29	0.96	0.78-1.18
Stomach	331	1.03	0.83-1.28	1.00	0.81-1.24	1.09	0.88-1.36	0.79	0.64-0.99
Cardia	253	1.22	0.95-1.56	1.03	0.80-1.30	1.07	0.84-1.37	0.82	0.64-1.04
Noncardia	78	0.59	0.37-0.93	0.94	0.60-1.47	1.17	0.75-1.82	0.72	0.46-1.14
Esophageal/gastric cardia	613	1.04	0.89-1.22	0.95	0.81-1.11	1.06	0.90-1.24	0.90	0.77-1.05
Other	101	0.94	0.64-1.39	1.24	0.84-1.84	0.98	0.66-1.45	0.80	0.54-1.18
Cerebrovascular	523	0.99	0.84-1.18	0.93	0.79-1.11	1.04	0.88-1.24	0.90	0.76-1.07
Other	812	1.04	0.91-1.20	1.00	0.87-1.14	0.94	0.82-1.08	0.96	0.84-1.11

*A = retinol, zinc; B = riboflavin, niacin; C = vitamin C, molybdenum; and D = beta carotene, vitamin E, selenium.

and D (beta carotene, vitamin E, selenium) are shown. Significantly ($P = .03$) lower total mortality rates were observed among persons receiving pills with beta carotene, vitamin E, and selenium (factor D), but not among those receiving the three other vitamin/mineral combinations. There was a 9% reduction in overall mortality (RR = 0.91; 95% CI = 0.84-0.99) among those receiving factor D. Cancer mortality among this group was reduced 13% (RR = 0.87; 95% CI = 0.75-1.00), with esophageal/gastric cardia rates reduced 10% (RR = 0.90; 95% CI = 0.77-1.05). The decrease in cancer mortality among those receiving beta carotene, vitamin E, and selenium was more pronounced for stomach (RR = 0.79) and other (RR = 0.80) cancers than for esophageal cancers (RR = 0.96), but differences between these RRs were not significant ($P > .10$). The lower risk for those receiving factor D was seen for both cardia (RR = 0.82) and noncardia (RR = 0.72) stomach cancers. When a 1-year lag (to allow time for an intervention effect to become apparent) was incorporated into the proportional hazards regression analysis, the differences between those receiving versus those not receiving beta carotene, vitamin E, and selenium became slightly more pronounced: The RR for total cancer mortality was 0.85 (95% CI = 0.73-0.98); for stomach cancer, it was 0.77 (95% CI = 0.61-0.98). This lag

effect is displayed in Figs. 1 and 2, which plot mortality from total cancer and stomach cancer by calendar quarter for those receiving versus those not receiving beta carotene, vitamin E, and selenium. Cancer rates overlapped until approximately 1 year (total cancer; Fig. 1) or 2 years (stomach cancer; Fig. 2) after the start of the intervention and then tended to diverge. None of the other factors showed a progressive benefit of the kind seen in Figs. 1 or 2. Mortality from noncardia stomach cancer was significantly ($P = .02$) lower among those receiving retinol and zinc, but cardia cancer rates were elevated and there was no overall reduction in stomach cancer death rates in this group.

Patterns for cancer incidence resembled those for cancer mortality (Table 6). In total, 1298 persons were diagnosed with cancer during the study period. Among these diagnoses, 49% were esophageal cancer, 42% were stomach cancer (34% cardia and 8% for noncardia cancers), and 9% were other cancers. As with the mortality findings, lowered incidences of total cancer (RR = 0.93; 95% CI = 0.83-1.03), esophageal/gastric cardia cancer (RR = 0.94; 95% CI = 0.84-1.06), and stomach cancer (RR = 0.84; 95% CI = 0.71-1.00) were observed among those receiving beta carotene, vitamin E, and selenium (Table 6). The reductions for stomach cancer were equivalent for cardia and noncardia

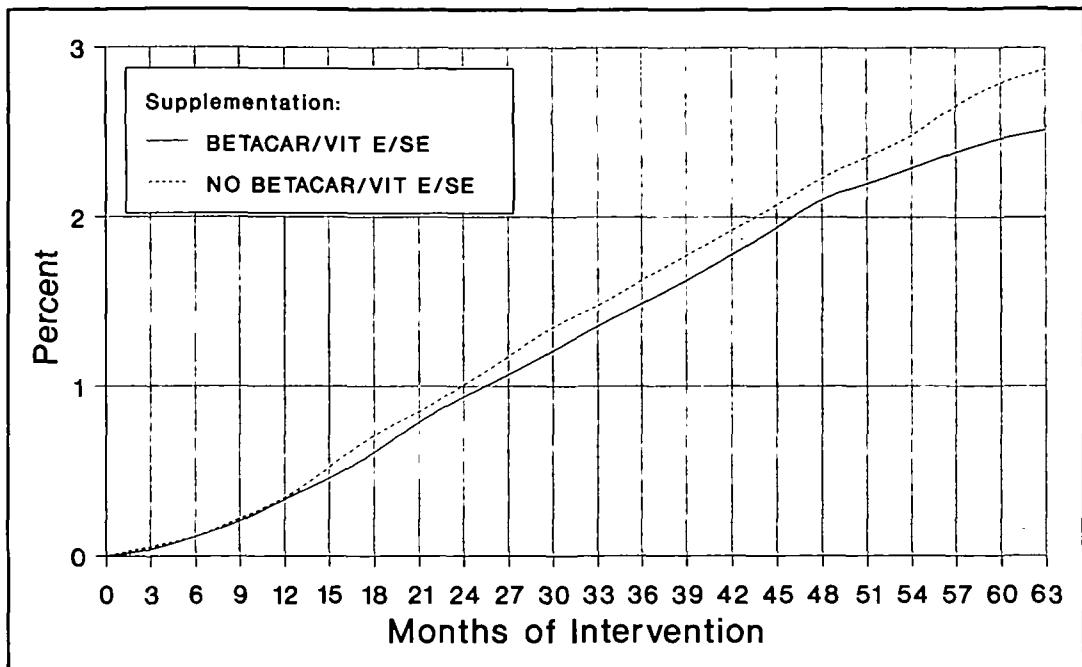


Fig. 1. Cumulative total cancer deaths as percent of study population, March 1986-May 1991.

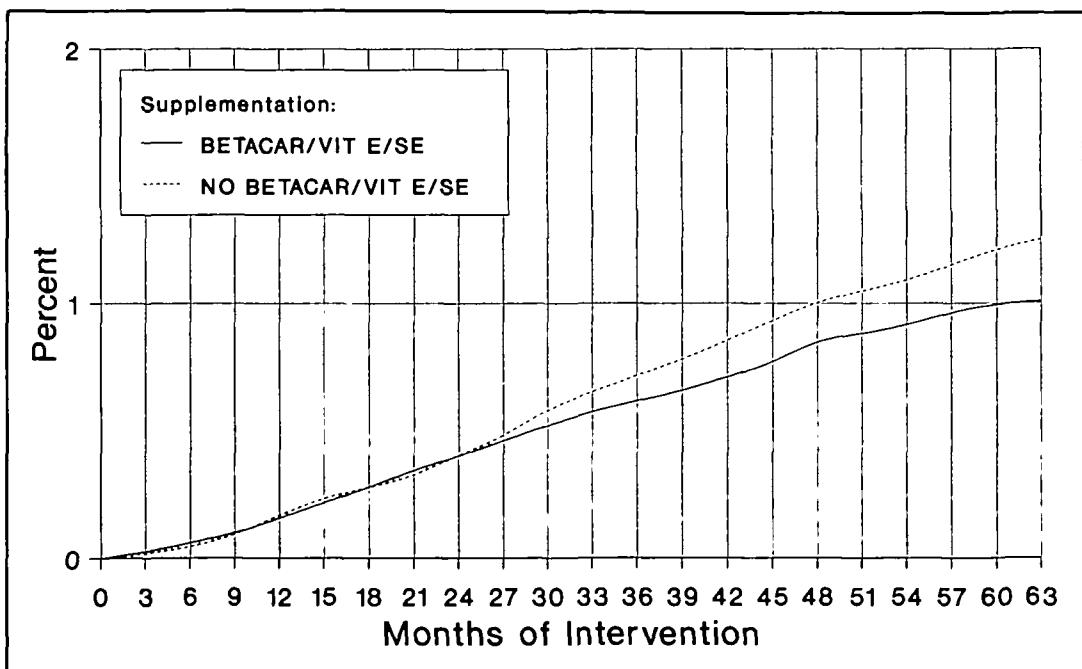


Fig. 2. Cumulative stomach cancer deaths as percent of study population, March 1986-May 1991.

tumors. Esophageal cancer incidence was lower among those receiving riboflavin and niacin ($RR = 0.86$, 95% CI = 0.74-1.01, $P = .06$), but rates of gastric cardia cancer were nonsignificantly elevated ($RR = 1.07$; 95% CI = 0.88-1.29). Total cancer incidence was 5% lower ($RR = 0.95$; 95% CI = 0.85-1.06) among those receiving versus those not receiving supplementation with riboflavin and niacin.

Discussion

The findings from this large randomized trial provide support for the hypothesis that intake of specific micro-

nutrients may inhibit cancer development. Reductions in total mortality and in cancer mortality and incidence, especially for stomach cancer, were observed over a 5 1/4-year period for the nearly 15000 individuals who received daily supplements containing beta carotene, vitamin E, and selenium. A reduction in esophageal cancer incidence was also suggested among those receiving riboflavin and niacin. No other nutrient combination demonstrated any clear beneficial effects on cancer rates.

Linxian County in north-central China offered unique advantages for this intervention trial, having a large and stable population with subclinical deficiencies of several

Table 6. RRs and 95% CIs of cancer incidence according to vitamin/mineral factor

Type of cancer	n	Factor*									
		A			B			C			D
		RR	95% CI	RR	95% CI	RR	95% CI	RR	95% CI	RR	95% CI
Total	1298	1.00	0.89-1.11	0.95	0.85-1.06	1.06	0.95-1.18	0.93	0.83-1.03		
Esophagus	640	1.07	0.92-1.25	0.86	0.74-1.01	1.06	0.91-1.24	1.02	0.87-1.19		
Stomach	539	0.96	0.81-1.14	1.04	0.88-1.23	1.10	0.92-1.30	0.84	0.71-1.00		
Cardia	435	1.02	0.85-1.24	1.07	0.88-1.29	1.07	0.90-1.29	0.85	0.70-1.02		
Noncardia	104	0.73	0.49-1.08	0.92	0.63-1.35	1.21	0.82-1.78	0.82	0.56-1.20		
Esophageal/cardia	1075	1.05	0.93-1.19	0.94	0.83-1.06	1.06	0.94-1.20	0.94	0.84-1.06		
Other	119	0.80	0.56-1.15	1.09	0.76-1.56	0.92	0.64-1.32	0.88	0.62-1.27		

*A = retinol, zinc; B = riboflavin, niacin; C = vitamin C, molybdenum; and D = beta carotene, vitamin E, and selenium.

nutrients and an extraordinarily high incidence of epithelial cancers thought to be influenced by diet and nutritional status. The random allocation within strata of individuals to the intervention groups guaranteed equity between the groups by age and sex, facilitated balance with respect to unmeasured factors, and helped to avoid confounding. The participant compliance was exceptional: Pill disappearance (implying apparent ingestion) exceeded 90%, and blood collections from randomly selected individuals every quarter provided biochemical confirmation of excellent compliance. The large numbers of events—over 2100 deaths and nearly 1300 cancers—yielded excellent power and precise estimation of effects.

There are several caveats, however, that should be considered in interpreting the trial findings. Some concern the study design. An advantage of the factorial design is the ability to test several hypotheses simultaneously. However, with four independent tests of main effects, the chance that one will be significant at the $P \leq .03$ level is .11 (i.e., $1 - .97^4$) as a result of the multiple comparisons. Secondly, the fractional factorial design confounds main effects with three-way interaction effects, so what we attribute to factor D might actually be an ABC interaction. Such interactions are typically rare and seem much less likely than an effect due to a single factor, however, and can likely be dismissed. Finally, the special features of the Linxian setting, a relatively remote, rural area of China with a population marginally deficient in a number of nutrients, suggest caution in extrapolating the findings to other populations.

This trial revealed a significant reduction in total mortality, due mostly to a lowered risk of cancer, among those receiving the combination of beta carotene, vitamin E, and selenium. There was a differential effect between mortality from stomach cancer (21% reduction) and other cancers (20% reduction) versus esophageal cancer (4% reduction), suggesting that the benefit may vary by site and/or cell type. However, the CIs for these RRs all overlapped; thus, we are reluctant to emphasize the site-specific differences. Nevertheless, it may be noteworthy that the stomach cancers were adenocarcinomas, while the esophageal cancers were nearly all squamous cell carcinomas.

Studies in experimental animals (6,7) have demonstrated the cancer inhibitory properties of beta carotene, vitamin E,

and selenium. For example, beta carotene has inhibited formation of UV-induced skin cancers, oral carcinomas caused by dimethylbenzanthracene exposure, and colon tumors that develop following dimethylhydrazine exposure. Vitamin E and selenium have also lowered the incidence of tumors in a number of experiments involving exposure to different carcinogens, although some studies (6,7) suggested either no effect or enhancement of carcinogenesis. Vitamin E has been shown to inhibit nitrosamine-induced esophageal cancer in mice (15).

The epidemiologic evidence is also consistent with a beneficial role of beta carotene, vitamin E, and selenium on cancer risk (5,6). It has repeatedly been shown that intake of fresh fruits and vegetables is associated with reduced risks of esophageal, stomach, and total cancer (5). Risks among persons having the highest intakes often are as low as one half of those having the lowest intakes for several foods, especially citrus and other fruits and fresh green, orange, and yellow vegetables. Although it has been difficult to identify specific components of these foods that may be responsible for the decreased risk, a role for beta carotene and possibly other carotenoids has been suggested by case-control studies of esophageal and stomach cancers, including studies in China (5,6,16-18). Difficulty in estimating dietary intakes of vitamin E and selenium has limited evaluation of their effects. However, in Italy, the largest case-control study (19) of stomach cancer conducted to date found that risks of cancers of the stomach, including gastric cardia, were more closely correlated with an index of dietary vitamin E than of beta carotene, although the opposite pattern was reported in Canada (20).

Further supporting evidence for a protective effect of beta carotene, vitamin E, and selenium comes from observations of inverse associations of blood levels of these nutrients with risk of several cancers, including those of the esophagus and stomach. Ecologic surveys (21-23) across rural Chinese counties found that plasma levels of selenium were inversely correlated with mortality rates for both esophageal and stomach cancers, while plasma beta carotene was significantly lower in counties with high stomach cancer rates. In Linxian, blood levels of beta carotene and alpha-tocopherol are consistently lower than Western norms, while levels of selenium are near normal or only marginally lower (8-11). In evaluation of nutrients in stored sera for persons who

subsequently developed stomach cancer, prediagnostic levels of serum beta carotene were depressed in studies in the United States, Great Britain, and Switzerland (24). Differences with respect to serum vitamin E and selenium in these studies were less pronounced. A study in Finland (25), however, found that serum levels of selenium were significantly lower in men who subsequently developed stomach cancer.

Only a few observational studies have evaluated risk of cancer in relation to use of vitamin or mineral supplements. In a U.S. hospital-based study (26) of 133 male esophageal cancer patients, risk was 50% lower among users of vitamin E supplements. A 50% reduction in risk of oral cancer also was reported among users of vitamin E supplements in a national study (27) that enrolled over 1000 American patients.

Several clinical trials have shown beneficial effects of beta carotene or vitamin E on precancerous lesions. In a 20-month randomized trial in Uzbekistan (28), daily doses of 40 mg of beta carotene together with weekly supplements of 100 000 IU of retinol and 80 mg of vitamin E were associated with endoscopically determined regression of chronic esophagitis (although the effect was not statistically significant) and with a significant reduction in oral leukoplakia. Several other trials (29-31) have also reported reversals in oral leukoplakia, thought to be a precursor to oral cancer, following supplementation with beta carotene or vitamin E. Furthermore, beta carotene combined with retinol lowered the frequency of buccal micronuclei among betel chewers in the Philippines (32), while beta carotene alone reduced the prevalence of micronuclei in the sputum of smokers in the Netherlands (33).

No other randomized clinical trials have reported on the effects on esophageal or stomach cancers of supplementation with beta carotene, vitamin E, or selenium. In the only reported cancer trial (34), daily 50-mg beta carotene supplementation was not found to be effective in inhibiting second primary basal or squamous cell skin cancers among patients with nonmelanoma skin cancer. In the smaller parallel trial that we conducted among persons with esophageal dysplasia in Linxian, described in a companion paper (12), total cancer mortality and incidence rates were similar between those receiving 6 years of supplementation with 26 vitamins and minerals (including beta carotene, vitamin E, and selenium) and those receiving placebo. Intervention trials involving beta carotene, vitamin E, and/or selenium and assessing cancer end points are ongoing in populations in the United States and Finland. Although the numbers of esophageal or stomach cancers likely to arise in these trials will be considerably smaller than in Linxian, evaluation of the effects of supplementation on these cancers should be possible.

The mechanisms by which beta carotene, vitamin E, or selenium inhibit cancer development are not clear, but might involve their antioxidant properties (6). These compounds, especially beta carotene, can quench free radicals and protect against oxidative damage to DNA. The nutrients also inhibit the endogenous formation of *N*-nitroso compounds, some of which are potent carcinogens in animal experiments and are

suspected risk factors for stomach and esophageal cancers in human populations, including Linxian's (35,36). Beta carotene, vitamin E, and selenium may also possess immunologic and other properties that influence carcinogenesis (6). We could not directly evaluate mechanistic pathways, although we are currently assessing differences in cytologically and endoscopically determined precancerous lesions as well as immune function by treatment group.

Despite its high (90%) power to detect reductions of 14% and 23% in total and cancer mortality, respectively, the trial failed to find significant protective effects on total, esophageal, or stomach cancer mortality during the 5½-year supplementation period for retinol and zinc, riboflavin and niacin, or vitamin C and molybdenum. A significant reduction in noncardia stomach cancer mortality was observed among those receiving retinol and zinc, but it was counterbalanced by an increase in gastric cardia cancer mortality in this group. A potential benefit of retinol on cancer risk has been postulated on the basis of its role in maintaining cell integrity and on the ability of certain retinoids to inhibit chemically induced tumors in laboratory animals, although in some experimental models, vitamin A and its analogues have enhanced carcinogenesis (6). Epidemiologic support for a beneficial effect of vitamin A, although suggestive at one time, has waned since the trial began. Several recent analytic studies have found no evidence of a protective effect of dietary or serum retinol for esophageal or stomach cancers, with the consensus suggesting that benefits are associated with the vitamin A precursor beta carotene (37). In clinical trials (38), however, high doses of the synthetic retinoid isotretinoin have proven effective in reducing the incidence of second primary cancers of the head and neck in oral cancer patients. Although human data on the relationship between zinc and cancer are limited, zinc was included because it enhances delivery of retinol to body tissues and because esophageal carcinogenesis is promoted in zinc-deficient rats (39). It is noteworthy that in a randomized trial (40) in a county neighboring Linxian, 13 months of supplementation with retinol (50 000 IU per week) and zinc (50 mg per week) along with high doses (200 mg per week) of riboflavin had no effect on the prevalence of precancerous esophageal lesions. The frequencies of buccal micronuclei also were similar in the treated and placebo groups, although esophageal micronuclei were reduced following supplementation with retinol, zinc, and riboflavin (41).

One of the characteristics of areas of the world (e.g., Linxian and parts of Iran and South Africa) with markedly elevated esophageal cancer rates is low dietary intake of B vitamins, particularly riboflavin and niacin (42). Indeed, riboflavin status as measured by erythrocyte glutathione reductase activity was severely depressed in Linxian when compared with the United States; over 90% of the Linxian population was termed deficient in this nutrient (8-11). Riboflavin deficiencies have induced esophageal hyperplasia in baboons and altered the metabolism of nitrosamines (43,44). Supplementation with riboflavin and niacin, along with zinc, magnesium, and molybdenum, inhibited esophageal carcinogenesis in corn-fed rats (45). In Uzbekistan,

however, supplementation with riboflavin for 20 months was not successful in reversing chronic esophagitis (28). We found that esophageal cancer incidence and mortality were 14% and 10% lower, respectively, among those receiving daily riboflavin and niacin supplements, with the 14% reduction in esophageal cancer incidence of borderline statistical significance. Total cancer incidence or mortality, however, was only slightly reduced among those receiving B vitamin supplementation, thus providing only weak support for a protective effect. On the other hand, the limited a priori evidence suggests that a benefit from riboflavin and niacin may be more pronounced for esophageal cancer. Thus, we view the finding of lowered esophageal cancer incidence as an encouraging sign worthy of additional investigation.

Vitamin C has been suggested as protective against several cancers, particularly stomach cancer (6). Although results of experimental studies are mixed, most case-control studies of stomach and esophageal cancers have shown that adult diets of the patients typically are low in intake of vitamin C-containing foods (5). Vitamin C can inhibit endogenous formation of nitrosamines, and it has antioxidant and other biological properties that may also inhibit carcinogenesis (46). Data from the Linxian trial, however, showed no evidence of reduced cancer mortality or incidence (in fact, the RRs were above 1.0) among persons receiving daily vitamin C and molybdenum supplements at doses approximately twice the U.S. RDAs.

The failure of this trial to find significant reductions in cancer mortality among those supplemented with retinol and zinc, riboflavin and niacin, or vitamin C and molybdenum could be related to the shortness (5½ years) of the intervention and follow-up. Indeed, if these nutrients had a protective effect on the early stages of carcinogenesis, our focus on concurrent events in adults taking the supplements would not be expected to show a mortality differential. The data suggest only that supplementation with these particular nutrients resulted in no demonstrable short-term mortality benefit. Continued monitoring of the participants will determine whether any reductions in cancer or other disease may emerge in the coming years.

Several reports have suggested that antioxidant nutrients may reduce the risk or progression of cerebrovascular disease, a common cause of death in Linxian, and perhaps lower the rate of hypertension, a strong risk factor (6,47-49). We found no significant treatment group differences, but mortality from cerebrovascular disease was 10% lower among those receiving supplements with the antioxidants beta carotene, vitamin E, and selenium. Although the effects of antioxidants on cerebrovascular disease may be caused by limiting neuronal tissue damage from cerebral ischemia, evidence is accumulating that antioxidants may inhibit atherosclerosis, especially of the coronary vessels, by reducing the oxidation of low-density lipoproteins (50). A lowered risk of cardiovascular disease has been reported among American men and women taking vitamin E supplements (51,52). Furthermore, in a randomized trial among U.S. physicians (53), beta carotene was found to reduce by 44% the risk of major coronary events in the

subset of participants with chronic angina. Elsewhere, plasma levels of carotene and vitamins E and C have been inversely related to risk of angina (54). Only 1% of the deaths among Linxian trial participants were attributed to ischemic heart disease, limiting evaluation of intervention effects, but we found little or no reduction in mortality from cardiovascular disease among those receiving beta carotene, vitamin E, and selenium.

In summary, when the Linxian trial observations are combined with the epidemiologic, experimental, and biological evidence at hand, it seems plausible that the lowered cancer rates represent a protective effect of beta carotene, vitamin E, and selenium intervention. Although pill supplementation ceased in the summer of 1991, continued follow-up of the Linxian participants over the coming years is planned. In this manner, persistence of the lowered mortality associated with beta carotene, vitamin E, and selenium and the reduced esophageal cancer incidence associated with riboflavin and niacin can be evaluated along with the potential long-term effects of each of the four nutrient combinations after intervention. Shorter-term trials are also planned in the context of a gastric screening project in another high-risk area of China to assess the effectiveness of beta carotene versus vitamin E versus selenium in inhibiting transitions from chronic atrophic gastritis to intestinal metaplasia and gastric dysplasia. Thus, while the Linxian results should be considered preliminary in nature, they offer a hopeful sign that vitamin/mineral supplementation may lower the risk of certain cancers and suggest lines of further research to evaluate the protective effects of specific micronutrients.

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Notes

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We thank the Linxian residents who participated in the trials, the Linxian county government, the Linxian People's Hospital, and the commune hospitals in Yaocun, Rencun, Donggang, and Hengshui for support. For guidance and dedicated field and laboratory work in carrying out the investigation, we thank Drs. Shihxin Lu, Kan Yang, Wen Wang, Xiao-nong Zou, Shufan Liu, Quping Yang, Guchen Yang, and Zhijian Chen of the Cancer Institute, Chinese Academy of Medical Sciences, and Qiong Shen of the Henan Medical University. For expert assistance in data management and processing, we thank Linda Cranston, Jack Cahill, Suzanne Huang Rexing, Linda Lannom, Anell Bond, Eric Mehl, Walt Hufford, Erika Wilson, Shelly Niwa, Cathy Agar, and Drew Nuland of Westat, Inc. For scientific advice and support we thank Drs. Peter Greenwald, Steven Mark, Christine Swanson, and B. J. Stone of the National Cancer Institute and Dr. Abby Ershow of the National Heart, Lung and Blood Institute. For expert advice and review of diagnostic materials we thank Drs. Klaus Lewin, Roberta Nieberg, Marvin Weiner, and Wilfred Weinstein from the University of California at Los Angeles and Dr. Zheng-Yan Wang from the Cancer Institute, Chinese Academy of Medical Sciences. For expert advice, suggestions, and review during the trials, we thank Dr. Paul Engstrom of

the Fox Chase Cancer Center (Philadelphia, Pa.), Dr. Pelayo Correa of the Louisiana State University (New Orleans), and Dr. Stephen Lagakos of the Harvard School of Public Health (Boston, Mass.), who served on the Data Safety and Monitoring Committee. We also acknowledge and thank

Hoffmann-La Roche for assistance in the provision of the vitamin/mineral supplements.

Manuscript received February 19, 1993, revised June 22, 1993; accepted July 1, 1993.

Nutrition Intervention Trials in Linxian, China: Multiple Vitamin/Mineral Supplementation, Cancer Incidence, and Disease-Specific Mortality Among Adults With Esophageal Dysplasia

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Background: A number of vitamins and minerals have been shown to influence carcinogenesis in experimental animals. In humans, epidemiologic evidence suggests that intake of fruits and vegetables may reduce risk of esophageal and other cancers. Vitamins and minerals in these foods may contribute to the reduced cancer risk. The people of Linxian, China, have persistently low intake of multiple nutrients and exhibit one of the world's highest rates of esophageal/gastric cardia cancer, with an exceptionally high risk of esophageal dysplasia. **Purpose:** To determine whether supplementation with multiple vitamins and minerals may reduce esophageal/gastric cardia cancer among persons with esophageal dysplasia, we conducted a 6-year prospective intervention trial in Linxian. **Methods:** Mortality and cancer incidence were ascertained from May 1985 through May 1991 for 3318 persons with cytologic evidence of esophageal dysplasia who were randomly assigned to receive, throughout that period, daily supplementation with 14 vitamins and 12 minerals or placebo. Doses were typically two to three times U.S. Recommended Daily Allowances. Compliance was assessed by counting unused pills monthly for all trial participants and by assaying nutrient levels in blood collected from samples of individuals randomly selected without replacement every 3 months throughout the trial. Cancers were identified through routine surveillance and by special cytology and endoscopy screenings after 2½ years and 6 years. **Results:** A total of 324 deaths occurred during the 6-year intervention period; 167 occurred in the control (placebo) group and 157 occurred in the supplement group. Cancer was the leading cause of death (54% of all deaths); 18% were due to cerebrovascular diseases and 29% to other causes. Cumulative esophageal/gastric cardia death rates were 8% lower (relative risk [RR] = 0.92; 95% confidence interval [CI] = 0.67-1.28) among individuals

receiving supplements rather than placebo, a nonsignificant ($P>.10$) difference. Risk of total mortality was 7% lower (RR = 0.93; 95% CI = 0.75-1.16; $P>.10$), total cancer 4% lower (RR = 0.96; 95% CI = 0.71-1.29; $P>.10$), cerebrovascular disease 38% lower (RR = 0.62; 95% CI = 0.37-1.06; $P = .08$), and other diseases 12% higher (RR = 1.12; 95% CI = 0.74-1.69; $P>.10$) among the treated group. Cumulative cancer incidence rates were nearly the same in the two groups. **Conclusions:** No substantial short-term beneficial effect on incidence or mortality for this type of cancer occurred following daily supplementation with multiple vitamins and minerals among adults with precancerous lesions of the esophagus. **Implications:** Although no statistically significant short-term benefits were observed, longer follow-up should be more informative about the effectiveness of this 6-year supplementation on cancer and other diseases among individuals with esophageal dysplasia. [J Natl Cancer Inst 85:1492-1498, 1993]

Rates of esophageal/gastric cardia cancer in Linxian, a rural county in Henan Province, north-central China, are among the highest in the world (1). The excess risk is especially pronounced among persons with esophageal dysplasia, a precancerous lesion affecting over 20% of adults in this area (2,3). The excess cancers occur not only as squamous cell carcinomas of the esophagus, but also as adenocarcinomas of the gastric cardia. Traditionally, both tumors have been called "esophageal cancer" in Linxian because of their proximity to one another and similarity in symptoms.

*See "Notes" section following "References."

Original article

Dietary glycemic load and colorectal cancer risk

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Summary

Background: Insulin and insulin-like growth factors can stimulate proliferation of colorectal cells. High intake of refined carbohydrates and markers of insulin resistance are associated with colorectal cancer. To test the insulin/colon cancer hypothesis, we determined whether the dietary glycemic index and the glycemic load are associated with colorectal cancer risk.

Design: A case-control study on colorectal cancer conducted in Italy. Cases included 1125 men and 828 women with histologically confirmed incident cancer of the colon or rectum. Controls were 2073 men and 2081 women hospitalized for acute conditions. We calculated average daily dietary glycemic index and glycemic load, and fiber intake from a validated food frequency questionnaire.

Results: Direct associations with colorectal cancer risk emerged for glycemic index (odds ratio (OR) in highest vs. lowest quintile = 1.7; 95% confidence interval (CI): 1.4–2.0) and glycemic load (OR = 1.8; 95% CI: 1.5–2.2), after allowance for sociodemographic factors, physical activity, number of daily meals, and intakes of fiber, alcohol and energy. ORs were more elevated for cancer of the colon than rectum. Overweight and low intake of fiber from vegetables and fruit appeared to amplify the adverse consequences of high glycemic load.

Conclusions: The positive associations of glycemic index and load with colorectal cancer suggest a detrimental role of refined carbohydrates in the etiology of the disease.

Key words: colon cancer, fiber, glycemic load, rectal cancer

Introduction

Carbohydrate foods consumed in isoglucidic amounts produce different glycemic and insulinemic responses depending upon the nature of the food (e.g., ratio of amylose to amylopectin, amount and type of fiber, particle size of starch granules) and type and extent of food processing (e.g., extrusion, flaking, grinding, and cooking) [1].

A role of diets with a high glycemic response and consequent hyperinsulinemia in colorectal carcinogenesis has been postulated [2, 3]. Several findings from an Italian case-control study support this hypothesis. Direct associations were found between colorectal cancer and intake of refined carbohydrates and energy [4], number of daily meals [5], body mass index (BMI) in men and, among women, central adiposity [6]. Conversely, inverse associations emerged for intake of vegetables, pulses, and fish [7], fibre [8], unsaturated-to-saturated fat ratio [4], and level of physical activity [9].

In order to test the insulin/colon cancer hypothesis, we decided to evaluate the dietary carbohydrate component using the glycemic index (GI), an indicator of

carbohydrate foods' ability to raise blood glucose levels, and the glycemic load score (GL), as a combination of quality as well as quantity of carbohydrates consumed, and thus a measure of dietary insulin demand.

Patients and methods

A case-control study of cancer of the colon and rectum was conducted between January 1992 and June 1996 in six Italian areas: the provinces of Pordenone and Gorizia in North-eastern Italy; the urban areas of Milan and Genoa and the provinces of Forlì, in the North; and Latina and the urban area of Naples in the South [7].

Cases had histologically confirmed colorectal cancer diagnosed no longer than one year prior to the interview (median 1 month) and with no previous diagnoses of cancer at other sites. Overall, 1225 subjects with cancer of the colon (688 men and 537 women, median age 62, range 19–74 years) and 728 with cancer of the rectum and rectosigmoid junction (437 men and 291 women, median age 62, range 23–74 years) were included.

Controls were in-patients with no history of cancer, in major teaching and general hospitals in the same areas where cases lived, and identified for acute, non-neoplastic, non-gynecological conditions, unrelated to hormonal or digestive tract diseases. All chronic conditions which may lead to lifestyle modifications (e.g., diabetes mellitus, cardiovascular diseases, etc.) were not eligible causes of hospital

Table 1. Glycemic index (GI) values for a few common foods and recipes.^{a,b}

Foods	GI
White bread ^c	100
Biscuits	95
Sugar	89
Pizza	86
Ice cream	83
Pasta/rice with tomato sauce	62
Apples and pears	58
Peas, beans, chick peas, lentils	57
Vegetable soup with noodles	54
Whole milk	49
Peaches, apricots and prunes	39
Tomatoes	13

^a Modified by references [17–19].

^b Meat- and fish-based recipes and cheese have a GI close to 0.

^c Standard food.

admission. Conversely, comorbidity from the above conditions did not represent an exclusion criterion. The control group included 2073 men and 2081 women aged 19–74 years (median age 58) belonging to the following diagnostic categories: trauma, mostly fractures and sprains (27%); other orthopedic disorders, such as low back and disc disorders (24%); acute surgical conditions (18%); eye diseases (24%); and other miscellaneous diseases, such as ear, nose, throat, skin and dental conditions (7%). About 4% of cases and controls approached during their hospital stay refused to be interviewed.

The same structured questionnaire and coding manual were used in each center, and all interviewers were centrally trained and routinely supervised. The questionnaire included information on socio-demographic characteristics, such as education and occupation, lifetime smoking and alcohol-drinking habits, physical activity, anthropometric measures at various ages, a problem-oriented personal medical history and family history of cancer. Body mass index (BMI) was computed as weight in kilograms divided by height in square meters. In order to compute waist-to-hip (W:H) ratio, the interviewer measured the circumference of waist (2 cm above the umbilicus) and hip (maximal protrusion). On account of medical or logistic problems, waist and/or hip circumferences were not measured in 25% of colorectal cancer cases and 19% of control subjects [6].

An interviewer-administered food-frequency questionnaire (FFQ) was used to assess subjects' habitual diet, including total energy. Average weekly frequency of consumption of foods or food groups, as well as complex recipes during the two years prior to cancer diagnosis or hospital admission (for controls) was elicited. The FFQ also included 15 questions aimed at assessing individual fat intake pattern and meal frequency. Satisfactory reproducibility [10, 11] and validity [12] of the FFQ have been reported. To compute energy and nutrient intake, an Italian food-composition data base was used [13]. Fiber, defined as non starch polysaccharides plus lignin, was divided according to the type of food from which it originated (i.e., vegetables and fruit or cereals) [14–16].

The GI is a qualitative assessment of foods based on the incremental glucose response and insulin demand they produce for a given amount of carbohydrates. We expressed GI as a percent of the glycemic response elicited by white bread as a standard food. For each case and control subject, we calculated daily average GI by summing the products of the carbohydrate content per serving for each food or recipe, times the average number of servings of that food per week, times its GI, all divided by the total amount of available carbohydrate weekly intake [17, 18]. A score for the daily average GL was computed as the GI, but without dividing by the total amount of carbohydrates. For these calculations we used the carbohydrate content of 50 foods or recipes since 28 foods or recipes, chiefly meat- and fish-based ones and cheese, contained a negligible amount of carbohydrates [13]. With

Table 2. Distribution of 1225 cases of colon cancer, 728 of rectal cancer and 4154 controls^a by sex, age group, years of education, and level of physical activity. Italy, 1992–1996.

Characteristic	Cancer cases		Controls, n (%)
	Colon, n (%)	Rectum, n (%)	
Sex			
Male	688 (56)	437 (60)	2073 (50)
Female	537 (44)	291 (40)	2081 (50)
Age group (years)			
< 40	55 (4)	26 (4)	347 (8)
40–49	114 (9)	67 (9)	732 (18)
50–59	321 (26)	197 (27)	1244 (30)
60–69	518 (42)	306 (42)	1356 (33)
≥ 70	217 (18)	132 (18)	475 (11)
Education (years)			
< 7	621 (51)	422 (58)	2276 (55)
7–11	331 (27)	181 (25)	1156 (28)
≥ 12	267 (22)	122 (17)	693 (17)
χ^2 ^b	48.66 ^c	0.41	
Physical activity (at the work place)			
Low	444 (36)	231 (32)	1378 (33)
Medium	451 (37)	258 (35)	1476 (36)
High	330 (27)	239 (33)	1299 (31)
χ^2 ^b	42.59 ^c	1.40	

^a Some figures do not add up to the total because of some missing values.

^b Compared to the control group, adjusted for age, sex, and center.

^c P < 0.05.

respect to GI values we chiefly used international tables [18]. In order to take into account Italian cooking habits (e.g., pasta 'al dente'), Italian sources were used for a few local recipes [19]. Food items for which a GI had not been determined were assigned the GI of the nearest comparable food (e.g., tangerines were assigned the GI of oranges). As an example, Table 1 shows GIs estimated for a few common foods and recipes.

Odds ratios (ORs) and the corresponding 95% confidence interval for quintiles of GI and GL were derived using unconditional multiple logistic regression models [20]. The regression equations included terms for age, sex, study center, years of education, occupational physical activity, number of daily meals, and intakes of fiber, alcohol and energy. Adjustment for energy was made using the residuals method [21]. GI and GL were also introduced as continuous variables, and the unit of measurement were 5 and 50, respectively.

Results

After allowance for age, sex, and center, patients with cancer of the colon, but not rectum, were significantly more educated and reported lower levels of physical activity than controls (Table 2).

GI was positively correlated to GL (Pearson correlation coefficient, $r = 0.59$), intake of bread ($r = 0.66$), cakes and sweets ($r = 0.28$), table sugar ($r = 0.23$), and energy ($r = 0.27$), but negatively correlated to intake of fruit ($r = -0.19$) and vegetables ($r = -0.12$). Correlation of GI with other dietary and non-dietary factors, including intake of different types of fat, alcoholic beverages, daily number of meals, BMI, level of physical activity, and smoking habits were weak (i.e., $r < 0.10$).

Table 3. Odds ratios (ORs) and 95% confidence intervals (CIs)^a of colorectal cancer by quintile of energy-adjusted daily glycemic index and glycemic load score, by subsite(s) and overall. Italy, 1992–1996.

Cancer site(s)	Number	OR (95% CI) Quintile					χ^2 (trend) <i>P</i>	Continuous (<i>5 units × day</i>)
		1 ^b	2	3	4	5		
<i>Glycemic index</i>								
<i>Upper limit</i>		70.7	73.8	76.5	79.6	—		
Colon	1225	1	1.4 (1.2–1.8)	1.7 (1.4–2.1)	1.6 (1.3–2.0)	1.9 (1.5–2.4)	29.5 <i>P</i> < 0.001	1.18 (1.11–1.26)
Rectum	728	1	1.0 (0.8–1.4)	1.4 (1.1–1.9)	1.3 (1.0–1.7)	1.4 (1.1–1.9)	9.6 <i>P</i> = 0.002	1.12 (1.04–1.21)
Colon and rectum	1953	1	1.3 (1.1–1.5)	1.6 (1.3–1.9)	1.5 (1.2–1.8)	1.7 (1.4–2.0)	32.8 <i>P</i> < 0.001	1.16 (1.10–1.22)
<i>Glycemic load</i>								
<i>Upper limit</i>		151	190	229	285	—		
Colon	1225	1	1.2 (1.0–1.6)	1.2 (1.0–1.5)	1.6 (1.3–2.0)	1.9 (1.5–2.4)	34.2 <i>P</i> < 0.001	1.26 (1.16–1.36)
Rectum	728	1	1.2 (0.9–1.5)	1.0 (0.7–1.3)	1.3 (1.0–1.7)	1.5 (1.1–1.9)	6.9 <i>P</i> = 0.01	1.09 (1.00–1.20)
Colon and rectum	1953	1	1.2 (1.0–1.5)	1.1 (0.9–1.4)	1.5 (1.2–1.8)	1.8 (1.5–2.2)	35.3 <i>P</i> < 0.001	1.20 (1.13–1.29)

^a Adjusted for age, sex, study center, years of education, occupational physical activity, number of daily meals, intakes of fiber, alcohol and energy.

^b Reference category.

Colorectal cancer risk increased with an increase in dietary GI (OR for highest *versus* lowest quintile: 1.7; 95% CI: 1.4–2.0) and GL (OR = 1.8; 95% CI: 1.5–2.2) (Table 3). The ORs were somewhat higher for colon cancer (1.9 for GI and 1.9 for GL in the highest quintile) than rectal cancer (1.4 and 1.5, respectively).

Findings were similar in separate strata of age, but the association for the combination of colorectal cancer was somewhat stronger among women (OR in highest quintile = 2.0 for GI and 2.6 for GL) than among men (ORs 1.5 for either GI and GL). This difference was probably accounted for by a greater proportion of subjects with colon cancer in the female than in the male sex. No material interaction or effect modification was observed between GI or GL and level of physical activity, alcohol drinking and unsaturated-to-saturated fatty acid ratio. One hundred sixteen (6%) cases and one hundred eighty-five (4%) control subjects reported a history of non-insulin dependent diabetes. OR in the highest intake quintile of GI and GL were, respectively, 2.1 and 2.4 among diabetic individuals and 1.7 and 1.8 among non-diabetic individuals.

We examined the joint effect of GL and different fiber intake by cross-classifying patients with both variables (Figure 1a and b). GL was directly associated with colorectal cancer risk in each stratum of fiber from vegetables and fruit (median intake 11.9 g per day). Similarly, low fiber intakes showed elevated ORs in each stratum of dietary GL (Figure 1a). The OR for the combination of the highest tertile of GL and the lowest tertile of fiber

from vegetables and fruit compared with the opposite extreme was 1.9 (95% CI: 1.5–2.4). GL and fiber from cereals (median intake 5.7 g per day) produced a different picture (Figure 1b). The OR for the combination of the highest tertile of GL and the lowest one of cereal fiber was 0.8 (95% CI: 0.5–1.5), whereas for the highest tertile of GL and the highest of cereal fiber was 1.5 (95% CI: 1.0–2.1). In fact GL and fiber from cereals were strongly correlated ($r = 0.81$).

We also assessed the combined influence of GL and the best indicators of visceral adipose tissue in our study, i.e., W:H ratio in women and BMI in men (Table 4). The OR was 2.8 (95% CI: 1.9–4.1) in the highest GL tertile in combination with the highest tertile of W:H ratio in women. Among men, the OR for the combination of the highest tertile of BMI and the highest GL tertile was 1.8 (95% CI: 1.3–2.7).

Discussion

High dietary GI and GL have been associated with increased risk of non-insulin dependent diabetes [22,23] and coronary heart disease [24], but the influence of GI and GL on colorectal cancer risk has been little studied [25]. Our present findings show that a diet that increases glycemic response is involved in the etiology of cancers of the colon-rectum, particularly of those which arise from the colon. The associations with GI and GL were independent from the presence of diabetes. Low intake

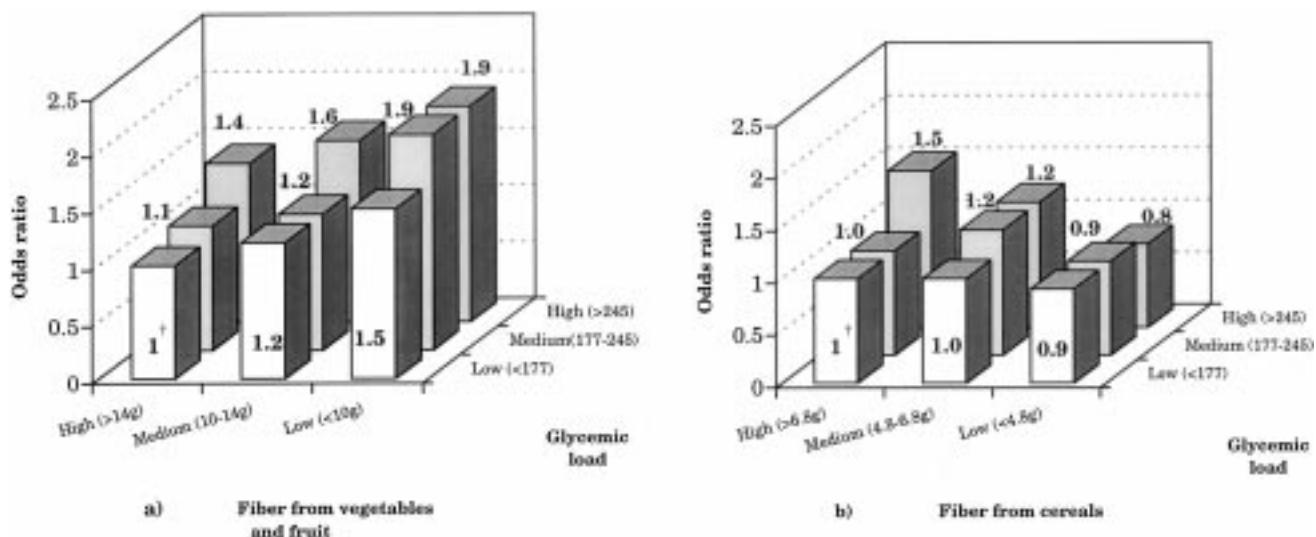


Figure 1. Odds ratio* of colorectal cancer by tertile of energy-adjusted glycemic load and fiber intake from different sources. Italy, 1992–1996.

* Adjusted for age, sex, study center, years of education, occupational physical activity, meal frequency, alcohol consumption, and energy intake.

† Reference category.

of fiber from vegetables and fruit and elevated BMI or W:H ratio appeared to contribute in enhancing colorectal cancer risk when the diet was high in GL.

The positive associations we observed between GI and GL and colorectal cancer were among the strongest reported so far for any dietary factor [7, 25], and were consistent in different strata of age, sex, and various risk covariates. The strong risk increase among individuals on a high-GL-low-fiber diet is in agreement with the property of fiber to flatten the glucose-response and has also been observed in individuals with non-insulin dependent diabetes [22, 23]. Fiber from vegetables and fruit, which accounted for 2/3 of fiber intake, seemed to exert a beneficial effect on colorectal cancer risk and, possibly, to mitigate the impact of high dietary GL. It is possible that fiber from cereals did not appear to be protective in our [8] as well as in other study populations [26] because it was derived chiefly from highly refined cereal products [27]. Data on the relationship between different types of fiber and glycemic response and insulin demand depend on the nature of fiber and its relation to the nutrients contained in the food [28]. When taken out of their botanical structure, isolated cereal fiber is not seen to reduce the rate of carbohydrate absorption while viscous fiber (i.e., guar) has been shown to decrease the rate of glucose absorption [29].

Whereas consumption of sugar and cakes is relatively low, Italy shows the highest consumption of carbohydrates from refined cereals among affluent countries [30], up to more than 300 g/day in one fifth of this study population. Interestingly, the highest intake quintile of white bread, whose GI is 100, was associated with an energy-adjusted OR of 1.6 (95% CI: 1.3–1.9) whereas the highest intake quintile of pasta, whose GI is only approximately 60, showed an OR of 1.2 (95% CI: 1.0–1.5).

Visceral adipose tissue has been associated with increased insulin resistance and colorectal cancer risk [31].

Table 4. Odds ratios (ORs) and 95% confidence intervals (CIs)^a of colorectal cancer by tertile of energy-adjusted daily glycemic load and waist-to-hip ratio in females, and body mass index in males. Italy, 1992–1996.

	OR (95% CI) Glycemic load			
	< 158	158–217	> 217	All ^b
Females				
Waist-to-hip ratio				
< 0.8	1 ^c	1.5 (1.1–2.1)	1.9 (1.4–2.8)	1 ^c
0.8–0.86	1.4 (0.9–2.0)	2.1 (1.5–3.1)	1.9 (1.3–2.7)	1.5 (1.2–2.0)
> 0.86	1.3 (0.9–1.8)	1.6 (1.1–2.4)	2.8 (1.9–4.1)	1.6 (1.2–2.1)
All ^b	1 ^c (1.1–1.8)	1.4 (1.1–2.2)	1.7 (1.3–2.2)	
Males				
Body mass index				
< 24.6	1 ^c	0.9 (0.7–1.3)	1.2 (0.9–1.7)	1 ^c
24.6–27.44	0.9 (0.6–1.3)	1.2 (0.8–1.6)	1.3 (0.9–1.8)	1.1 (0.9–1.3)
> 27.44	1.1 (0.8–1.5)	1.3 (0.9–1.8)	1.8 (1.3–2.7)	1.3 (1.1–1.6)
All ^b	1 ^c (0.9–1.4)	1.1 (1.1–1.8)	1.4 (1.1–1.8)	

^a Adjusted for age, study center, years of education, occupational physical activity, number of daily meals, intakes of fiber, alcohol and energy, and recent weight loss.

^b Adjusted also for glycemic load, waist-to-hip ratio or body mass index as appropriate.

^c Reference category.

Men have more visceral adipose tissue than women. Thus, BMI was a good surrogate of visceral adipose tissue in the male sex while W:H ratio had to be considered among women [6]. Assessment of BMI and W:H ratio in colorectal cancer patients is hampered by weight loss in the proximity of cancer diagnosis. We had, however, information on BMI changes over time, and we were able to allow for recent weight loss [6].

In addition to dietary findings, several lines of evidence suggest a role of insulin in colorectal carcinogenesis. Non-insulin dependent diabetes mellitus is associated with increased risk of colorectal cancer [32, 33]. Insulin is considered to be a growth factor and has been shown to increase insulin-like growth factor (IGF)-I, which is involved in long-term regulation of energy metabolism [2]. Insulin and IGF receptors have been found in both normal and malignant cells of colonic mucosa, and can stimulate proliferation of human colorectal cells [2, 34]. Tall individuals [35] and those with acromegaly, who have elevated levels of growth hormone and IGF-I [36], have an increased incidence of colorectal cancer. Circulating levels of IGF-I and II and IGF binding protein (IGF BP)-III, which modulate access of IGF-I to IGF-receptors, were related to subsequent colorectal cancer risk [37, 38].

We used a validated questionnaire [10, 12] and had access to a large number of colorectal cancer patients and control subjects who, in the vast majority, agreed to participate in the study. The control group included a broad range of acute conditions unrelated to diet modifications. Reported associations with GI and GL were not restricted to any major nosological category of control, and held true when cases and controls with a history of diabetes were excluded. Allowance was possible for a number of potential confounding factors, including number of daily meals, energy and alcohol intake, and physical activity. Weaknesses of the present findings include, however, the limitations of current GI estimates. Many of them have been derived from small samples and variations of estimates in mixed-meal situations are unclear [17,18]. Notwithstanding, the present findings lend indirect support to the hypothesis that increased glycemic response and insulin resistance are associated with colorectal cancer, most notably with that of the colon.

Acknowledgements

This work was supported by the contributions of the Italian Association for Research on Cancer, Milan, Italy.

The authors wish to thank Drs A. Giacosa, M. Montella, E. Conti, and Prof. D. Amadori for their help in study coordination, and Mrs L. Mei for editorial assistance.

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Received 10 August 2000; accepted 29 September 2000.

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Total and Cancer Mortality After Supplementation With Vitamins and Minerals: Follow-up of the Linxian General Population Nutrition Intervention Trial

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- Background** The General Population Nutrition Intervention Trial was a randomized primary esophageal and gastric cancer prevention trial conducted from 1985 to 1991, in which 29584 adult participants in Linxian, China, were given daily vitamin and mineral supplements. Treatment with “factor D,” a combination of 50 µg selenium, 30 mg vitamin E, and 15 mg beta-carotene, led to decreased mortality from all causes, cancer overall, and gastric cancer. Here, we present 10-year follow-up after the end of active intervention.
- Methods** Participants were assessed by periodic data collection, monthly visits by village health workers, and quarterly review of the Linxian Cancer Registry. Hazard ratios (HRs) and 95% confidence intervals (CIs) for the cumulative effects of four vitamin and mineral supplementation regimens were calculated using adjusted proportional hazards models.
- Results** Through May 31, 2001, 276 participants were lost to follow-up; 9727 died, including 3242 from cancer (1515 from esophageal cancer and 1199 from gastric cancer). Participants who received factor D had lower overall mortality (HR = 0.95, 95% CI = 0.91 to 0.99; $P = .009$; reduction in cumulative mortality from 33.62% to 32.19%) and gastric cancer mortality (HR = 0.89, 95% CI = 0.79 to 1.00; $P = .043$; reduction in cumulative gastric cancer mortality from 4.28% to 3.84%) than subjects who did not receive factor D. Reductions were mostly attributable to benefits to subjects younger than 55 years. Esophageal cancer deaths between those who did and did not receive factor D were not different overall; however, decreased 17% among participants younger than 55 (HR = 0.83, 95% CI = 0.71 to 0.98; $P = .025$) but increased 14% among those aged 55 years or older (HR = 1.14, 95% CI = 1.00 to 1.30; $P = .47$). Vitamin A and zinc supplementation was associated with increased total and stroke mortality; vitamin C and molybdenum supplementation, with decreased stroke mortality.
- Conclusion** The beneficial effects of selenium, vitamin E, and beta-carotene on mortality were still evident up to 10 years after the cessation of supplementation and were consistently greater in younger participants. Late effects of other supplementation regimens were also observed.

J Natl Cancer Inst 2009;101:507–518

With incidence rates exceeding 100 per 10 000 person-years, the people of Linxian, China, have some of the highest rates of esophageal squamous cell carcinoma and gastric cardia adenocarcinoma in the world (1,2). Several studies in the early 1980s showed that nutritional deficiencies were common in this area, suggesting a link between these deficiencies and the high cancer rates (3). The Linxian General Population Nutrition Intervention Trial (NIT), a large-scale, randomized, double-blind, primary prevention trial, was designed to test the efficacy of four combinations of vitamins and minerals in reducing esophageal and gastric cardia cancer incidence and mortality in Linxian (4–6). The results of this trial, which started March 1, 1986, and concluded May 31, 1991, showed that supplementation with the antioxidant combination of selenium, vitamin E, and beta-carotene statistically significantly reduced total mortality, total cancer mortality, and gastric cancer mortality (5). The identification of statistically significant effects

raises questions about the durability of these effects and also potential good or bad late effects related to supplementation.

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See “Funding” and “Notes” following “References.”

DOI: 10.1093/jnci/djp037

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CONTEXT AND CAVEATS

Prior knowledge

The undernourished population of Linxian, China, has high rates of cancer of the esophagus and gastric cardia. In the General Population Nutrition Intervention Trial of 1985–1991, 29 584 Linxian villagers aged 40–69 years were given daily supplements of one or more of four vitamin and mineral combinations. “Factor D,” which contained selenium, vitamin E, and beta-carotene, was associated with reduced mortality overall, from cancer, and from gastric cancer.

Study design

For 15-year follow-up, villagers were interviewed monthly concerning their health and registered cancer deaths were reviewed to look for sustained and delayed effects of the vitamin supplements. Hazard ratios for death by cancer, stroke and other causes were calculated for the four supplement combinations.

Contribution

Ten years after the end of the trial, participants who took factor D still had a 5% reduction in total mortality and 11% reduction in gastric cancer; these effects were concentrated among participants younger than 55 years. Esophageal cancer decreased 17% in participants younger than 55 years, but increased 14% in those older than 55 years.

Implications

Sustained benefits were associated with a combination of selenium, vitamin E, and beta-carotene supplementation. More subtle long-term effects were also observed for other vitamin supplements.

Limitations

It is not known which items in the supplement combination are responsible for the combined effect. Because the study was performed in a nutritionally deprived population with high rates of esophageal and gastric cancer, the findings might not be applicable to other populations. It is possible that the findings could be biased by a general improvement in nutrition among the participants over the postintervention period.

From the Editors

Since the conclusion of active trial treatment in 1991, follow-up has continued on all participants to collect data on cancer incidence and all-cause mortality. Here we report mortality results for the total 15.25 years of the trial and posttrial follow-up (through May 31, 2001) for the original a priori trial endpoints, that is, the effects of the intervention agents on esophageal and gastric cardia cancer mortality, and for all-cause mortality.

Subjects and Methods

Design of the Trial and Posttrial Follow-up

The design and conduct of the Linxian General Population NIT and its extended follow-up have been described elsewhere (4–6). In brief, participants were recruited in 1985 from four northern communes in Linxian, a rural county in Henan Province. Residents 40–69 years of age with no history of cancer or debilitating disease were eligible for this trial and were asked to enroll. In all, 29 584

subjects, 60% of those invited, were randomly assigned in the trial. These individuals were interviewed for medical history, family history of cancer, diet, and alcohol and tobacco consumption; were given a brief medical exam; and were asked to donate 10-mL blood sample.

The nine nutrients studied in this trial and their daily doses were retinol (5000 IU, as retinol palmitate), zinc (22.5 mg, as zinc oxide), riboflavin (3.2 mg), niacin (40 mg), ascorbic acid (120 mg), molybdenum (30 µg, as molybdenum yeast complex), selenium (50 µg, as selenium yeast), alpha-tocopherol (30 mg), and beta-carotene (15 mg). Doses ranged from one to two times US Recommended Daily Allowances. An independent study of each of these nutrients and vitamins, although desirable, was not practical. Therefore, these nine nutrients were combined into four regimens or factors: retinol and zinc (factor A); riboflavin and niacin (factor B); vitamin C and molybdenum (factor C); and selenium, vitamin E, and beta-carotene (factor D).

The trial had a one-half 2^4 fractional factorial design, so participants were randomly assigned by a computer-generated random numbers list to take one of eight combinations of the four factors. These eight intervention groups (treatment arms) were defined by the following combinations of factors: ABCD, AB, AC, AD, BC, BD, CD, or placebo. With this design, half the subjects received and half did not receive each of the four factors (eg, half received and half did not receive factor A).

Supplements were distributed in coded pill bottles from March 1, 1986, to May 1, 1991. Throughout the trial, the pill codes were kept in a secured file at the study data management center in the United States and were available only to the study data manager. During the trial period, village health workers visited participants monthly to deliver pills, assess pill compliance, and ascertain vital and disease status. Diagnostic materials (case records, pathology slides, and X-rays) for 85% of the cancer cases in the trial period were available and were reviewed by a panel of American and Chinese experts. In the subsequent 10 years posttrial, village health workers or study interviewers continued to contact participants monthly. For new cancer diagnoses and deaths, diagnostic materials were collected and cancer diagnoses were verified by the panel of American and Chinese experts (1991 to 1996) or senior Chinese diagnosticians from Beijing (1996–2001). Throughout the trial and the posttrial follow-up, case ascertainment was considered complete and loss to follow-up minimal ($n = 276$, or <1%). Outcomes for this study were based on data from the full 15.25 years of follow-up, from March 1, 1986 (the start of intervention), through May 31, 2001 (10 years after the intervention ended). Due to delayed ascertainment of outcomes, the number of deaths reported here for the intervention period is higher than that in our original report.

Compliance, measured by pill disappearance rates and biochemical measurements, was excellent. The overall pill disappearance rate was 93%, with no differences by treatment arm (range = 92% to 93%). Blood was analyzed in quarterly surveys of a sample of participants for retinol, beta-carotene, ascorbic acid, and glutathione reductase activation coefficient as a measure of riboflavin status, and during the trial period all values were consistently statistically significantly different between treated and untreated individuals (all P values < .001) (4,5).

The conduct of the Linxian General Population NIT was approved by the institutional review boards of the Cancer Institute of Chinese Academy of Medical Sciences and the US National Cancer Institute, and written informed consent was obtained from all participants for participation. The trial was registered as ClinicalTrials.gov number, NCT00342654.

Statistical Methods

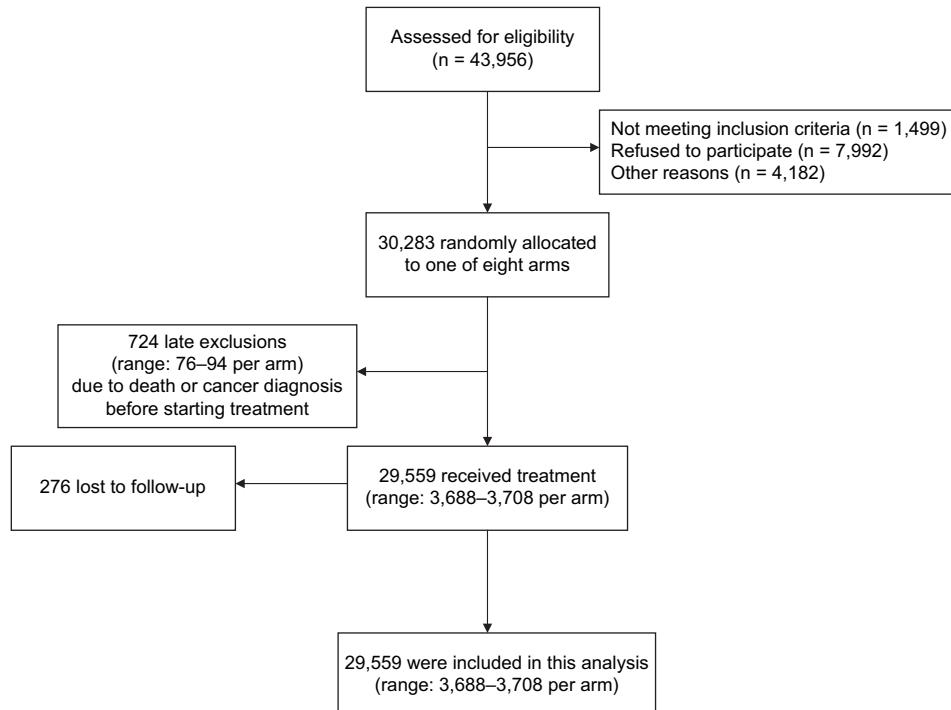
The main outcomes of this study were esophageal and gastric cardia cancer mortality and total mortality. Participants were censored at their last known follow-up date, date of death, or the administrative closure of follow-up for the study (May 31, 2001), whichever came first. The 15.25-year follow-up period was analyzed as a whole and in three 5-year periods: the trial period (March 1, 1986, to May 31, 1991) when the intervention agents were given and two other periods (June 1, 1991, to May 31, 1996, and June 1, 1996, to May 31, 2001) when active follow-up continued but no additional intervention was performed. Among the cancers, both esophageal squamous cell carcinoma and gastric cardia adenocarcinoma occur at epidemic rates in this population, share some etiologic risk factors, and before widespread use of endoscopy and biopsy, were diagnosed as a single disease referred to as “esophageal cancer” or “hard of swallowing disease” (7). Through 2001, esophageal adenocarcinoma was nonexistent in Linxian. We present data for the effects of the supplements on mortality of esophageal squamous cell carcinoma, gastric cardia adenocarcinoma, gastric noncardia adenocarcinoma, and the combination of esophageal and gastric cardia cancer (the original hard of swallowing disease).

We tabulated baseline frequencies and percentages of participants by demographics. We compared risk between those who received each factor and those who did not. In the analysis of factorial trials, this kind of analysis is known as “at the margins analysis”

and has the most power to examine the effect of each factor (8). Cox proportional hazards models were used to calculate hazard ratios (HRs) and 95% confidence intervals (95% CIs) for each factor, adjusting for the other three treatment factors, sex, age (continuous), and commune (four communes). These analyses were conducted on 29 559 of 29 584 initial study participants; 25 were lost to follow-up before the intervention began (Figure 1). Interactions between each of the factors and age group or sex were examined by including appropriate terms in the Cox models. This testing of interactions between each of the treatments (factors) and age and sex was planned a priori (9,10). The 55-year age cut point was chosen because it is the midpoint in the 40- to 69-year age distribution of the trial population. Kaplan-Meier estimates of cumulative event rates were plotted to compare time to death for each factor, for all subjects, and by sex and age group (11). In addition to “pure” risks determined from Cox models and Kaplan-Meier estimates, we also estimated selected “crude” risks without adjustment for covariates (ie, cumulative incidence), analogous to the approach of Fine and Gray (12). The pure cumulative risks slightly exceeded the crude cumulative risks, as expected (data not shown). Indeed, the estimated pure risks exceeded the crude risk by no more than 0.01 for total mortality, total cancer mortality, gastric cancer mortality, and esophageal cancer mortality. To estimate the calendar time-specific hazard ratios for the figures, we calculated smoothed (moving) hazard ratio estimates using a generalized additive model with a local regression (loess) smoother with span 0.4 and a test-based confidence band.

The assumption of a constant treatment hazard ratio across the three study periods was verified for each of the analyses by examining treatment by time period interactions. All *P* values are two-sided and *P* values less than .05 were considered statistically significant. Analyses were conducted using SAS version 9.1.3 service pack 4 (SAS Institute, Inc, Cary, NC); figures were produced

Figure 1. CONSORT flow diagram of the Linxian General Population Trial.



using S-Plus 6.2 for Windows and Sigma Plot 8.0 (Systat Software, Inc, San Jose, CA).

Results

Through May 31, 2001, there were 381954 total person-years of follow-up over a median of 15.25 years of observation. The baseline demographic characteristics, smoking and alcohol use, and family history of esophageal cancer for all subjects are shown in Table 1. As expected, there were no statistically significant differences between any of these baseline characteristics by treatment group assignments. Smoking prevalence was comparable and alcohol consumption was much less common than in the United States. A family history of esophageal cancer was substantially more common than in the West.

Total and cause-specific numbers of deaths for the entire study period and for the three 5-year periods are shown in Table 2. A total of 9727 deaths were reported during the 15.25 years of follow-up. Deaths due to cancer and cerebrovascular diseases each accounted for approximately one-third of the total deaths. The most common specific causes of death were cerebrovascular events ($n = 2984$), esophageal cancer ($n = 1515$), and gastric cancer ($n = 1199$). Total numbers of deaths were 2528 in the trial period (follow-up period 1), 3555 in follow-up period 2, and 3644 in follow-up period 3.

Adjusted hazard ratios (95% confidence intervals) for the associations between each intervention factor and total and cause-specific deaths for the entire study period and for each of the 5-year follow-up periods are also shown in Table 2. Subjects given factor A (retinol and zinc) had a marginally increased risk of total mortality for the total follow-up period ($HR = 1.04$, 95% CI = 1.00 to 1.09; $P = .035$) compared with subjects not given factor A. In the

Table 1. Baseline demographic characteristics, smoking and alcohol consumption, and family history of cancer

Characteristic	All participants (% of total)	Range for the eight treatment arms
No. of participants	29 559	3688–3708
Age group (y)		
<50	12 365 (42%)	42%
50–59	10 258 (35%)	34%–35%
≥60	6936 (23%)	23%–24%
Sex		
Female	16 378 (55%)	55%–56%
Male	13 181 (45%)	44%–45%
Smoking*,†		
Nonsmoker	20 613 (70%)	70%–71%
Smoker	8842 (30%)	29%–30%
Alcohol drinking†,‡		
Nondrinker	22 539 (77%)	76%–77%
Drinker	6915 (23%)	23%–24%
Family history of esophageal cancer		
No	20 800 (71%)	70%–72%
Yes	8651 (29%)	28%–30%

* Ever smoking cigarettes for 6 or more months.

† Data on smoking ($n = 104$), drinking ($n = 105$), and family history ($n = 108$) were not available for some subjects.

‡ Ever drinking any alcoholic beverage in the last 12 months.

factor A group, the hazard ratio point estimate for total deaths remained consistently higher than unity in all three follow-up periods, with period-specific estimates of 1.03, 1.05, and 1.05, respectively, although none of the period-specific estimates was statistically significant. This increased risk was mainly due to the effect of factor A on noncancer deaths: The hazard ratio for cerebrovascular deaths was 1.08 (95% CI = 1.00 to 1.16; $P = .045$) and for other causes of death was 1.06 (95% CI = 0.99 to 1.13; $P = .088$). The hazard ratio point estimates associated with these causes of death remained higher than unity (although not statistically significant) for all three follow-up periods. Neither sex (interaction $P = .372$) nor age group (interaction $P = .757$) statistically significantly modified the effect of factor A on total mortality. Factor A was also associated with period-specific protective effects for other cancer deaths in period 2 ($HR = 0.76$, 95% CI = 0.58 to 1.00; $P = .050$) and for cardia cancer deaths in period 3 ($HR = 0.73$, 95% CI = 0.57 to 0.93; $P = .012$), although neither effect was consistent in the other time periods.

Intervention with factor B (riboflavin and niacin) was not associated with total deaths in the overall ($HR = 0.98$, 95% CI = 0.94 to 1.02; $P = .318$) or period-specific analyses when compared with lack of factor B, and hazard ratio point estimates remained close to 1 in all three follow-up periods (Table 2). This factor was not associated with cause-specific deaths overall or in any follow-up period, except for a marginally statistically significant protective effect on total cancer deaths in follow-up period 2 ($HR = 0.89$, 95% CI = 0.80 to 1.00; $P = .043$). The effect of factor B on total deaths was not statistically significantly modified by sex (interaction $P = .177$) or age group (interaction $P = .109$).

Factor C (vitamin C and molybdenum) was not associated with total deaths in overall ($HR = 0.97$, 95% CI = 0.94 to 1.01; $P = .177$) or period-specific analyses when compared with lack of factor C (Table 2). However, this factor was inversely associated with overall risk of cerebrovascular deaths ($HR = 0.92$, 95% CI = 0.86 to 0.99; $P = .023$), an effect not seen during the trial period but which became apparent during later follow-up. There were no statistically significant associations between factor C and total cancer mortality, esophageal or gastric cancer mortality, or other causes of mortality in the overall or period-specific analyses, although a marginally statistically insignificant increase in the combined esophageal and cardia cancer mortality endpoint was noted in the overall analysis ($HR = 1.08$, 95% CI = 1.00 to 1.17; $P = .052$). Sex did not modify the effect of factor C on total mortality (interaction $P = .290$), but there was a statistically significant interaction with age (interaction $P = .003$) such that younger (<55 years at random assignment) participants had reduced risk ($HR = 0.89$, 95% CI = 0.83 to 0.96; $P = .001$) and older (≥ 55 years at random assignment) participants were unaffected ($HR = 1.01$, 95% CI = 0.97 to 1.06; $P = .607$). Statistically significant age group interactions were also seen for factor C with total cancer mortality and esophageal cancer mortality; younger patients were unaffected but older participants had elevated hazard ratios (Table 3).

Factor D (selenium, vitamin E, and beta-carotene) reduced total mortality ($HR = 0.95$, 95% CI = 0.91 to 0.99; $P = .009$; reduction in cumulative mortality from 33.62% in the no-factor D group to 32.19% in the factor D group). Moving hazard ratio curves (Figure 2)

Table 2. Hazard ratios and 95% confidence intervals for death by cause and vitamin and mineral treatment factors*

Cause of death by study period	N	Vitamin and mineral treatment factor†,‡			
		Factor A (vs no factor A), HR (95% CI)	Factor B (vs no factor B), HR (95% CI)	Factor C (vs no factor C), HR (95% CI)	Factor D (vs no factor D), HR (95% CI)
Total study period (1986–2001)					
Total deaths	9727	1.04 (1.00 to 1.09)‡	0.98 (0.94 to 1.02)	0.97 (0.94 to 1.01)	0.95 (0.91 to 0.99)‡
Cancer	3242	1.00 (0.93 to 1.07)	0.96 (0.90 to 1.03)	1.04 (0.97 to 1.11)	0.95 (0.89 to 1.02)
Esophageal	1515	1.07 (0.97 to 1.19)	0.93 (0.84 to 1.02)	1.09 (0.98 to 1.20)	1.01 (0.91 to 1.11)
Gastric	1199	0.97 (0.87 to 1.09)	0.99 (0.88 to 1.10)	1.05 (0.94 to 1.18)	0.89 (0.79 to 1.00)‡
Cardia	873	0.95 (0.84 to 1.09)	1.00 (0.87 to 1.14)	1.07 (0.94 to 1.23)	0.89 (0.78 to 1.01)
Noncardia	326	1.02 (0.82 to 1.27)	0.95 (0.88 to 1.03)	0.99 (0.80 to 1.24)	0.90 (0.72 to 1.12)
Esophageal and cardia	2388	1.03 (0.95 to 1.11)	0.95 (0.88 to 1.03)	1.08 (1.00 to 1.17)	0.96 (0.89 to 1.04)
Other cancer	528	0.87 (0.73 to 1.03)	1.02 (0.86 to 1.21)	0.89 (0.75 to 1.05)	0.94 (0.79 to 1.11)
Cerebrovascular	2984	1.08 (1.00 to 1.16)‡	1.02 (0.95 to 1.10)	0.92 (0.86 to 0.99)‡	0.98 (0.91 to 1.05)
Other	3501	1.06 (0.99 to 1.13)	0.96 (0.90 to 1.03)	0.96 (0.90 to 1.03)	0.92 (0.86 to 0.98)‡
Trial period (1986–1991)					
Total deaths	2528	1.03 (0.95 to 1.11)	0.99 (0.91 to 1.07)	1.00 (0.93 to 1.08)	0.91 (0.84 to 0.99)‡
Cancer	992	0.99 (0.87 to 1.12)	1.03 (0.91 to 1.16)	1.04 (0.92 to 1.18)	0.91 (0.81 to 1.04)
Esophageal	448	0.97 (0.81 to 1.17)	0.90 (0.75 to 1.08)	1.06 (0.88 to 1.28)	1.00 (0.84 to 1.21)
Gastric	406	1.05 (0.86 to 1.27)	1.08 (0.89 to 1.31)	1.06 (0.87 to 1.28)	0.81 (0.66 to 0.98)‡
Cardia	297	1.15 (0.92 to 1.45)	1.07 (0.85 to 1.34)	1.10 (0.88 to 1.39)	0.83 (0.66 to 1.04)
Noncardia	109	0.81 (0.56 to 1.18)	1.09 (0.75 to 1.59)	0.94 (0.65 to 1.37)	0.75 (0.51 to 1.10)
Esophageal and cardia	745	1.04 (0.90 to 1.20)	0.96 (0.84 to 1.11)	1.08 (0.93 to 1.25)	0.93 (0.81 to 1.07)
Other cancer	138	0.86 (0.62 to 1.20)	1.37 (0.98 to 1.93)	0.91 (0.65 to 1.28)	0.96 (0.69 to 1.35)
Cerebrovascular	643	1.06 (0.91 to 1.24)	0.93 (0.80 to 1.09)	0.99 (0.85 to 1.16)	0.90 (0.77 to 1.05)
Other	893	1.05 (0.92 to 1.20)	0.98 (0.86 to 1.12)	0.97 (0.85 to 1.10)	0.92 (0.81 to 1.05)
Follow-up period 2 (1991–1996)					
Total deaths	3555	1.05 (0.98 to 1.12)	0.97 (0.91 to 1.03)	0.95 (0.89 to 1.02)	0.99 (0.93 to 1.06)
Cancer	1245	1.02 (0.91 to 1.14)	0.89 (0.80 to 1.00)‡	1.04 (0.93 to 1.16)	0.99 (0.89 to 1.11)
Esophageal	594	1.15 (0.98 to 1.35)	0.91 (0.77 to 1.07)	1.04 (0.89 to 1.22)	1.05 (0.89 to 1.23)
Gastric	436	1.00 (0.83 to 1.21)	0.91 (0.75 to 1.10)	1.11 (0.92 to 1.34)	0.95 (0.79 to 1.14)
Cardia	321	0.99 (0.80 to 1.24)	0.90 (0.72 to 1.12)	1.11 (0.89 to 1.38)	0.96 (0.77 to 1.20)
Noncardia	115	1.02 (0.71 to 1.47)	0.94 (0.65 to 1.35)	1.13 (0.78 to 1.63)	0.91 (0.63 to 1.31)
Esophageal and cardia	915	1.09 (0.96 to 1.24)	0.90 (0.79 to 1.03)	1.06 (0.94 to 1.21)	1.02 (0.89 to 1.16)
Other cancer	215	0.76 (0.58 to 1.00)	0.81 (0.62 to 1.07)	0.90 (0.69 to 1.18)	0.93 (0.71 to 1.22)
Cerebrovascular	1037	1.07 (0.95 to 1.21)	1.11 (0.98 to 1.26)	0.89 (0.79 to 1.01)	1.10 (0.97 to 1.24)
Other	1273	1.05 (0.94 to 1.17)	0.94 (0.84 to 1.05)	0.92 (0.83 to 1.03)	0.90 (0.81 to 1.01)
Follow-up period 3 (1996–2001)					
Total deaths	3644	1.05 (0.99 to 1.12)	0.99 (0.92 to 1.05)	0.97 (0.91 to 1.04)	0.94 (0.88 to 1.00)‡
Cancer	1005	0.98 (0.87 to 1.11)	0.99 (0.86 to 1.13)	1.04 (0.92 to 1.18)	0.94 (0.83 to 1.06)
Esophageal	473	1.08 (0.90 to 1.29)	0.97 (0.81 to 1.16)	1.17 (0.98 to 1.40)	0.95 (0.80 to 1.14)
Gastric	357	0.86 (0.70 to 1.06)	0.99 (0.80 to 1.21)	0.98 (0.79 to 1.20)	0.91 (0.74 to 1.13)
Cardia	255	0.73 (0.57 to 0.93)‡	1.05 (0.82 to 1.34)	1.00 (0.78 to 1.28)	0.86 (0.67 to 1.10)
Noncardia	102	1.30 (0.88 to 1.93)	0.84 (0.57 to 1.24)	0.91 (0.62 to 1.35)	1.06 (0.72 to 1.57)
Esophageal and cardia	728	0.94 (0.81 to 1.09)	1.00 (0.86 to 1.15)	1.11 (0.96 to 1.28)	0.92 (0.80 to 1.06)
Other cancer	175	1.02 (0.75 to 1.37)	1.08 (0.80 to 1.45)	0.85 (0.63 to 1.15)	0.93 (0.69 to 1.25)
Cerebrovascular	1304	1.08 (0.97 to 1.20)	1.00 (0.90 to 1.12)	0.90 (0.81 to 1.01)	0.94 (0.84 to 1.05)
Other	1335	1.08 (0.97 to 1.20)	0.96 (0.86 to 1.07)	1.00 (0.89 to 1.11)	0.93 (0.84 to 1.04)

* HR = hazard ratio; CI = confidence interval.

† Factor A = vitamin A (5000 IU/d) + zinc (22.5 mg/d); factor B = riboflavin (3.2 mg/d) + niacin (40 mg/d); factor C = ascorbic acid (120 mg/d) + molybdenum (30 µg/d); factor D = selenium (50 µg/d) + vitamin E (30 mg/d) + beta-carotene (15 mg/d). HRs (95% CIs) adjusted for the other three treatments factors, age (continuous), sex, and commune (four communes).

‡ P < .05.

show the smoothed hazard ratios remained less than 1.0 for most of the 15.25 years of observation, indicating a protective effect during most of this period. The estimated hazard ratios for the three follow-up periods were 0.91 (95% CI = 0.84 to 0.99; $P = .023$), 0.99 (95% CI = 0.93 to 1.06; $P = .723$), and 0.94 (95% CI = 0.88 to 1.00; $P = .046$), respectively (Table 2). The hazard ratios for the three major group causes of mortality for the full 15.25 years of follow-up

were also less than 1.0 but were not uniformly statistically significant: 0.95 (95% CI = 0.89 to 1.02; $P = .148$) for cancer, 0.98 (95% CI = 0.98 to 1.05; $P = .613$) for cerebrovascular events, and 0.92 (95% CI = 0.86 to 0.98; $P = .013$) for other causes. The majority of the overall effect was attributed to a reduced risk of death from gastric cancer and causes of death other than cancer or cerebrovascular diseases.

Table 3. Hazard ratios for total and cause-specific mortality by age group and vitamin and mineral treatment factor for the total 15½ year follow-up for endpoints and factors with statistically significant or close to statistically significant interactions*

Cause of death and treatment factor†	Age group‡		<i>P</i> for age group interaction
	<55 years at random assignment, HR (95% CI)	≥55 years at random assignment, HR (95% CI)	
Total mortality (n = 9727)			
Factor C (vs no factor C)	0.89 (0.83 to 0.96)	1.01 (0.97 to 1.06)	.002
Factor D (vs no factor D)	0.88 (0.82 to 0.95)	0.98 (0.93 to 1.03)	.023
Total cancer mortality (n = 3242)			
Factor C (vs no factor C)	0.93 (0.83 to 1.03)	1.12 (1.02 to 1.22)	.016
Factor D (vs no factor D)	0.85 (0.76 to 0.95)	1.02 (0.94 to 1.12)	.019
Esophageal cancer mortality (n = 1515)			
Factor C (vs no factor C)	0.96 (0.81 to 1.12)	1.18 (1.04 to 1.35)	.058
Factor D (vs no factor D)	0.83 (0.71 to 0.98)	1.14 (1.00 to 1.30)	.003

* HR = hazard ratio; CI = confidence interval.

† Factor C = ascorbic acid (120 mg/d) + molybdenum (30 µg/d); factor D = selenium (50 µg/d) + vitamin E (30 mg/d) + beta-carotene (15 mg/d).

‡ HRs (95% CIs) adjusted for the other three treatments factors, age (continuous), sex, and commune (four communes).

The effect of factor D on total mortality was not modified by sex (interaction $P = .843$), but was modified by age group (interaction $P = .024$). Total and age-specific cumulative event and moving hazard ratio curves presented in Figure 2 show cumulative mortality by factor D. The hazard ratios were 0.95 (95% CI = 0.91 to 0.99; $P = .009$) for all study subjects, 0.88 (95% CI = 0.82 to 0.95; $P < .001$)

for subjects younger than 55 years old at study entry, and 0.98 (95% CI = 0.94 to 1.03; $P = .367$) for subjects 55 or older at study entry (Table 3). Therefore, virtually the entire effect of factor D on total mortality was due to effects in individuals younger than 55 years.

Similar cumulative event rate curves and moving hazard ratio curves for total cancer mortality (Figure 3), total gastric cancer

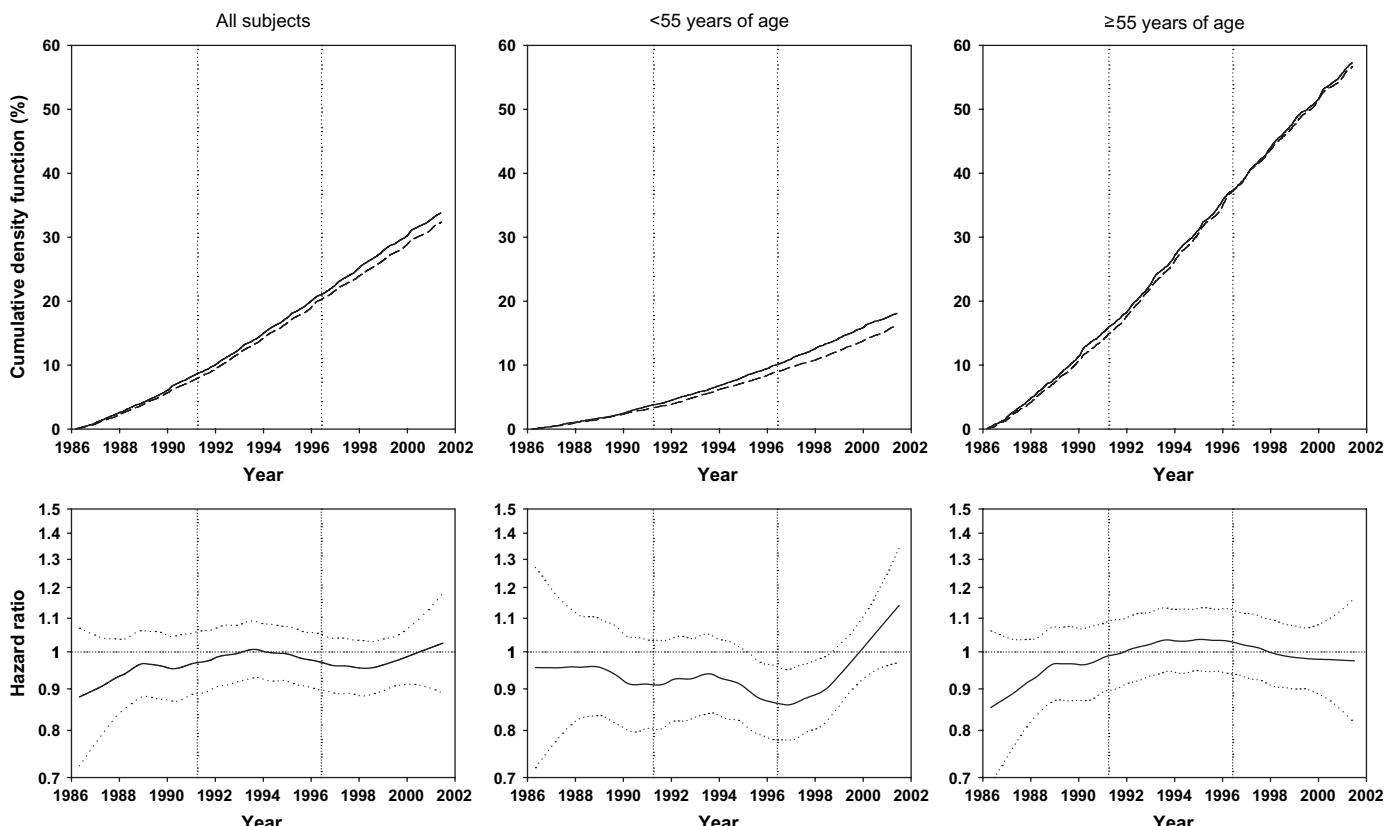


Figure 2. Effects of factor D (a combination of 50 µg selenium, 30 mg vitamin E, and 1.5 mg beta-carotene daily) on total mortality for all subjects, subjects younger than 55 years at random assignment, and subjects 55 years and older at random assignment, as shown by cumulative event rates (cumulative density function, as percentages) from

Kaplan-Meier estimates and smoothed (moving) hazard ratio curves. In Kaplan-Meier-based curves, **dashed lines** represent participants who received factor D; **solid lines** represent participants who did not receive factor D. In smoothed hazard ratio curves, **dotted lines** represent 95% confidence intervals around the hazard ratios.

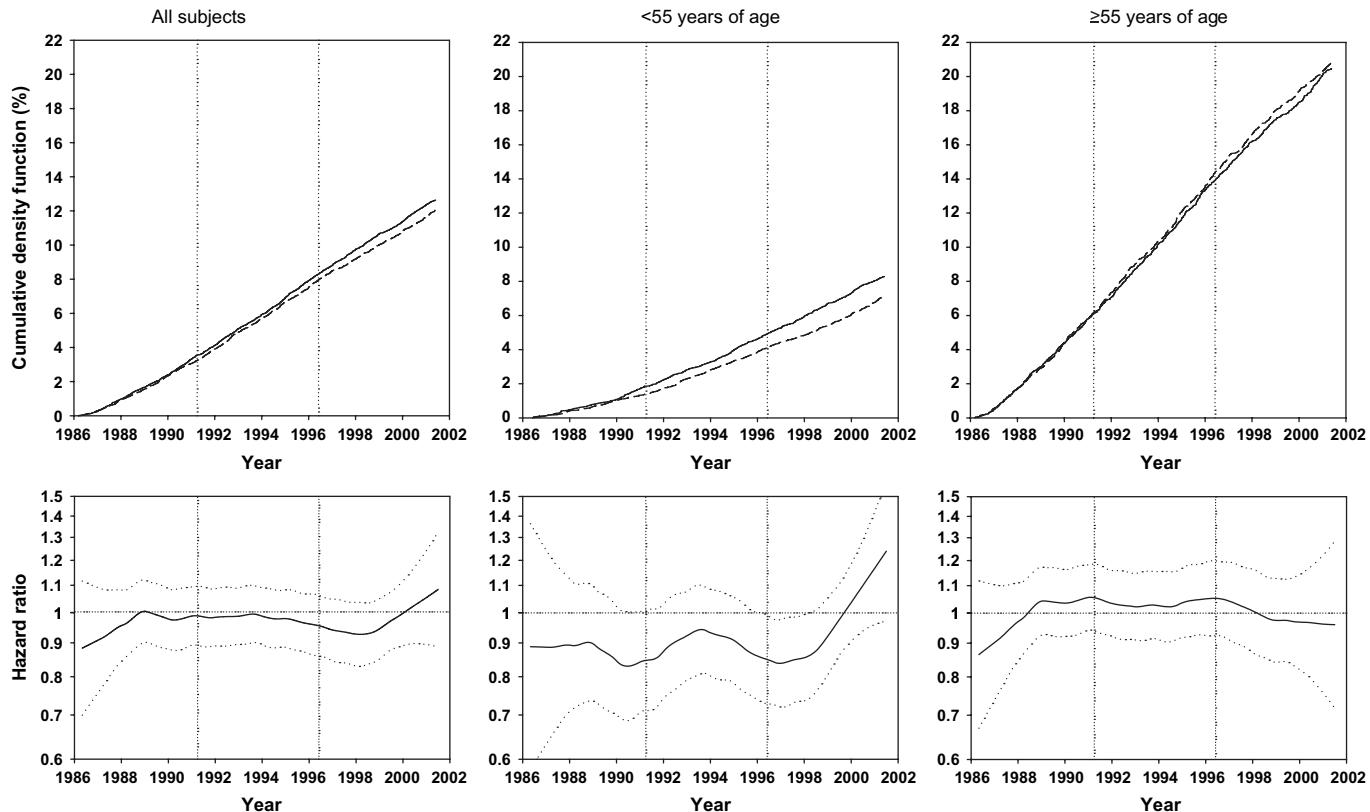


Figure 3. Effects of factor D on total cancer mortality for all subjects, subjects younger than 55 years at random assignment, and subjects 55 years and older at random assignment, as shown by cumulative event rates (as percentages) from Kaplan-Meier estimates and smoothed (moving) hazard

ratio curves. In Kaplan-Meier-based curves, **dashed lines** represent participants who received factor D; **solid lines** represent participants who did not receive factor D. In smoothed hazard ratio curves, **dotted lines** represent 95% confidence intervals around the hazard ratios.

mortality (Figure 4), and esophageal cancer mortality (Figure 5) all show that the effects of factor D were predominantly or exclusively in subjects younger than 55 years. The hazard ratios for total cancer mortality associated with factor D were 0.95 (95% CI = 0.89 to 1.02; $P = .148$) for all subjects, 0.85 (95% CI = 0.76 to 0.95; $P = .003$) for those younger than 55, and 1.02 (95% CI = −0.94 to 1.12; $P = .976$) for those 55 or older (Table 3). Corresponding hazard ratios for gastric cancer mortality were 0.89 (95% CI = 0.79 to 1.00; $P = .043$), 0.83 (95% CI = 0.69 to 1.00; $P = .046$), and 0.93 (95% CI = 0.80 to 1.07; $P = .307$). Cumulative crude gastric cancer mortality for all subjects was 4.28% in the no-factor D group compared with 3.84% in the factor D group, an overall reduction of 0.44%. For esophageal cancer mortality, effect modification by age was even more pronounced. There was no overall association between factor D and esophageal cancer mortality for all subjects ($HR = 1.01$, 95% CI = .91 to 1.11; $P = .905$); however, in subjects younger than 55 years, factor D esophageal cancer mortality decreased ($HR = 0.83$, 95% CI = 0.71 to 0.98; $P = .025$), whereas in individuals aged 55 years or older, it increased ($HR = 1.14$, 95% CI = 1.00 to 1.30; $P = .047$) (Table 3).

Discussion

The initial results of the Linxian General Population NIT, published in 1993, showed no association between factors A, B, or C and overall mortality, total cancer mortality, or mortality from

esophageal or gastric cancers (5). However, factor D, which included selenium, vitamin E, and beta-carotene, statistically significantly reduced total mortality, total cancer mortality, and mortality from gastric cancer (5). An important question remained, however, whether the preventive effects of factor D would last beyond the trial period. The results of the continued follow-up show that hazard ratios, as indicated by moving hazard ratio curves, remained less than 1.0 for each of these endpoints for the majority of the follow-up period; 10 years after completion of the trial, the group that received factor D still showed a 5% reduction in total mortality and an 11% reduction in gastric cancer mortality. Overall, one in 70 people who took factor D was spared death from all causes, and one in 227 was spared death from gastric cancer.

Stratification of results by sex and age was planned a priori. There were no statistically significant interactions with sex. However, when stratified by age, factor D had a strong protective effect in individuals younger than 55 years but almost no effect on subjects aged 55 years or older. This pattern was seen consistently for total mortality, total cancer mortality, gastric cancer mortality, and esophageal cancer mortality. Indeed, the effect of factor D on esophageal cancer was reversed by age, showing a protective effect for younger but a harmful effect for older individuals.

Because this trial provided selenium, vitamin E, and beta-carotene as one factor, it was not possible to disentangle the effects of these three supplements. However, observational case-cohort studies using subjects in this cohort and patients with upper

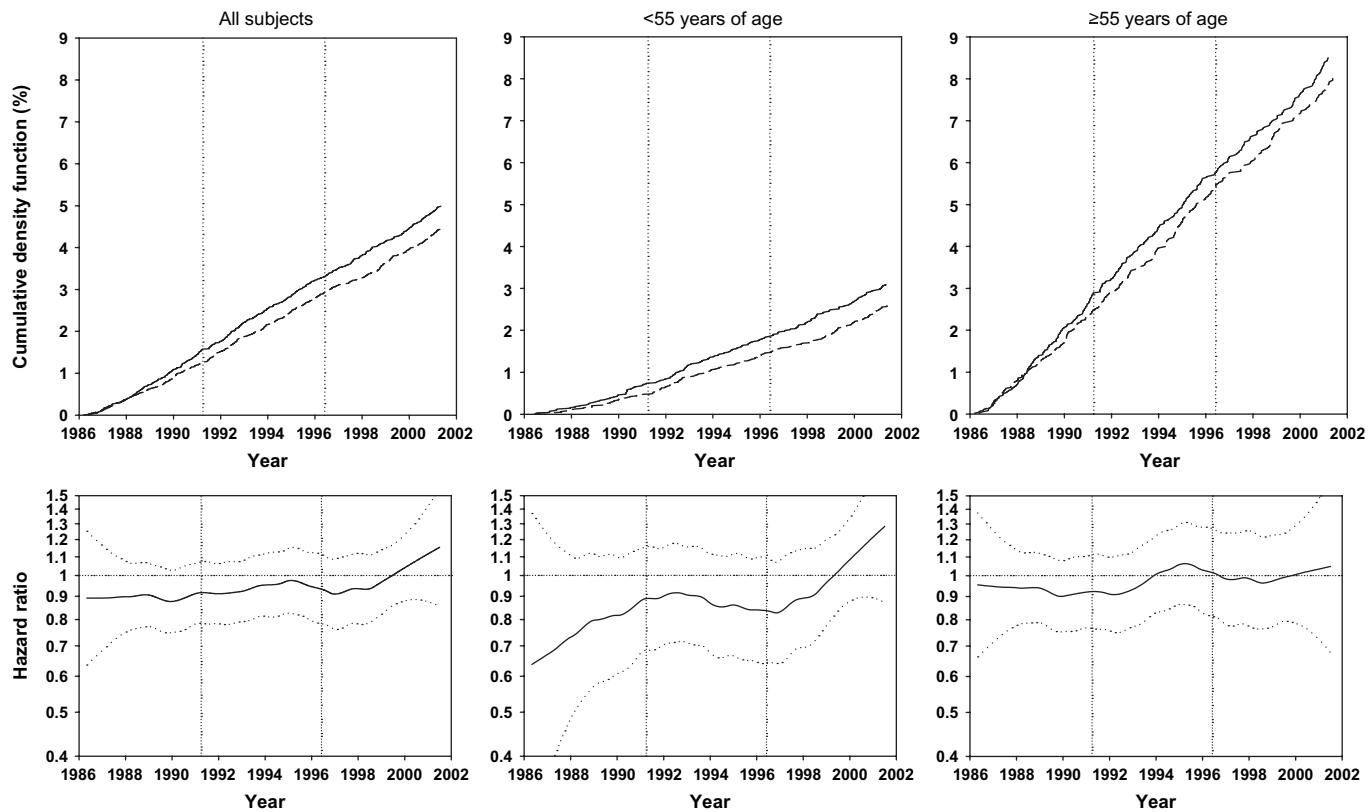


Figure 4. Effects of factor D on total gastric cancer mortality for all subjects, subjects younger than 55 years at random assignment, and subjects 55 years and older at random assignment, as shown by cumulative event rates (cumulative density function, as percentages) from Kaplan-Meier estimates and smoothed (moving) hazard

ratio curves. In Kaplan-Meier-based curves, **dashed lines** represent participants who received factor D; **solid lines** represent participants who did not receive factor D. In smoothed hazard ratio curves, **dotted lines** represent 95% confidence intervals around the hazard ratios.

gastrointestinal tract cancers that developed during the intervention period showed inverse associations between risk of esophageal cancer and baseline serum levels of selenium and alpha-tocopherol, but not beta-carotene (13–15). Higher baseline serum selenium also was associated with reduced risk of gastric cardia cancer (13). These results suggest that the protective effects seen in the randomized trial were due to the selenium and vitamin E components. In a subcohort of 1103 subjects from this trial followed through May 31, 2001, higher baseline serum selenium levels also were associated with statistically significant reductions in esophageal and gastric cardia cancer mortality (16). A separate randomized controlled trial in Linxian (17) gave further support for a preventive effect of selenium in subjects with preexisting esophageal squamous dysplasia, the precursor lesion of esophageal squamous cell carcinoma. Compared with control subjects, those with mild dysplasia who received 10 months of daily supplementation with 200 µg of selenomethionine were more likely to have regression and less likely to have progression of their esophageal squamous dysplasia.

In addition to evaluating the durability of the beneficial effects observed during the trial period for factor D, we also evaluated other postintervention events in this trial to look for late effects, and several were noted. When the full 15.25 years of follow-up were considered, nutritional supplementation with factor A (vitamin A and zinc) was associated with increased total mortality, mainly due to an increase in stroke deaths among subjects given

factor A compared with those who were not given factor A, whereas supplementation with factor C (vitamin C and molybdenum) was associated with a decrease in stroke deaths and with a slight increase in esophageal/cardia cancer deaths.

Increased mortality among factor A recipients was not expected, given the low retinol levels of the population at the start of the trial, the modest doses of both vitamin A and zinc, and the generally null or protective results from previous observational studies in this cohort relating high serum retinol and tissue zinc concentrations to various cancer endpoints (15,18). In fact, a previous analysis of stroke (the main contributor to the observed increase in total mortality) in this trial showed a protective effect for persons who took the combination of factor A and factor D (HR = 0.71, 95% CI = 0.50 to 1.00, for group AD vs placebo) (19).

We were surprised that factor C, a combination of vitamin C and molybdenum, appeared to be associated with increased risk for the combined esophageal and cardia cancer endpoint, given the well-known role of vitamin C as an antioxidant and inhibitor of carcinogenic N-nitroso compound production in the stomach (20). Furthermore, many epidemiological studies have shown reduced risk of these cancers in persons with high consumption of fruits and vegetables and rich sources of vitamin C, and the only prospective study of plasma vitamin C and gastric cancer showed a protective effect for high concentrations (21). No similar prospective studies of plasma vitamin C values in esophageal cancer are known.

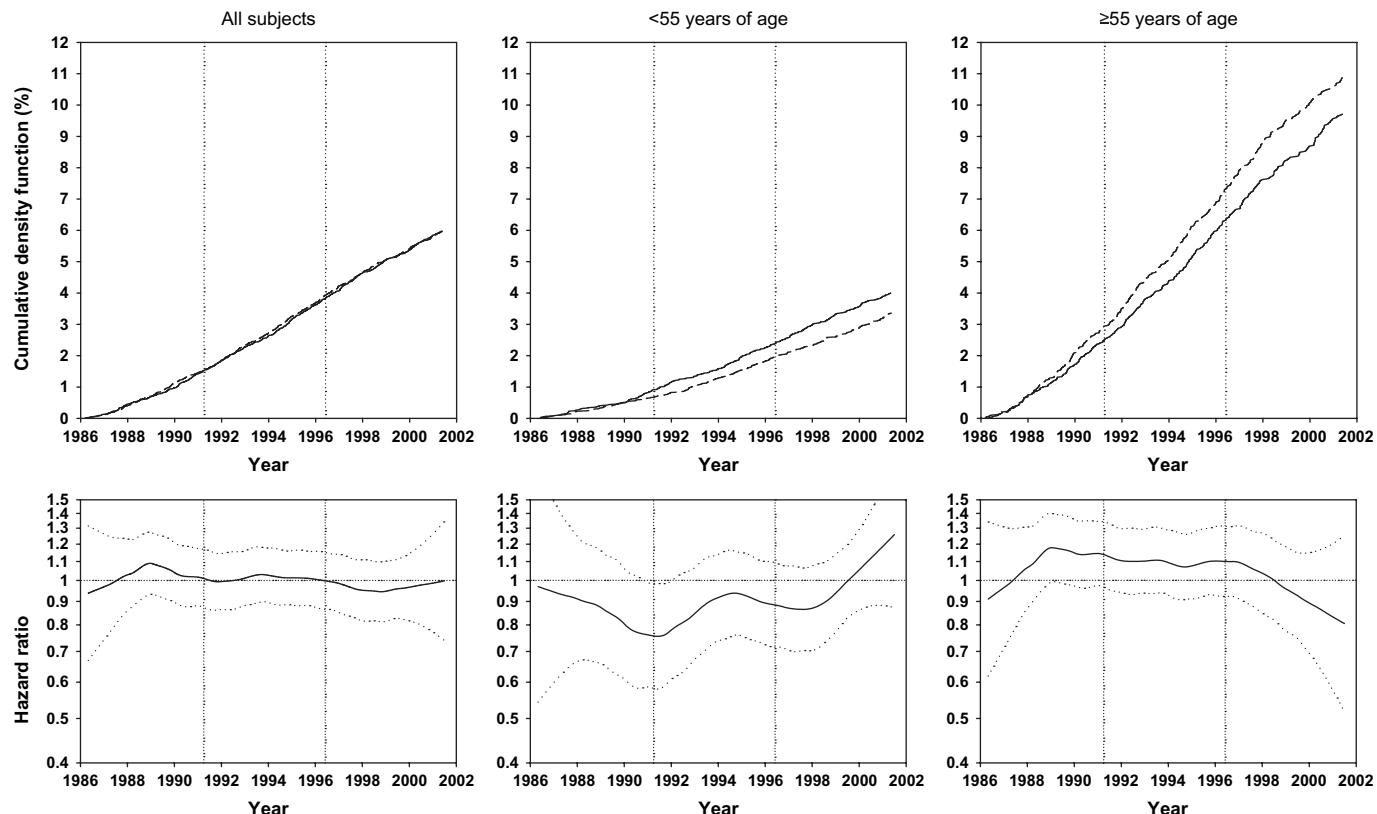


Figure 5. Effects of factor D on esophageal cancer mortality for all subjects, subjects younger than 55 years old at random assignment, and subjects 55 years and older at random assignment, as shown by cumulative event rates (cumulative density function, as percentages) from Kaplan-Meier estimates and smoothed (moving) hazard ratio

curves. In Kaplan-Meier-based curves, **dashed lines** represent participants who received factor D; **solid lines** represent participants who did not receive factor D. In smoothed hazard ratio curves, **dotted lines** represent 95% confidence intervals around the hazard ratios.

The decreased risk of stroke among trial participants who received factor C was not wholly unexpected. Higher plasma vitamin C levels have been associated with reduced risk of stroke in prospective epidemiological studies (22–24), although randomized trials that have included vitamin C as part of an antioxidant vitamin treatment arm have not shown any effect (25,26). There are no data on molybdenum and stroke.

The effects of vitamin and mineral supplements on reduction of total mortality and cancer mortality have been heavily debated over the past 25 years. The results of this study need to be interpreted in the context of other trials of vitamin and mineral supplementation. By the 1980s, it was established that antioxidants could quench free oxygen radicals and potentially reduce the risk of cancer by preventing DNA damage by these radicals. Observational epidemiological studies showed inverse associations between cancer incidence and dietary intake of several vitamins and minerals, but more definitive evidence awaited the completion of randomized trials (27). It was generally assumed that prescribing pills would be a more convenient and acceptable way to prevent cancer than proscribing carcinogens. These facts and assumptions motivated the design and conduct of the first generation of randomized controlled cancer prevention trials, including the Linxian General Population NIT, to reduce cancer risk using vitamins and minerals. The results of the largest and most informative of these trials (ie, those with more than 10 000 participants) were often contrary to the initial expectations. Beta-carotene supplementation increased

total mortality in both the Alpha-Tocopherol, Beta-Carotene (ATBC) Cancer Prevention Study (28) and the beta-CAratene and Retinol Efficacy Trial (CARET) Study (29), whereas no mortality benefit for beta-carotene was seen in either The Physicians' Health Study (PHS) (30) or the Women's Health Study (31). The most likely explanation for the unexpected findings from these four large trials conducted in the West is a mismatch of the design of the trials with the population attributes: each of these trials tested pharmacological doses of micronutrients in already well-nourished populations (32).

A recent meta-analysis of randomized trials of antioxidant supplements for prevention of cancer and other diseases (33) combined the results of these large-scale studies with many smaller studies and concluded that treatment with beta-carotene, vitamin A, or vitamin E likely increased total mortality, and the effect of vitamin C or selenium on total mortality needed further study. The results of this study agree in part with the meta-analysis conclusions. In this trial, factor A (which included vitamin A) increased mortality, factor C (which included vitamin C) was not associated with overall mortality, and factor D (which included selenium) decreased mortality. However, beta-carotene and vitamin E, two supplements that were associated with increased mortality risk in the meta-analysis, were in factor D, which reduced mortality in this trial. Several potential explanations exist for these apparently discrepant results. First, the protective effect of selenium may have been stronger than the possible deleterious effects of beta-carotene

and vitamin E in this trial, so the overall effect of factor D was beneficial. This hypothesis is supported by medium-sized trials that have shown beneficial effects for selenium in reducing mortality and cancer risk (34–36).

A second possible explanation for these discrepancies is that baseline nutritional status of the populations studied influenced the supplementation effects. The people of Linxian are nutritionally deficient (3,37), so vitamin and mineral supplements may be more beneficial to them than to other populations that have been studied. The results of the Dysplasia NIT (38), a medium-sized randomized nutrition intervention trial that was conducted among subjects with cytologically diagnosed esophageal squamous dysplasia in Linxian at the same time as the General Population NIT, showed that supplementation with 26 minerals and vitamins was associated with a non-statistically significant 7% reduction in mortality risk. Results from the Nutritional Prevention of Cancer Trial showed that the benefits for selenium supplementation on total cancer mortality (39) and the development of prostate cancer (36) were essentially limited to participants with lower selenium levels at the start of the trial. Further support for this hypothesis comes from the data of the meta-analysis of antioxidant supplement trials and total mortality itself (33). We classified the 68 study populations included in this analysis as Western ($n = 58$), East Asian ($n = 8$), or other ($n = 2$) and found statistically significant heterogeneity between the results of the studies performed in Western and East Asian populations. The Western studies had a combined odds ratio (OR) of 1.04 (95% CI = 1.01 to 1.06) and the East Asian studies had a combined OR of 0.92 (95% CI = 0.84 to 1.02) (χ^2 for heterogeneity $P = .02$). Because nearly all of the events in the East Asian group came from Linxian, which we know has borderline or deficient nutrition, this difference in meta-analysis results may well reflect differences in the baseline nutritional status of the populations evaluated.

A third possible explanation for the heterogeneity of results observed among studies that have evaluated the association of vitamin supplements and total mortality or gastrointestinal cancer risk is effect modification by the stage of disease at study entry. Our results show that only individuals younger than 55 years benefited from factor D. This result may indicate greater benefit earlier in the course of carcinogenesis and is consistent with a “point of no return,” beyond which supplementation with vitamins is not useful and may be harmful, preferentially benefiting the developing tumor more than the host. This hypothesis may help explain why observational studies, which reflect long-term intake of vitamins and vitamin-containing fruits and vegetables, have usually shown beneficial associations, whereas trials, which have largely been conducted in older patients, have sometimes shown harmful effects from vitamin interventions.

Participants in the ATBC and CARET studies, in addition to being older than those in the Linxian general population trial (ages 50–69 years for ATBC and 45–74 years for CARET), were heavy smokers and some were exposed to asbestos, both powerful carcinogenic exposures that may have put them beyond the point in the disease process that they could benefit from supplements. Detailed analyses of both of these studies have shown that the increased risk associated with vitamin use was almost exclusively seen in current (as opposed to former) smokers (40) and in those who smoked

most (41). In contrast, the PHS study, which included fewer than 10% smokers, showed no adverse effect of beta-carotene (30).

In addition to this report, three other cancer prevention nutrition intervention trials have reported results from continued follow-up after the termination of intervention. Follow-up of the participants of the ATBC Study for up to 8 years after the end of the intervention showed that both the harmful effects of beta-carotene (ie, increased total mortality and lung cancer incidence) and the beneficial effect of vitamin E (ie, decreased prostate cancer incidence) disappeared, albeit slowly (42). However, analyses of cerebral infarcts (80% of all strokes) among vitamin E recipients in the ATBC Study showed reversed effects during the trial (relative risk [RR] = 0.86, 95% CI = 0.75 to 0.99) (43) and the 6 years post-trial (RR = 1.13, 95% CI = 1.00 to 1.27) (44). After 6 years of postintervention follow-up in the CARET study, the relative risk of total mortality remained greater than 1.0, but this elevated risk diminished and was no longer statistically significant (45). Lung cancer mortality, however, was still statistically significantly increased. The Calcium Polyp Prevention Trial reported that the protective effect of calcium supplementation on colorectal adenoma recurrence found during the trial period (RR = 0.81, 95% CI = 0.74 to 0.98) (46) continued up to 5 years after supplementation ended and was, if anything, stronger after than during the intervention itself (RR = 0.63, 95% CI = 0.46 to 0.87) (47).

Durability of cancer prevention effects after cessation of intervention has also been observed with nonnutritional agents. In fact, the most consistent example of a sustained cancer prevention effect reported to date from any cancer prevention agent tested in trials is for tamoxifen in the primary prevention of breast cancer. Posttrial follow-up from three tamoxifen trials (48–50) consistently found benefit after the conclusion of active treatment, and in one trial (50), statistically significant reduction in risk (among patients with estrogen receptor-positive tumors) was seen only after treatment had ceased.

This study has several strengths. It was a randomized double-blind design and had excellent compliance and long-term follow-up with virtually complete ascertainment of cases in a well-defined population.

This study also has limitations. Interventions with factors containing multiple agents do not allow evaluation of the effects of individual agents alone, nor were we able to evaluate more than one dose for each of the agents supplemented. The people of Linxian are deficient in many micronutrients, which may limit the generalizability of these results to well-nourished populations. If the protective effects of this study are due to replacement of essential nutrients in a nutritionally deprived population, then similar interventions might be useful in similarly deprived populations in the West, including the United States, although populations with low rates of esophageal and gastric cancer mortality are unlikely to avert as many deaths as high-rate populations such as that in Linxian. Finally, the smoothed hazard ratios that we presented were intended to provide an alternative visual representation of the effects at specific points in time and to complement the cumulative view offered by the Kaplan-Meier curves. These smoothed hazard ratios should be interpreted with caution, however, because the confidence intervals around these curves nearly always include 1.0. Thus, such curves are affected by the play of chance and may be

biased by choice of smoothing parameters, edge effects, and other factors.

It should be noted that the follow-up period occurred during a time of dramatic economic progress in China as a whole. Although documented improvements in dietary intakes in Linxian during follow-up were modest (37), more substantial undocumented changes almost certainly occurred. Effects of dietary improvements should have been evenly distributed across all participants in the various randomized treatment groups in the trial. Thus, if the effects of the supplements and the dietary micronutrient intake are additive, any dietary changes that might have occurred should not bias the treatment group effects in the postintervention period. If instead the benefit from supplementation was to correct a deficiency state to exceed some minimum required threshold, then, if all people started to become less deficient because of dietary improvements over time, the observed treatment effects would be expected to weaken. It is all the more remarkable then that benefits persisted despite this likely improved nutrition and its attendant attenuation of treatment effects. It is also possible that improved diet may have modified effects in the postintervention period, including the enhanced benefit widely observed in younger participants and the emergence of late effects, most notably the benefit for factor C on cerebrovascular deaths.

In summary, 10 years of postintervention follow-up of participants in this cancer prevention trial demonstrated the durability of previously observed beneficial effects on mortality from supplementation with selenium, vitamin E, and beta-carotene. The persistence of risk reduction for up to 10 years after treatment in this trial reinforces the validity of the original trial findings and is consistent with an emerging new paradigm in cancer prevention, namely, that prevention may be achievable with short-term as opposed to life-long treatment. Striking age interactions were seen, suggesting that supplements may be more beneficial in younger age groups. Late beneficial and harmful effects on mortality not observed during the trial period of supplementation were also seen for other supplementation groups. Durability and late effects should be examined in other prevention trials.

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Funding

National Cancer Institute contracts (N01-SC-91030 and N01-RC-47701 to the Cancer Institute, Chinese Academy of Medical Sciences); the Intramural Research Program of the Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health; the Cancer Institute, Chinese Academy of Medical Sciences.

Notes

The authors declare that they have no conflict of interest. The authors are solely responsible for the design of the study; the collection and analysis of data and the interpretation of the results; the preparation of the manuscript; and the decision to submit the manuscript for publication. The authors wish to thank the many citizens of Linxian who have faithfully participated in these studies over the past 20 years.

Manuscript received July 5, 2008; revised January 8, 2009; accepted February 2, 2009.

International Research Conference on Food, Nutrition & Cancer

Effect of White Versus Red Meat on Endogenous N-Nitrosation in the Human Colon and Further Evidence of a Dose Response¹

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ABSTRACT N-Nitroso compounds are found in the colon and are formed endogenously because amines and amides are produced by bacterial decarboxylation of amino acids in the large gut. They can be N-nitrosated in the presence of a nitrosating agent. To test the hypothesis that increased nitrogenous residues from red meat would increase endogenous N-nitrosation, thus accounting for the epidemiologic association between red meat consumption and colorectal cancer, we fed increased levels of red meat and measured apparent total N-nitroso compounds (ATNCs) in fecal samples in a series of studies of volunteers maintained under controlled conditions. A result of these studies is that we have shown a consistent dose response to red meat consumption. Fiber, in the form of vegetables, bran or resistant starch, does not reduce the level of ATNCs formed, although transit time is reduced and fecal weight are increased. Here we show that the equivalent amount (420–600 g) of meat as white meat has no effect on fecal ATNCs in 12 volunteers ($P = 0.338$). At dosages of 0, 60, 120, 240 and 420 g of red meat/d, mean levels of ATNC output are highly correlated with dose of meat: for concentration ATNC versus dose of meat in g/d, $r = 0.972$, $\beta = 0.252$ ng/g (SE 0.035); for total ATNC output versus dose of meat in g/d, $r = 0.963$, $\beta = 2.605$ μ g/d (SE 0.419). The effects of nonmeat protein and of heme on increased N-nitrosation and the genotoxic effects of the ATNCs produced are presently being investigated. *J. Nutr.* 132: 3522S–3525S, 2002.

KEY WORDS: • white meat • red meat • cancer • colon • N-nitroso compounds

Up to 80% of colorectal cancer cases have been attributed to diet, suggesting that this cancer, the second most common in Western countries, is a preventable disease (1). Armstrong and Doll (2) attributed much of the international variation in large bowel cancer incidence among countries to dietary differences, especially meat and fat consumption. If meat is associated with increased risk, lower rates for cancer would be expected in vegetarians; in a meta-analysis of five cohorts, non-meat eaters were not at lower risk than meat eaters (3). However, in the largest study of these vegetarians versus meat eaters, meat was associated with increased risk of colorectal cancer (3,4). Two systematic reviews of meat consumption in relation to colorectal cancer incidence were recently published. In 13 prospective studies an increase of 100 g all meat or red meat was associated with a significant 12–17% increased risk of colorectal cancer, and a significant 49% increased risk was found for a daily increase of 25 g processed meat (5). In 34

case-control studies and 14 cohort studies average relative risk was 1.35 [confidence interval ³(CI 1.21–1.51)] for red meat and 1.31 (CI 1.13–1.51) for processed meat (nitrite treated, cured or smoked, including lunch meats) and meat products and there was no significant association with total meat (6). In contrast, white meat consumption has been associated with decreased risk in two prospective studies (7,8).

One explanation for the association between meat and colorectal cancer is the presence of N-nitroso compounds (NOCs) formed endogenously within the colon. NOCs are formed because the amines and amides produced primarily by bacterial decarboxylation of amino acids can be N-nitrosated in the presence of a nitrosating agent (9,10). A number of facultative and anaerobic colonic bacteria can catalyze the formation of NOCs at optimum pH 7.5 (11–13). In the anaerobic large bowel, nitrate is reduced during dissimilatory nitrate metabolism by the colonic flora to nitrite from which nitrosating agents may be formed. Supplements of nitrate have therefore been shown to elevate fecal NOCs (14). N-Nitrosation in the colon has been demonstrated in animals and shown to be dependent on the presence of gut flora (15).

Meat increases the level of nitrogenous residues reaching the colon (16) so that meat might be expected to increase colonic level of NOCs. We previously showed that fecal NOC

¹ Presented as part of a symposium, "International Research Conference on Food, Nutrition & Cancer," given by the American Institute for Cancer Research and the World Cancer Research Fund International in Washington, D.C., July 11–12, 2002. This conference was sponsored by BASF Aktiengesellschaft; California Dried Plum Board; The Campbell Soup Company; Danisco Cultor; Galileo Laboratories, Inc.; Mead Johnson Nutritionals; Roche Vitamins, Inc.; and Yamamoto/Shaklee/INOBYS. Guest editors for this symposium were Helen Norman and Ritva Butrum, American Institute for Cancer Research, Washington, D.C. 20009.

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³ Abbreviations used: ATNC, apparent total N-nitroso compound; CI, confidence interval; NOC, N-nitroso compound.

TABLE 1

Effect of 420–600 g/d of red meat on fecal N-nitroso compound levels compared with the effects of 60 g/d red meat and 420–600 g/d of white meat in 12 male volunteers

Item	60 g meat		High red meat		High white meat		<i>P</i> , ^{1,2}
	Means	SD	Means	SD	Means	SD	
ATNCs, $\mu\text{g/g}^3$	572	349	2104	1524	759	528	0.014 ¹ 0.001 ² 0.338 ³
ATNCs, $\mu\text{g/d}$	70.4	40.5	249	167	87	55	0.009 ¹ 0.009 ² 0.408 ³
Fecal weight, g/d	131	49	121	32	124	41	0.690 ¹ 0.390 ² 0.514 ³
Mean transit time, h	64	28	66	33	72	41	0.242 ¹ 0.705 ² 0.299 ³

¹ High white meat vs high red meat.

² High red meat vs low red meat.

³ High white meat vs low meat.

ATNCs, apparent total N-nitroso compounds.

excretion increases during high-red meat diets (17,18) and that a dose response exists (19). We also showed that an increase in fermentable carbohydrate entering the colon in the form of vegetables, bran or resistant starch did not reduce levels of NOCs produced, although these dietary factors will decrease transit time, increase fecal weight and dilute the contents of the large bowel, thus reducing cancer risk (17,18,20).

We have been unable to show an effect of white meat on endogenous N-nitrosation but have only studied two individuals so far (17). Hence the effect of white meat in 12 individuals, at two different levels, is reported here. In addition, the effect of 120 g red meat on endogenous N-nitrosation in nine volunteers is added to previously published data showing a dose-response effect to 60, 240 and 480 g of red meat/d (19).

MATERIALS AND METHODS

Subjects and diets. Eighteen healthy male volunteers (aged 24–74 y) were studied in a metabolic suite. During this time only food that was provided from a standardized menu of normal food was permitted to be eaten. All diets were constant in fat and nonstarch polysaccharide (dietary fiber) and adjusted for the energy needs for each subject with extra bread, low-fat margarine and marmalade. Energy requirements ranged from 10 to 12.5 MJ/d. In protocol 1, seven subjects were studied for three 10-d dietary periods of 60 g red meat, 600 g red meat (as beef and pork) and 600 g white meat (as chicken, turkey or white cod). In protocol 2, five subjects were studied for three 15-d periods. The diets were the same as in protocol 1 except that 420 g red and white meat rather than 600 g was used. In protocol 3, nine subjects were fed 60 and 120 g red meat for 15 d each. To equalize the energy content of the diets, a glucose polymer drink and cream were substituted for meat on the 60- and 120-g diets. All other items of food on each diet of the protocols were the same. Deionized water was given throughout for drinking and used in cooking to keep nitrate intake constant. Diets were randomly assigned by using a crossover design and subjects were their own control. Permission for the studies was given by the Dunn Human Nutrition Unit and Addenbrookes Hospital ethics committees.

Protocols. All fecal samples were collected and stored at -20°C except for those collected on days 8–10 in protocol 1 and 13 and 14 in protocols 2 and 3. These samples were processed to prepare homogenates within 20 min of excretion. Each sample was diluted

fourfold with ultrapure deionized water, homogenized in a stomacher (Colworth 3500, Seward) for 20 min and centrifuged at 4500 rpm for 10 min. Each supernatant was filtered, distributed into aliquots and stored at -20°C . Fecal homogenates were analyzed for NOCs and nitrite by the release of nitric oxide after chemical denitration via thermal energy analysis (21). NOCs detected by this group-selective method are referred to as apparent total NOCs (ATNCs). All samples collected during the study were weighed and radiographed, and recoveries of radiopaque fecal markers (22) were noted.

RESULTS

In protocol 1, 96% of the total fecal markers administered were recovered (i.e., 4% of markers were present in specimens not collected after the experimental period). In protocol 2, the mean number of markers recovered in the fecal samples by x-ray analysis was 99.0%, and the mean marker recovery from each volunteer ranged from 98.8% to 99.2%. In protocol 3, mean marker recovery was 97.7% (range 99.9–99.6%).

The mean results for all 12 subjects from protocols 1 and 2 are shown in Table 1. Both the concentration of ATNCs and the output per day were significantly higher when the diets with 420–600 g red meat were fed compared with the diet with 60 g meat ($P = 0.001$, $P = 0.009$, respectively). However, ATNC levels when the diets with 420–600 g white meat were fed were not significantly different from the low-meat diet ($P = 0.338$ and $P = 0.408$, respectively, for concentration and output per day) and were significantly lower than those obtained with the diet with 420–600 g red meat ($P = 0.014$, $P = 0.009$, respectively, for concentration and output).

Figure 1 shows individual values of fecal ATNC concentration as a percentage change from the 60-g red meat diet. All subjects increased their fecal ATNC concentration on the high-red meat diet, but there was a large individual variation in response. Four subjects exhibited a fourfold increase, whereas eight subjects increased levels by 1–2.5 times. ATNC levels in all subjects decreased on changing from the high-red meat diet to the high-white meat diet to levels found on the low-meat diet except for two subjects consuming the 600-g high-white meat diet in whom levels increased on changing to the white meat diet.

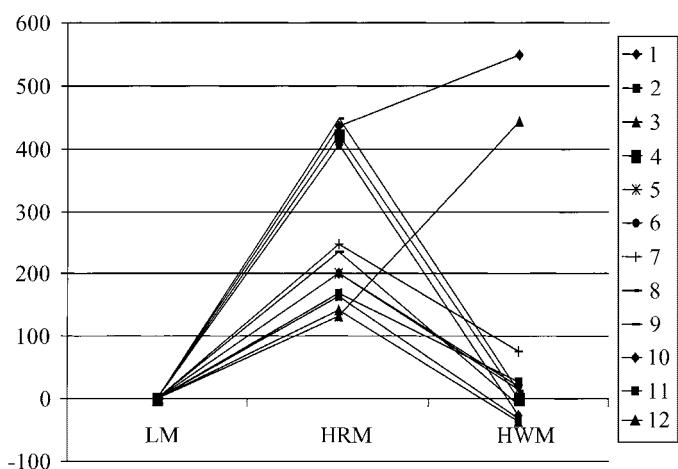


FIGURE 1 Individual changes in fecal ATNC concentration in 12 individuals fed a low-meat (60 g) diet, a high-red meat (420–600 g) diet and a high-white meat (420–600 g) diet. Subjects 1–7 were in group 1 (600 g meat), and subjects 8–12 were in group 2 (420 g meat).

In protocol 3, mean levels of ATNC concentration were $764 \mu\text{g} \cdot \text{kg}^{-1}$ (SD 698) on the low-meat diet and $1164 \mu\text{g} \cdot \text{kg}^{-1}$ (SD 1555; $P = 0.205$) on the high meat diet. ATNC output on the low-meat diet was $77 \mu\text{g}/\text{d}$ (SD 27) compared with $125 \mu\text{g}/\text{d}$ (SD 125) on the high-meat diet ($P = 0.209$). Figure 2 shows mean values for fecal NOC concentration using combined results from those already published (19) and the results from these nine individuals studied in protocol 3 ($n = 17$ for the 60-g level). Although differences between the 60- and 120-g levels in protocol 3 were not significant, mean levels of fecal ATNCs on the 120-g red meat diet were intermediate between those obtained from the 60-g and 240-g diets (Fig. 2). Mean levels of NOC output were highly correlated with dose of meat: for ng of ATNCs/g versus dose of g of meat/d , $r = 0.972$, $b = 0.252$ (SE 0.035), and for $\mu\text{g of ATNCs/d}$ versus dose of g of meat/d , $r = 0.963$, $b = 2.605$ (SE 0.419).

DISCUSSION

The influence of red meat on fecal ATNC excretion has now been shown in more than 50 healthy male volunteers, all of whom were studied in this study and four previous studies from our laboratory in a metabolic suite where diet could be carefully controlled (17–20). The direction of increase with increasing meat is consistent in nearly all individuals. Furthermore, Figure 2 shows there is a dose response that occurs at normal levels of 120 g of meat/d in addition to the higher levels of 240 and 420 g/d published elsewhere (19). Under these controlled conditions, the dose of red meat was highly predictive of average fecal ATNCs, with R^2 values of 0.97 and 0.96 for concentration and output per day, respectively. At the higher levels of meat consumption, concentrations of ATNCs are of the same order of magnitude as the concentration of tobacco-specific NOCs in cigarette smoke (11,17). We previously showed that fermentable carbohydrate does not change fecal NOC output (17,18,20). In this study, the nonstarch polysaccharide (fiber) contents of the diets were the same and diet had no effect on fecal weight and mean transit time (Table 1).

A high-red meat diet containing 600 g of meat/d provides only $13 \mu\text{g}$ of preformed ATNCs/d (18). Fecal ATNC levels exceeded this value in all subjects studied so far, showing that

fecal ATNC excretion during the study was due to endogenous intestinal formation. This formation is not due to an increase in the amount of nitrosating agents, such as nitrate, because nitrate levels have been kept constant throughout to avoid interference from this factor. An equivalent amount of protein as white meat in this study had no effect; therefore increased endogenous production of nitric oxide from oxidation by nitric oxide synthase of the extra L-arginine present in the high-meat diets is unlikely to account for any increase (23). The 420-g red meat diet would have provided $\sim 7.3 \text{ g arginine}$ compared with 1.04 g from the 60-g diet (24).

Despite the consistent response to meat, there is substantial individual variation in the extent of response (Fig. 1). This individual variation remains despite the highly controlled conditions under which studies are carried out. The individual variation may arise from individual differences in gut flora, with high responders harboring high populations of nitric oxide-producing bacteria. Alternatively, individual differences in iron or protein absorption would alter the amount of precursors available for N-nitrosation entering the colon. Iron is required for nitrate reductase activity, which is responsible for bacterially mediated N-nitrosation (11–13).

Although red meat resulted in the expected increase in endogenous N-nitrosation in this study, the same amount of white meat had no effect in 10 of 12 volunteers; therefore mean fecal ATNC levels were not significantly different from those found in a diet with 60 g of meat. In vitro work has shown that the heme proteins myoglobin and hemoglobin in meat react with nitric oxide under anaerobic conditions to form nitrosating agents with the ability to nitrosate phenol (25). Under certain conditions, hemes are known to be nitrosated and act as nitrosating agents (26). The formation of N-nitrosoarginine by heme enzymes under anaerobic conditions was also demonstrated (27). Red meat is a much richer source of heme iron than is white meat; therefore the lack of effect of white meat may be due to a comparative absence of heme. Present studies are investigating the effect of heme on endogenous N-nitrosation (28).

Many classes of NOC have been identified, including nitrosamines, nitrosamides and nitrosoguanidines, and a number of these are known to cause DNA damage after the formation of alkylating agents during NOC metabolism. Methylnitrosourea was shown to induce G→A transitions at codons 12

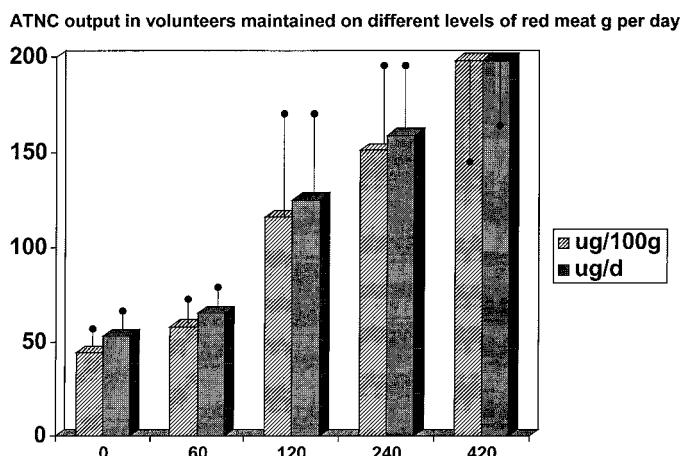


FIGURE 2 Dose response to 0, 60, 240 and 420 g of meat/d and to 120 g of meat/d (from reference 19 and this study). Eight subjects were studied at the 0-, 240- and 420-g level, 9 at the 120-g level and 17 at the 60-g level. Mean and SEM bars are shown.

and 13 in rat colon tumors and is used to induce colon cancer in rat models (29). Endogenous N-nitrosation may thus be the mechanism behind the increased risk of colorectal cancer from red meat, but further work is required to establish the genotoxicity and carcinogenicity of these compounds present in the colon, especially when large amounts of red meat are consumed.

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Influence of a Diet Very High in Vegetables, Fruit, and Fiber and Low in Fat on Prognosis Following Treatment for Breast Cancer

The Women's Healthy Eating and Living (WHEL) Randomized Trial

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CONSIDERABLE EVIDENCE FROM preclinical studies indicates that plant-derived foods contain anticarcinogens.¹ A comprehensive review of the literature found that a diet high in vegetables and

For editorial comment see p 335.

Context Evidence is lacking that a dietary pattern high in vegetables, fruit, and fiber and low in total fat can influence breast cancer recurrence or survival.

Objective To assess whether a major increase in vegetable, fruit, and fiber intake and a decrease in dietary fat intake reduces the risk of recurrent and new primary breast cancer and all-cause mortality among women with previously treated early stage breast cancer.

Design, Setting, and Participants Multi-institutional randomized controlled trial of dietary change in 3088 women previously treated for early stage breast cancer who were 18 to 70 years old at diagnosis. Women were enrolled between 1995 and 2000 and followed up through June 1, 2006.

Intervention The intervention group ($n=1537$) was randomly assigned to receive a telephone counseling program supplemented with cooking classes and newsletters that promoted daily targets of 5 vegetable servings plus 16 oz of vegetable juice; 3 fruit servings; 30 g of fiber; and 15% to 20% of energy intake from fat. The comparison group ($n=1551$) was provided with print materials describing the "5-A-Day" dietary guidelines.

Main Outcome Measures Invasive breast cancer event (recurrence or new primary) or death from any cause.

Results From comparable dietary patterns at baseline, a conservative imputation analysis showed that the intervention group achieved and maintained the following statistically significant differences vs the comparison group through 4 years: servings of vegetables, +65%; fruit, +25%; fiber, +30%, and energy intake from fat, -13%. Plasma carotenoid concentrations validated changes in fruit and vegetable intake. Throughout the study, women in both groups received similar clinical care. Over the mean 7.3-year follow-up, 256 women in the intervention group (16.7%) vs 262 in the comparison group (16.9%) experienced an invasive breast cancer event (adjusted hazard ratio, 0.96; 95% confidence interval, 0.80-1.14; $P=.63$), and 155 intervention group women (10.1%) vs 160 comparison group women (10.3%) died (adjusted hazard ratio, 0.91; 95% confidence interval, 0.72-1.15; $P=.43$). No significant interactions were observed between diet group and baseline demographics, characteristics of the original tumor, baseline dietary pattern, or breast cancer treatment.

Conclusion Among survivors of early stage breast cancer, adoption of a diet that was very high in vegetables, fruit, and fiber and low in fat did not reduce additional breast cancer events or mortality during a 7.3-year follow-up period.

Trial Registration clinicaltrials.gov Identifier: NCT00003787

JAMA. 2007;298(3):289-298

www.jama.com

fruit probably decreases breast cancer risk and that a diet high in total fat possibly increases risk.² However, evidence of an association between a diet

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high in vegetables and fruit and low in total fat and prevention of cancer progression has been mixed in epidemiological studies.³⁻¹⁷ An interim analysis of data from the Women's Intervention Nutrition Study (WINS), which assessed the effect of a dietary intervention designed to reduce fat intake on relapse-free survival in breast cancer patients,¹⁸ found that the intervention was associated with a marginally statistically significant improvement in relapse-free survival. To our knowledge, no other clinical trials investigating dietary change and breast cancer survival have been reported.

The Women's Healthy Eating and Living (WHEL) Study was a randomized trial assessing whether a dietary pattern very high in vegetables, fruit, and fiber and low in fat reduces the risks of recurrent and new primary breast cancer and all-cause mortality among women with previously treated early stage breast cancer. The study was based on the recommendations of a national committee of experts called to respond to a 1993 challenge grant from a private philanthropist who believed that the role of diet in preventing cancer progression deserved scientific study to enable cancer survivors to make decisions without having "to rely on folklore, rumor and hearsay."¹⁹

METHODS

Details of the study design, eligibility criteria, randomization procedures, and dietary intervention have been reported previously.^{20,21} In brief, we compared 2 dietary patterns: an intervention group that was intensively counseled to adopt a dietary pattern very high in vegetables, fruit, and fiber and low in fat²¹ and a comparison group advised to follow the 5-A-Day diet.^{22,23} The study tested primary hypotheses of whether the intervention dietary pattern was associated with (1) a longer breast cancer event-free interval and (2) increased overall survival among women previously treated for early stage breast cancer. Based on the 6 epidemiological studies that had been published at the time of the trial de-

sign, we estimated the likely effect size of this multicomponent diet.²⁰ Following Lachin and Foulkes,²⁴ we determined that a sample size of 3000 would have 82% power to detect a 19% reduction in additional breast cancer events in the intervention group (expected comparison group rate=24%) and a 24% reduction in all cause mortality (expected comparison group rate=15%).

Participants

Participants were enrolled at 7 study sites between 1995 and 2000. Eligibility criteria included diagnosis of a primary operable invasive breast carcinoma categorized using American Joint Committee on Cancer (edition IV) criteria as stage I (≥ 1 cm), stage II, or stage IIIA within the past 4 years; age at diagnosis between 18 and 70 years; treatment with axillary dissection and total mastectomy or lumpectomy followed by primary breast radiation; no current or planned chemotherapy; no evidence of recurrent disease or new breast cancer since completion of initial local treatment; and no other cancer in the past 10 years. Eligible women were randomly assigned to either the study dietary pattern or the comparison group (FIGURE 1). The institutional review boards at the 7 clinical sites approved the study protocol and consent forms, and all participants provided written informed consent.

Dietary Intervention

The intensive intervention was delivered primarily by telephone counseling, supplemented with 12 cooking classes in the first year and monthly newsletters throughout the study. Trained counselors²¹ followed a computer-assisted protocol that was based on social cognitive theory²⁵ and had 3 phases of decreasing intensity. During the first phase (3-8 calls in 4-6 weeks), counselors focused on building self-efficacy to implement the study targets, which consisted of daily intake of 5 vegetable servings plus 16 oz of vegetable juice, 3 fruit servings, 30 g of fiber, and 15% to 20% of energy intake

from fat. Phase 2 (through 5 months) focused on self-monitoring and dealt with barriers to adherence. Phase 3 (through study completion) focused on retaining motivation for the study dietary pattern and preventing setbacks. During the first year of the intervention, participants received an average of 18 counseling calls, attended an average of 4 of 12 offered cooking classes, and received 12 study newsletters. By 4 years, a key point for assessing long-term effect, these participants had received an average of 31 calls and 48 newsletters.

Women randomized to the comparison group were provided with print materials (from the US Department of Agriculture²⁶ and the National Cancer Institute^{22,23}) describing a diet with a recommended daily intake of 5 servings of vegetables and fruit, more than 20 g of fiber, and less than 30% total energy intake from fat. The comparison group attended an average of 1 of 4 offered cooking classes in the first year and received 24 newsletters tailored to the comparison group during the first 4 years.

Data Collection

Dietary Assessment. Dietary intake was assessed by sets of 4 prescheduled 24-hour dietary recalls conducted by telephone on random days over a 3-week period, stratified for weekend vs weekdays.²⁰ These dietary recalls were scheduled for all participants at baseline, 1 year, 4 years, and 6 years and on 50% random samples at 6, 24, and 36 months. We report data for participants completing follow-up assessments. We also conducted additional analyses as follows: for participants who did not complete follow-up assessments we imputed estimates by assuming that they changed dietary intake in a manner similar to comparison group respondents, using the expectation maximization algorithm in SAS software, version 9.1 (SAS Institute Inc, Cary, North Carolina).²⁷ As previously described,²⁰ dietary assessors completed a training program and used the multipass software-driven recall protocol of the Nutritional Data Sys-

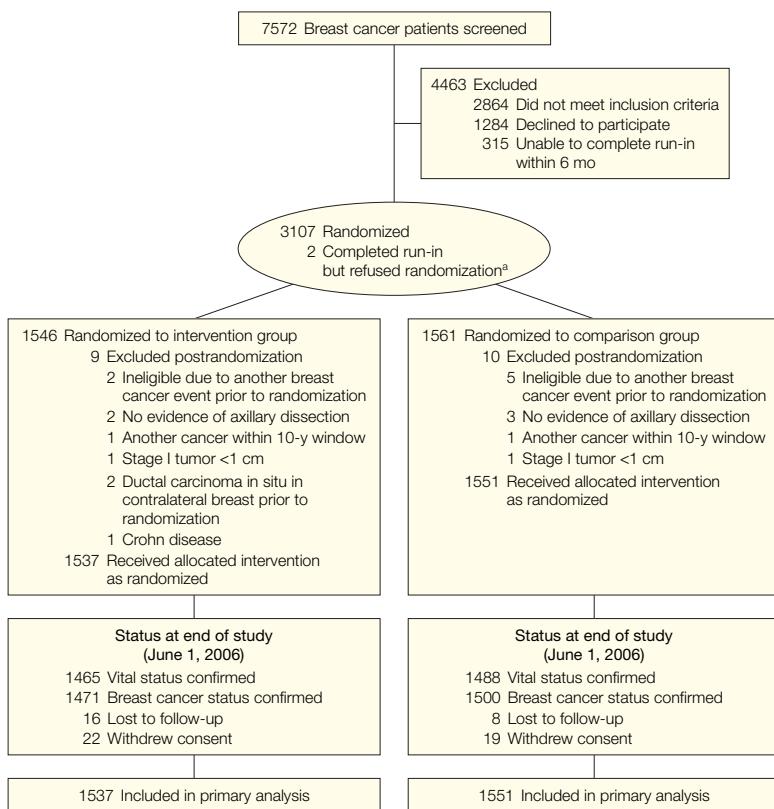
tem software (NDS-R, 1994–2006, University of Minnesota, Minneapolis). To assess overall adherence, we developed an adherence score.²⁰ A completely adherent participant would score 600 points: 300 points reflected vegetable consumption and the remaining 300 points were divided equally among fiber, fruit, and energy from fat.

Other Data Collection. We collected a detailed family history of cancer at baseline and used the mutation prevalence tables from Myriad Genetic Laboratories (Salt Lake City, Utah)²⁸ to classify families with at least a 10% risk of a *BRCA1/2* mutation as high-risk. Clinic visits conducted at baseline, 1 year, 2 or 3 years (randomly determined), 4 years, and 6 years included measured weight and venipuncture. Separated blood samples were stored in cryovials in -80°C freezers for later analysis. Participants were mailed a set of study questionnaires to complete and bring to each clinic visit, including a health status questionnaire with questions on recent physician visits and 2 questionnaires used in the Women's Health Initiative: the Personal Habits Questionnaire,²⁹ which included a 9-item measure of physical activity validated for our study³⁰ and from which we calculated metabolic equivalent task minutes per week,³¹ and the Thoughts and Feelings Questionnaire,³² which included measures of psychosocial functioning. Each participant self-reported race/ethnicity as 1 of 8 categories.

Assessment of Study Outcomes

Primary study end points were (1) the combined outcome of invasive breast cancer recurrence or new primary breast cancer and (2) death due to any cause. Recurrences were further classified as local/regional or distant metastasis. Carcinoma in situ was not counted as a study outcome. The breast cancer event-free interval was defined as the time from date of enrollment to development of a new breast cancer event. Follow-up time was censored at the time of the participant's death, at the last documented staff contact date, or at the study completion date (June 1, 2006).

Figure 1. Participant Flow



During semiannual telephone interviews, clinical site staff queried study participants regarding the occurrence of outcome events, any hospitalization, or new or existing medical diagnoses. Any report of a breast cancer event or death triggered a confirmation interview and collection of medical records and/or death certificates. Two independent oncologists reviewed the medical records (pathology reports and physician notes) to confirm reported recurrences. In cases of disagreement, the coordinating center pathologist adjudicated the outcome. Finally, we searched the National Death Index using Social Security number, name, and date of birth.

Statistical Analysis

Participants were randomly assigned to the intervention or comparison group using a random permuted-block design stratified by tumor stage, age, and clinical site. The allocation of participants was

conducted by the clinical site coordinator running the study's randomization computer program, which automatically stamped the assigned study group in the database. An independent data and safety monitoring committee conducted a blind review of potential post-randomization exclusions.²⁰

Baseline group comparisons of demographic, anthropometric, clinical, and dietary measures were conducted with 2-sided *t* tests, rank-sum tests, or χ^2 tests. We based the primary analysis of disease-free survival on the intention-to-treat principle using time-to-event methods. A 2-sided log-rank test was performed with $P < .041$ considered statistically significant (to account for interim analyses undertaken at the request of the data and safety monitoring committee). Both unadjusted and adjusted hazard ratios were computed. We fit a Cox model stratified by stage, age, and clinical site.

The frequencies of antiestrogen therapy use and bilateral oophorectomies differed marginally between study groups at baseline; therefore, these covariates were also included in the model. However, the antiestrogen therapy variable did not satisfy the proportional hazards assumption; hence, the analysis was stratified by this covariate.³³ Thus, the final model was stratified by stage of initial tumor, age at randomization, clinical site, and antiestrogen use and was adjusted for oophorectomy status.

The a priori analysis plan²⁰ included fitting a Cox proportional hazards model to evaluate the effect of the intervention on key covariates. These included stage of disease (classified as I, II, or III), age at randomization (<55 years vs ≥55 years), hormone receptor characteristics of initial tumor, body mass index, and years from diagnosis to randomization. Product terms between randomization assignment and indicator variables for covariate categories were included in Cox regres-

sion models. Interactions between randomization group and each covariate were formally tested for significance with likelihood ratio tests. The results are presented as hazard ratios and 95% confidence intervals.

In additional analyses, we examined possible interactions between study group and the baseline dietary factors targeted by the intervention (vegetable, fruit, fiber, and fat intakes) to address whether the effects of the dietary intervention might vary by baseline intake level.

Analyses were conducted in the statistics software package R, version 2.3.1 (R Foundation, Vienna, Austria; <http://www.r-project.org>) or SAS, version 9.1.

RESULTS

Recruitment and Baseline Characteristics

Study staff screened 7572 potential participants and randomized 3107 between March 1995 and November 30, 2000 (Figure 1). There was no difference in postrandomization exclusions by study group (9 vs 10). The final study sample included 1537 women in the dietary intervention group and 1551 in the study comparison group.

The study end date was June 1, 2006. Number and frequency of reported physician visits did not differ significantly between groups at any point during the study. We confirmed vital status on the study end date (Figure 1) for 95% of the intervention group and 96% of the comparison group. Breast cancer status was confirmed for 96% of the intervention group and 97% of the comparison group.

Randomization achieved highly comparable groups (TABLE 1) with regard to demographics (ie, age, minority status, and education), breast cancer characteristics (ie, stage, grade, nodal involvement, hormone receptor status, time from breast cancer diagnosis to randomization, and eligibility for BRCA1/2 testing), and treatment (ie, surgery and radiation). Slight imbalances were observed between groups in bilateral oophorectomy, antiestrogen use, and chemotherapy treatment, all

Table 1. Baseline Characteristics of WHEL Study Participants by Study Group^a

Characteristics	Intervention (n = 1537)	Comparison (n = 1551)
Age at study entry, mean (SD), y	53.3 (8.9)	53.0 (9.0)
College graduate	853 (55.5)	820 (52.9)
Race/ethnicity		
White	1306 (85.0)	1328 (85.6)
African American	61 (4.0)	57 (3.7)
Hispanic	87 (5.7)	78 (5.0)
Asian American	46 (3.0)	50 (3.2)
Mixed/other	37 (2.4)	38 (2.5)
Cancer stage at diagnosis		
I	585 (38.1)	606 (39.1)
II	876 (57.0)	867 (55.9)
IIIA	76 (4.9)	78 (5.0)
Nodal status ^b		
Negative	879 (57.2)	896 (57.8)
1-3 positive nodes	436 (28.4)	448 (28.9)
>3 positive nodes	221 (14.4)	207 (13.4)
Hormone receptor status ^b		
ER+/PR+	955 (62.1)	948 (61.1)
ER+/PR-	197 (12.8)	169 (10.9)
ER-/PR+	52 (3.4)	77 (5.0)
ER-/PR-	300 (19.5)	319 (20.6)
Initial treatment		
Mastectomy	812 (52.8)	801 (51.6)
Breast-sparing surgery	725 (47.2)	750 (48.4)
Radiation	937 (61.0)	962 (62.0)
Adjuvant chemotherapy	1095 (71.2)	1064 (68.6)
Ever antiestrogen use	1067 (69.4)	1012 (65.3)
Tumor grade		
I (well differentiated)	239 (15.6)	245 (15.8)
II (moderately differentiated)	620 (40.3)	620 (40.0)
III (poorly differentiated)	551 (35.9)	557 (35.9)
Unspecified	127 (8.3)	129 (8.3)
Prior bilateral oophorectomy	223 (14.6)	177 (11.4)
Time from diagnosis to randomization, mean (SD), mo	23.6 (12.5)	23.5 (12.5)
Baseline eligibility for BRCA testing	132 (8.6)	123 (7.9)

Abbreviations: ER, estrogen receptor; PR, progesterone receptor.

^aData are expressed as No. (%) of participants unless otherwise indicated. There were no significant between-group differences in baseline characteristics based on χ^2 test for categorical variables or t test for continuous variables, except for ever antiestrogen use ($P = .03$) and prior bilateral oophorectomy ($P = .01$).

^bNumbers do not total 3088 because of missing data.

of which favored an intervention effect; however, no between-group difference was observed in the percentage of women who received at least 1 of these therapies (intervention, 93.6%; comparison, 92.3%; $P=.12$). Fourteen percent of women self-identified as minority race/ethnicity, and these were fairly equally divided among African Americans, Hispanics, and Asian Americans.²⁰

Dietary Changes Between Groups

In analyses of dietary change, only participants without a study end point at the time of assessment were included. A high proportion of women completed the dietary assessments (TABLE 2). At baseline, no between-group differences were observed in intakes of vegetables, fruit, or fiber or energy intake from fat, with both groups consuming a daily mean of more than 7 servings of vegetables and fruit. No

between-group differences were observed in measured mean body weight or in energy intake.

In the comparison group, consumption of vegetables, fruit, or fiber changed only modestly over the 6 years following randomization, while relative energy intake from fat increased 13%, reflecting an identified secular trend (J.P.P., V.A.N., L.N., et al, unpublished data, May 2007). In the intervention group, the dietary pattern changed substantially and a large ($P<.001$) between-group difference was achieved and maintained for each dietary target across the 6 years of the intervention. From no difference at baseline, the overall adherence score was 91% higher in the intervention group at 6 months and remained 61% higher than the comparison group at 6 years. Details of changes in dietary targets are presented elsewhere (J.P.P., V.A.N., L.N., et al, unpublished data,

May 2007). Using the more conservative imputed data approach,²⁷ at 1 year, the intervention group had increased average total vegetable and fruit intake to 12 servings/d. This change in total vegetable and fruit intake reflected a major increase in vegetable intake, averaging 7.8 vegetable servings/d at 1 year and remaining relatively high at 6 servings/d at the 4-year follow-up.

At 4 years, relative differences in mean intake between study groups were +65% for vegetable servings, +25% for fruit servings, +30% for fiber, and -13% for energy intake from fat. All differences were statistically significant at $P<.001$. Total plasma carotenoid concentration, a biomarker of vegetable and fruit intake, was 73% higher in the intervention group than the comparison group at 1 year and 43% higher at 4 years, differences that were statistically significant ($P<.001$). In addi-

Table 2. Dietary Pattern and Body Weight by Group^a

	Baseline	6 mo	12 mo	24 mo	36 mo	48 mo	72 mo
Intervention group							
Eligible sample, No.	1537	738	1463	715	676	1355	1308
Response rate, %	99.9	91.3	88.3	85.5	84.8	83.0	77.9
Comparison group							
Eligible sample, No.	1551	765	1484	699	713	1363	1313
Response rate, %	99.8	96.9	93.0	90.6	89.5	88.6	86.2
Total vegetable servings/d							
Intervention	3.9 (0.05)	8.4 (0.13)	7.8 (0.09)	7.1 (0.13)	6.6 (0.13)	6.4 (0.09)	5.8 (0.09)
Comparison	3.8 (0.05)	3.9 (0.07)	3.9 (0.05)	3.7 (0.07)	3.7 (0.07)	3.7 (0.05)	3.6 (0.05)
Total fruit servings/d							
Intervention	3.5 (0.05)	4.4 (0.08)	4.2 (0.06)	3.9 (0.08)	3.8 (0.09)	3.6 (0.06)	3.4 (0.07)
Comparison	3.4 (0.05)	3.6 (0.08)	3.4 (0.05)	3.3 (0.08)	2.9 (0.07)	2.8 (0.05)	2.6 (0.05)
Fiber, g/d							
Intervention	21.1 (0.21)	30.9 (0.40)	29.0 (0.28)	27.6 (0.41)	26.1 (0.42)	25.2 (0.29)	24.2 (0.30)
Comparison	21.2 (0.20)	21.4 (0.30)	21.0 (0.22)	20.5 (0.30)	20.0 (0.30)	19.3 (0.21)	18.9 (0.24)
Energy from fat, %							
Intervention	28.5 (0.18)	21.2 (0.26)	22.7 (0.20)	24.5 (0.29)	25.4 (0.32)	27.1 (0.24)	28.9 (0.25)
Comparison	28.7 (0.18)	27.8 (0.27)	28.4 (0.19)	29.2 (0.30)	30.6 (0.30)	31.4 (0.22)	32.4 (0.22)
Adherence score ^b							
Intervention	286 (3)	574 (7)	533 (6)	485 (8)	461 (8)	435 (6)	396 (6)
Comparison	283 (3)	301 (5)	292 (4)	282 (5)	268 (5)	262 (3)	246 (3)
Energy intake, kcal/d							
Intervention	1719 (10)	1619 (14)	1603 (10)	1592 (15)	1523 (15)	1552 (11)	1538 (11)
Comparison	1717 (11)	1615 (15)	1605 (11)	1606 (15)	1601 (16)	1574 (11)	1559 (12)
Body weight, kg							
Intervention	73.5 (0.42)	NA ^c	73.0 (0.45)	74.2 (0.71)	73.9 (0.73)	74.2 (0.51)	74.1 (0.54)
Comparison	73.3 (0.43)	NA ^c	73.8 (0.47)	74.0 (0.68)	74.9 (0.74)	74.1 (0.50)	73.7 (0.53)

^aData are expressed as mean (SE) unless otherwise indicated.

^bAdherence score was calculated as 30 points for each vegetable or fruit serving (excluding white potatoes, juice, and iceberg lettuce); 10 points for each ounce of vegetable juice; 5 points for each percentage point of energy from fat below 40% to a maximum of 100 points for 20% energy from fat; and 7.7 points for each gram of fiber per 1000 kcal above 5 g/1000 kcal. Perfect adherence was 600 points.

^cData for body weight are not applicable (NA) because it was not measured at 6 months.

tion, a subsample study identified changes in plasma triacylglycerol and high-density lipoprotein cholesterol concentrations that were specific to the intervention group, supporting self-reported changes in carbohydrate and fat intakes.³⁴ Study groups differed by less than 80 kcal/d in energy intake and by less than 1 kg in body weight at any study point.

Breast Cancer Event-Free Survival

During the study, 518 participants had a breast cancer event (TABLE 3), rep-

resenting 256 participants (16.7%) in the intervention group and 262 participants (16.9%) in the comparison group. The disease-free survival curves were virtually identical across groups (FIGURE 2). The unadjusted hazard ratio is presented in Figure 2. The hazard ratio after adjustment for antiestrogen use, oophorectomy status, and stratification factors (including tumor stage, clinic site, and age) at baseline was 0.96 (95% confidence interval, 0.80-1.14; $P=.63$). The likelihood ratio test statistics for group interactions with age, body mass index, physical activity, energy intake, characteristics of the original tumor (including hormone receptor status), and years from diagnosis to study entry were not statistically significant, nor were significant effects of the intervention on mortality observed for any subgroups of women classified by major covariates (TABLE 5).

Group Effects by Baseline Quartiles of Dietary Intake

Within each quartile of the targeted dietary components, the intervention group achieved significant change from baseline (TABLE 6). However, there was no evidence of a consistent pattern of an intervention effect for either breast cancer events or mortality according to any baseline diet subgroup, and the findings that are statistically significant in 2 of the 40 compared strata are what one might expect due to chance.

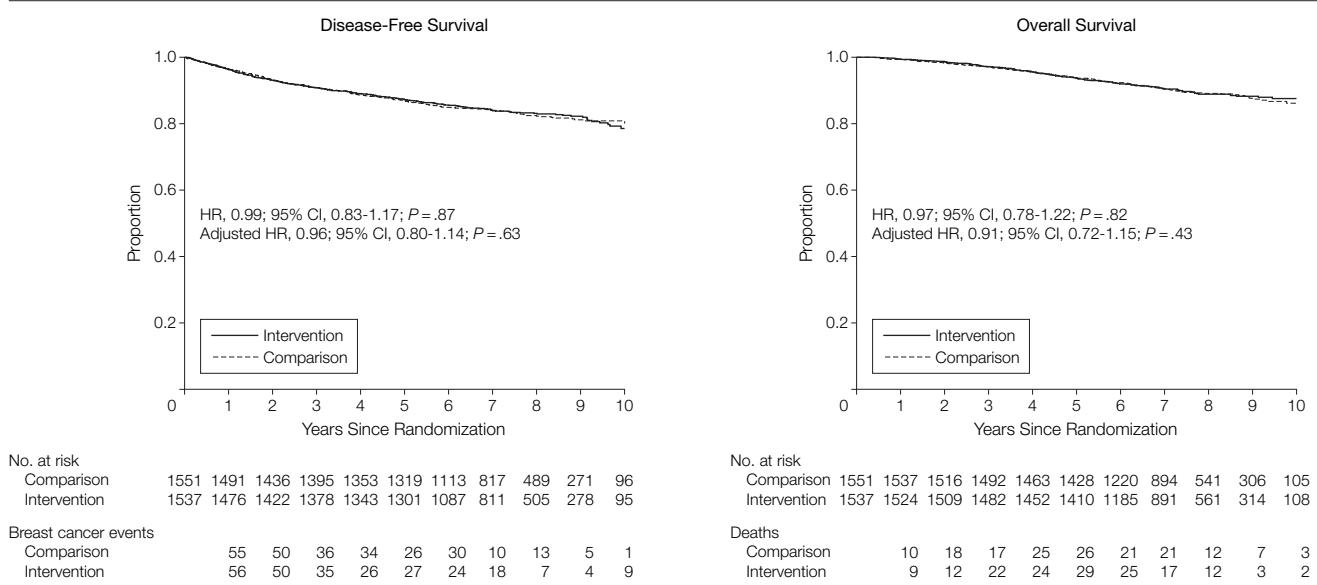
Table 3. Study Events

Study Outcomes	No. of Events	
	Intervention	Comparison
Confirmed breast cancer event	256	262
Local	35	28
Regional	10	10
Distal	168	189
New primary	43	35
Confirmed deaths	155	160
Breast cancer	127	135
Other cancer	12	15
Heart disease	2	5
Other	14	5

Overall Mortality

There were 315 deaths reported within the study period, with 155 (10.1%) in the intervention group and 160 (10.3%) in the comparison group (Figure 2). More than 80% of all deaths were due

Figure 2. Kaplan-Meier Estimates of Disease-Free Survival and All-Cause Mortality by Diet Group



CI indicates confidence interval; HR, hazard ratio. Adjusted HRs were adjusted for tumor stage, clinic site, age at randomization, antiestrogen use, and oophorectomy status.

COMMENT

In this randomized trial of a dietary intervention to achieve a substantial change to a diet very high in vegetables, fruit, and fiber and low in fat, the risk of developing additional breast cancer events and survival were not altered in women previously treated for early stage disease. No significant benefit in recurrence was observed overall among population subgroups characterized by demographic characteristics, baseline diet, or initial tumor types. Although breast cancer mortality rates in the United States declined during the study period,³⁵ the similarity in survival patterns between the WHEL Study groups suggests that continued follow-up would not alter the study results.

It is unlikely that our results were materially affected by bias in assessing the main study end points. Follow-up of participants was nearly complete and did not differ between study groups, and we considered only reports of recurrence and new primary tumors that were validated by medical record review. We observed large and sustained between-group differences in vegetable and fruit intake, as assessed by self-report and plasma carotenoid concentrations, a biomarker of vegetable and fruit intake. We also observed significant differences in fiber and fat intake. Although weight has been associated with health outcomes, we observed less than a 1-kg difference in average weight between WHEL Study groups at any time point. While psychosocial factors have been linked with health outcomes, we found no between-group differences for depression, social support, or quality of life during year 1, when the intervention was most intense.³⁶ Therefore, we believe that our investigation provides an adequate test of whether the study dietary pattern (very high in vegetables, fruit, and fiber and low in fat) provided an added benefit over the dietary pattern of the comparison group women.

Many WHEL Study participants had likely changed their dietary pattern since receiving a diagnosis of breast cancer³⁷; 75% were consuming at least 5 servings of vegetables and fruit a day

Table 4. Intervention Effects on Additional Breast Cancer Events by Baseline Clinical and Demographic Characteristics

	No./Total		HR (95% CI) ^a	P Value ^b
	Intervention	Comparison		
Age at randomization, y				
<55	167/908	161/917	1.05 (0.84-1.30)	.35
≥55	89/629	101/634	0.89 (0.67-1.18)	
Cancer stage at diagnosis				
I	50/585	55/606	0.94 (0.64-1.38)	.74
II	178/876	182/867	0.97 (0.79-1.19)	
IIIA	28/76	25/78	1.19 (0.69-2.04)	
Hormone receptor status ^c				
ER+/PR+	140/955	145/948	0.95 (0.76-1.20)	.85
ER+/PR-	37/197	32/169	0.97 (0.60-1.56)	
ER-/PR+	11/52	18/77	0.89 (0.42-1.88)	
ER-/PR-	64/300	62/319	1.14 (0.80-1.61)	
Time from diagnosis to randomization, y				
≤1	59/352	65/350	0.88 (0.62-1.26)	.50
>1 to 2	83/488	90/508	0.95 (0.71-1.28)	
>2 to 3	64/375	52/372	1.26 (0.87-1.82)	
>3 to 4	50/322	55/321	0.90 (0.61-1.32)	
Tumor differentiation				
I (well differentiated)	20/239	22/245	0.90 (0.49-1.65)	.75
II (moderately differentiated)	94/620	100/620	0.93 (0.70-1.24)	
III (poorly differentiated)	121/551	114/557	1.09 (0.85-1.41)	
Unspecified	21/127	26/129	0.83 (0.47-1.48)	
No. of positive nodes ^c				
0	93/879	117/896	0.80 (0.61-1.06)	.07
1-3	83/436	69/448	1.25 (0.91-1.72)	
4-6	39/116	31/115	1.29 (0.80-2.06)	
≥7	41/105	45/92	0.75 (0.49-1.15)	
Tumor size, cm ^c				
0 to <2	82/752	88/769	0.94 (0.70-1.28)	.22
2 to <3	88/421	86/441	1.09 (0.81-1.47)	
3 to <4	30/174	41/160	0.62 (0.39-1.00)	
4 to <5	21/79	20/72	0.90 (0.49-1.67)	
≥5	35/109	27/106	1.32 (0.80-2.18)	
Body mass index ^d				
<25	107/652	112/678	0.99 (0.76-1.29)	.17
25 to <30	81/475	68/480	1.22 (0.88-1.69)	
≥30	68/410	82/393	0.79 (0.57-1.09)	
Physical activity, MET-min/wk ^c				
≤210	68/397	66/350	0.90 (0.64-1.27)	.56
211-615	62/368	71/383	0.91 (0.64-1.27)	
616-1290	65/375	54/374	1.22 (0.85-1.74)	
>1290	49/351	60/387	0.88 (0.60-1.28)	
Energy intake, kcal/d ^c				
≤1430	63/377	73/396	0.89 (0.64-1.25)	.69
1431-1680	59/384	64/386	0.90 (0.63-1.28)	
1681-1980	64/390	54/379	1.16 (0.81-1.66)	
>1980	70/383	70/386	1.04 (0.75-1.45)	

Abbreviations: CI, confidence interval; ER, estrogen receptor; HR, hazard ratio; MET, metabolic equivalent task; PR, progesterone receptor.

^aHazard ratios of group effect were derived from Cox model stratified by covariate categories.

^bP values based on likelihood ratio test for group × covariate interaction.

^cNumbers do not total 3088 because of missing data.

^dCalculated as weight in kilograms divided by height in meters squared.

at randomization, an intake that is considerably higher than that observed in other populations of breast cancer sur-

vivors.^{38,39} However, we observed little evidence of recurrence benefit in the quartile of the intervention group that

Table 5. Intervention Effects on All-Cause Mortality by Baseline Demographic and Clinical Characteristics

	No./Total		HR (95% CI) ^a	P Value ^b
	Intervention	Comparison		
Age at randomization, y				
<55	84/908	85/917	0.99 (0.74-1.34)	
≥55	71/629	75/634	0.95 (0.69-1.32)	.85
Cancer stage at diagnosis				
I	33/585	32/606	1.07 (0.66-1.73)	
II	102/876	107/867	0.94 (0.71-1.23)	.90
IIIA	20/76	21/78	0.96 (0.52-1.77)	
Hormone receptor status ^c				
ER+/PR+	79/955	84/948	0.92 (0.68-1.26)	
ER+/PR-	25/197	20/169	1.03 (0.57-1.85)	.88
ER-/PR+	7/52	10/77	1.08 (0.41-2.83)	
ER-/PR-	44/300	42/319	1.13 (0.74-1.73)	
Time from diagnosis to randomization, y				
≤1	46/352	47/350	0.97 (0.64-1.45)	
>1 to 2	51/488	58/508	0.90 (0.62-1.32)	
>2 to 3	34/375	28/372	1.28 (0.77-2.11)	
>3 to 4	24/322	27/321	0.86 (0.50-1.49)	.72
Tumor differentiation				
I (well differentiated)	13/239	7/245	1.80 (0.72-4.52)	
II (moderately differentiated)	58/620	64/620	0.90 (0.63-1.28)	
III (poorly differentiated)	75/551	74/557	1.04 (0.75-1.43)	.32
Unspecified	9/127	15/129	0.62 (0.27-1.42)	
No. of positive nodes ^c				
0	58/879	67/896	0.89 (0.62-1.26)	
1-3	48/436	39/448	1.27 (0.83-1.94)	.20
4-6	21/116	19/115	1.09 (0.59-2.03)	
≥7	28/105	35/92	0.64 (0.39-1.05)	
Tumor size, cm ^c				
0 to <2	42/752	51/769	0.83 (0.55-1.26)	
2 to <3	59/421	50/441	1.27 (0.87-1.85)	
3 to <4	17/174	28/160	0.53 (0.29-0.96)	
4 to <5	13/79	12/72	0.94 (0.43-2.06)	
≥5	23/109	19/106	1.21 (0.66-2.22)	.13
Body mass index ^d				
<25	57/652	61/678	0.97 (0.67-1.38)	
25 to <30	48/475	44/480	1.11 (0.74-1.67)	.70
≥30	50/410	55/393	0.86 (0.59-1.27)	
Physical activity, MET-min/wk ^c				
≤210	43/397	47/350	0.80 (0.53-1.22)	
211-615	45/368	45/383	1.04 (0.69-1.58)	
616-1290	36/375	35/374	1.02 (0.64-1.63)	
>1290	25/351	27/387	1.01 (0.58-1.73)	.81
Energy intake, kcal/d ^c				
≤1430	44/377	48/396	0.95 (0.63-1.43)	
1431-1680	32/384	40/386	0.77 (0.49-1.23)	
1681-1980	34/390	31/379	1.08 (0.66-1.75)	
>1980	45/383	40/386	1.17 (0.77-1.79)	.62

Abbreviations: CI, confidence interval; ER, estrogen receptor; HR, hazard ratio; MET, metabolic equivalent task; PR, progesterone receptor.

^aHazard ratios of group effect were derived from Cox model stratified by covariate categories.

^bP values based on likelihood ratio test for group × covariate interaction.

^cNumbers do not total 3088 because of missing data.

^dCalculated as weight in kilograms divided by height in meters squared.

was consuming less than 5 daily servings of vegetables and fruit at baseline, despite a major change in dietary pattern that was specific to the intervention group. Previously, in longitudinal analyses of the comparison group only, we observed a possible threshold effect on recurrence for low levels of baseline plasma carotenoid concentration¹³ and also that a baseline combination of 5 fruits and vegetables a day and physical activity equivalent to walking at a moderate pace for 30 minutes, 6 d/wk, was associated with lower mortality.¹⁴ However, in this analysis of data from the randomized trial, a major increase over the 5-a-day dietary pattern was not associated with reduced breast cancer events or mortality.

We suggest caution in applying our findings to groups of women other than those represented in our study, which was confined to women who had already completed initial therapy for breast cancer and excluded women with diagnoses after age 70 years and those with stage 1 tumors smaller than 1 cm. Also, only 14% of our study population was self-identified as from African American, Hispanic, and Asian American racial/ethnic groups.

Our finding that reducing dietary fat intake did not benefit breast cancer outcomes appears at odds with the interim analyses from the Women's Intervention Nutrition Study (WINS), which concluded that reducing dietary fat intake was marginally associated with longer relapse-free survival of breast cancer patients, an effect most noted in the subgroup with estrogen-negative tumors. However, differential follow-up between intervention and comparison groups may have influenced the WINS finding.⁴⁰ Furthermore, the reduced body weight observed only in the intervention group might partially account for the improved relapse-free survival in WINS.⁴¹ In addition, although WINS reported an 8% to 9% between-group difference in energy intake from fat maintained through 5 years, they reported a higher rate of missing dietary intake data in the intervention group. At the

3-year point, data were available for 67% of the intervention group vs 74% of the comparison group. At 5 years, data were available for 39% of the intervention group and 44% of the comparison group. If even moderate proportions of nonresponders increased their fat intake, the between-group effect could well be less than the absolute 4% difference that we observed. Finally, it is important to note that the women enrolled in WINS differed from those in the WHEL trial regarding prognosis following the original diagnosis, age, and treatment regimen.

Although the WHEL Study's intervention diet focused mainly on increasing vegetable, fruit, and fiber intake, there was a significant between-group difference in fat intake. However, this difference may not have been sufficient to test the dietary fat hypothesis adequately. Unlike the changes observed for intakes of vegetables, fruit and fiber, the smallest dietary fat change was made by participants in the quartile that was furthest from the study target at baseline. Nonetheless, our analyses did not suggest an effect across quartiles of fat intake at baseline, nor did our results indicate an intervention effect in subgroups defined by hormone receptor status, as was seen in WINS.

The absence of an observed effect on breast cancer events or all-cause mortality over a 7.3-year follow-up period in this study does not rule out the possibility of improved longer-term survivorship within this cohort. We did not explore the possibility that increased exercise and weight loss might benefit breast cancer survivors. Finally, our study did not address whether consuming the high-vegetable/fruit/fiber and low-fat diet of our study intervention early in life would alter risk of primary breast cancer.

In conclusion, during a mean 7.3-year follow-up, we found no evidence that adoption of a dietary pattern very high in vegetables, fruit, and fiber and low in fat vs a 5-a-day fruit and vegetable diet prevents breast cancer recurrence or death among women with previously treated early stage breast cancer.

Table 6. Intervention Effects on Additional Breast Cancer Events and All-Cause Mortality by Baseline Quartiles of Dietary Intake

Variables by Baseline Quartile ^a	No. of Participants at Baseline (Difference, %) ^b	Breast Cancer Events ^c		Deaths ^c	
		No.	HR (95% CI) ^d	No.	HR (95% CI) ^d
Vegetables and fruit, servings/d					
≤4.94	772 (+60)	152	0.97 (0.71-1.34)	88	0.75 (0.49-1.14)
4.95-6.74	771 (+52)	122	1.23 (0.86-1.75)	76	1.25 (0.80-1.97)
6.75-8.92	768 (+53)	144	0.93 (0.67-1.30)	83	1.28 (0.83-1.98)
>8.92	770 (+54)	99	0.83 (0.56-1.24)	67	0.76 (0.47-1.23)
Vegetables, servings/d					
≤2.55	772 (+82)	162	1.13 (0.83-1.54)	101	1.05 (0.71-1.55)
2.56-3.54	770 (+75)	127	0.98 (0.70-1.39)	67	0.85 (0.52-1.38)
3.55-4.80	769 (+74)	118	0.85 (0.59-1.22)	76	0.85 (0.54-1.33)
>4.80	770 (+68)	110	0.97 (0.67-1.40)	70	1.19 (0.74-1.90)
Fruit, servings/d					
≤1.76	771 (+40)	139	1.02 (0.73-1.42)	85	0.75 (0.49-1.15)
1.77-2.93	771 (+21)	129	1.02 (0.72-1.44)	77	1.02 (0.65-1.60)
2.94-4.38	772 (+27)	126	1.11 (0.78-1.58)	77	1.60 (1.02-2.51)
>4.38	767 (+27)	123	0.81 (0.57-1.16)	75	0.76 (0.48-1.19)
Fiber, g/d					
≤15.6	771 (+40)	135	0.97 (0.70-1.36)	83	0.69 (0.45-1.07)
15.7-19.9	772 (+33)	132	1.07 (0.76-1.51)	83	1.12 (0.72-1.72)
20.0-25.2	769 (+28)	131	1.07 (0.76-1.51)	87	1.36 (0.89-2.08)
>25.2	769 (+28)	119	0.83 (0.58-1.20)	61	0.80 (0.48-1.34)
Fat, % of energy per d					
≤23.8	782 (-17)	106	0.72 (0.49-1.06)	59	0.73 (0.44-1.22)
23.9-28.6	768 (-13)	149	1.27 (0.92-1.76)	91	1.61 (1.06-2.45)
28.7-33.4	766 (-15)	122	0.86 (0.60-1.23)	78	0.73 (0.46-1.14)
>33.4	765 (-10)	140	1.13 (0.81-1.58)	86	0.98 (0.64-1.49)

Abbreviations: CI, confidence interval; HR, hazard ratio.

^aSeven participants (4 in comparison group and 3 in intervention group) were missing baseline dietary data.

^bPercentage relative difference in mean dietary intake ([intervention – control]/control) between intervention and comparison groups at 4 years.

^cLikelihood ratio test for group × diet quartile interactions was not significant at $P < .05$ level for any diet component (except energy from fat and mortality; $P = .04$).

^dHazard ratios were derived from Cox model stratified by quartiles of dietary intake.

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Obtained funding: Pierce, Caan, Greenberg, Bardwell, Gold, Hollenbach, Jones, Marshall, Newman, Ritenbaugh, Wasserman, Stefanick.

Administrative, technical, or material support: Flatt, Rock, Kealey, Al-Delaimy, Carlson, Faerber, Hajek, Karanja, Madlensky, Newman.

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Financial Disclosures: None reported.

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Funding/Support: The WHEL Study was initiated with the support of the Walton Family Foundation and continued with funding from National Cancer Institute grant CA 69375. Some of the data were collected from general clinical research centers (National Institutes of Health grants M01-RR00070, M01-RR00079, and M01-RR00827).

Role of the Sponsor: The funding sponsors had no role in the design, protocol development, or conduct of the trial or in data collection, data analysis, or manuscript preparation.

Additional Contributions: We thank the WHEL Study's data and safety monitoring committee (Brian Henderson, MD, Ross Prentice, PhD, Marion Nestle, MPH, PhD, and Charles Loprinzi, MD) and Sharon Ross, PhD (National Cancer Institute project officer) for their assistance with review of the article. We also acknowledge Kaylene Grove, BS, BA, Christine Hayes, MA, and Hollie Ward, BA, Cancer Prevention and Control Program, UCSD, for their administrative support and assistance with manuscript preparation. Finally, we are especially grateful to our dietary counseling team and WHEL Study participants for their sustained commitment and dedication to this long-term trial.

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Heme Iron from Meat and Risk of Colorectal Cancer: A Meta-analysis and a Review of the Mechanisms Involved

Nadia M. Bastide, Fabrice H.F. Pierre, and Denis E. Corpet

Abstract

Red meat and processed meat intake is associated with a risk of colorectal cancer, a major cause of death in affluent countries. Epidemiological and experimental evidence supports the hypothesis that heme iron present in meat promotes colorectal cancer. This meta-analysis of prospective cohort studies of colon cancer reporting heme intake included 566,607 individuals and 4,734 cases of colon cancer. The relative risk of colon cancer was 1.18 (95% CI: 1.06–1.32) for subjects in the highest category of heme iron intake compared with those in the lowest category. Epidemiological data thus show a suggestive association between dietary heme and risk of colon cancer. The analysis of experimental studies in rats with chemically-induced colon cancer showed that dietary hemoglobin and red meat consistently promote aberrant crypt foci, a putative precancer lesion. The mechanism is not known, but heme iron has a catalytic effect on (i) the endogenous formation of carcinogenic N-nitroso compounds and (ii) the formation of cytotoxic and genotoxic aldehydes by lipoperoxidation. A review of evidence supporting these hypotheses suggests that both pathways are involved in heme iron toxicity.

Cancer Prev Res; 4(2); 177–84. ©2011 AACR.

Introduction

Cancer of the colon and rectum, taken together, are the third most common type of cancer worldwide (1). In most publications, colon and rectal cancer are studied together and the term colorectal cancer (CRC) is used, which we also use here, except when the publications refer specifically to colon or rectal cancer. CRC is the second most common cause of cancer death in affluent countries. Dietary modifications might reduce this cancer burden by up to 70% (2). Three recent meta-analyses showed that total meat intake is not related to risk but that intake of red or processed meat is associated with a modest, but significant risk of CRC (3–5). Processed meat intake appears to be more closely linked with the risk of CRC than fresh red meat intake. In its 2007 report, the World Cancer Research Fund panel recommended that one should limit intake of red meat and avoid processed meat (1).

Several mechanisms may explain the relationship between the risk of CRC and the intake of red or pro-

cessed meat. First, meat cooked at high temperature contains mutagenic heterocyclic amines. But heterocyclic amines might not be major players in CRC risk, as: (i) consumption of chicken is a major contributor to intake of heterocyclic amines, but is not associated with the risk (6); and (ii) doses of heterocyclic amines that induce cancer in animals are 1,000 to 100,000 times higher than the dose ingested by humans (7). A second hypothesis suggests that the high saturated fat content of red and processed meat increases the risk of CRC. But several studies, including a recent meta-analysis, showed no effect of saturated fat on colorectal carcinogenesis (8–11). A third hypothesis concerns the carcinogenic N-nitroso compounds (NOC), which can be formed in the gastrointestinal tract by N-nitrosation of peptide derived amines or amides. The role of NOC in human cancer is discussed in the following text. Other more unlikely hypotheses involve the high protein, cholesterol, and salt content of red or processed meat. For a review of all these mechanisms, see ref. 12.

Sesink and colleagues suggested that heme iron, in the form of hemin [chloroporphyrin IX iron(III)] a ferric form of heme, may explain the link between the risk of colon cancer and red meat intake, and the lack of a link with white meat intake (13). Epidemiological and experimental evidence support heme toxicity. Heme consists of an iron atom present at the center of a large heterocyclic organic ring called a porphyrin (Fig. 1). Heme is included in so-called hemoprotein, that is, hemoglobin, myoglobin (both involved in the oxygen supply), and in cytochromes (which catalyze electron transfer reactions). Red meat

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Note: Supplementary data for this article are available at Cancer Prevention Research Online (<http://cancerprevres.aacrjournals.org/>)

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doi: 10.1158/1940-6207.CAPR-10-0113

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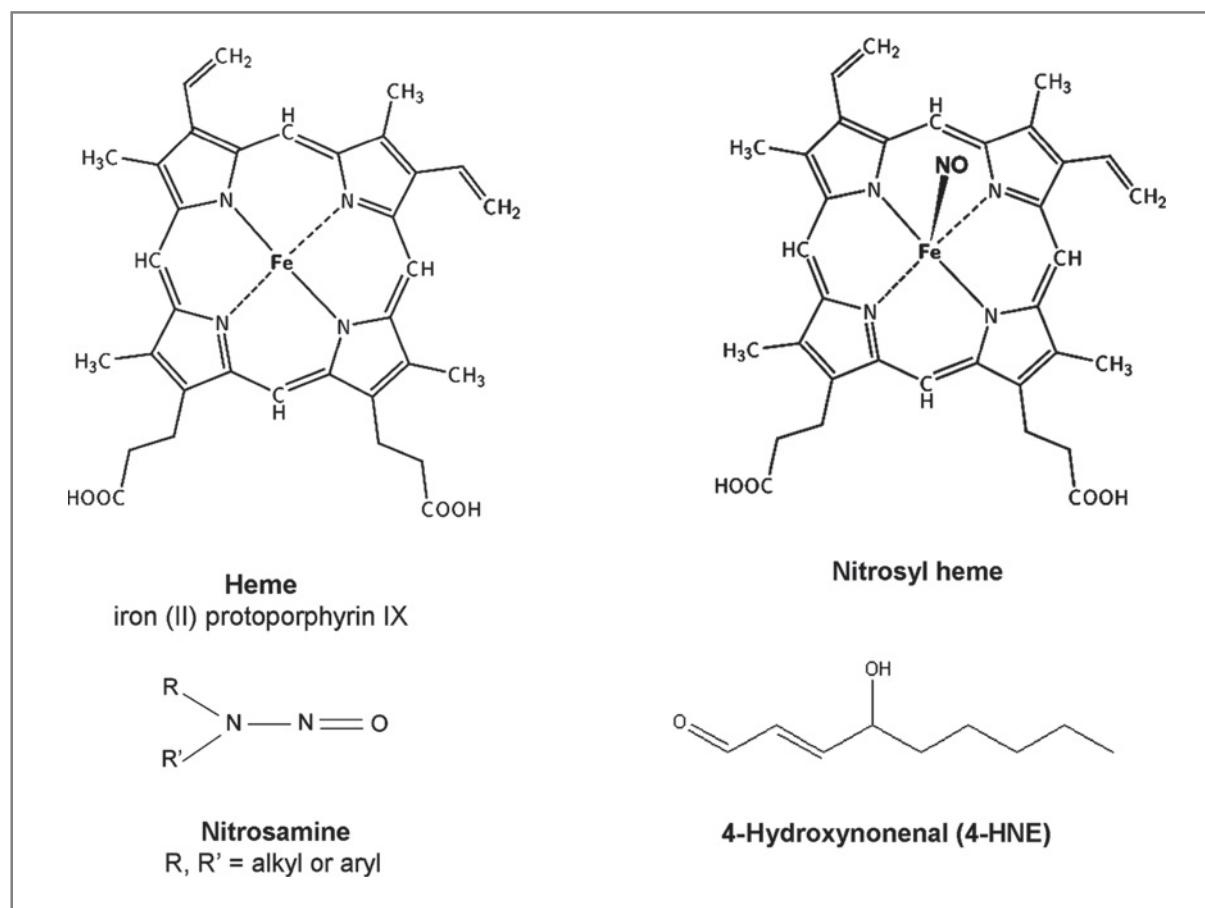


Figure 1. Structure of molecules cited in the review.

(such as beef, veal, lamb, mutton, pork, and offal) owes its dark red color to the presence of a high concentration of myoglobin, and the heme content of red meat is 10-fold higher than that of white meat (such as chicken; ref. 14). In processed red meat, heme iron is nitrosylated, because curing salt contains nitrate or nitrite (Fig. 1; ref. 12).

The aims of the present mini-review were: (i) to conduct a meta-analysis of epidemiological cohort studies on heme intake and the risk of colon cancer; (ii) to review experimental evidence supporting the aforementioned heme hypothesis; and (iii) to understand the mechanism of action of heme in carcinogenesis.

Heme iron intake and risk of colon cancer: a meta-analysis of prospective cohort studies

The objective of this part of the review was to assess, through meta-analysis, the magnitude of the relation between heme iron intake and colon cancer. As most studies do not report data on rectal cancer, we decided to limit our analysis to colon cancer. The methodological procedure is described in the Supplementary Material to this article.

The characteristics of the 5 prospective cohort studies included in the meta-analysis are summarized in Supplementary Data (Table S1). This meta-analysis included data on 566,607 individuals and 4,734 cases of colon cancer. Although 1 cohort study found no association between heme and cancer (15), 3 found that a high intake of heme iron was linked with a higher risk of colon cancer (16–18), and 1 found a positive, but not significant, association between heme iron and colon cancer (19; Fig. 2). In the Lee and colleagues study, the relative risk (RR) for both proximal and distal colon was 1.53 (95% CI: 0.99–2.38). In the Balder and colleagues study, the association was positive in the 2 genders combined (RR = 1.35, 95% CI: 1.03–1.77; ref. 17). The summary RR of colon cancer in all 5 studies was 1.18 (95% CI: 1.06–1.32) for subjects in the highest category of heme iron intake compared with those in the lowest category (Fig. 2). This meta-analysis showed a consistent association between high intake of heme iron and increased risk of colon cancer.

Two studies out of 5 considered calcium in the adjustments for the RR (16–18), and showed the strongest

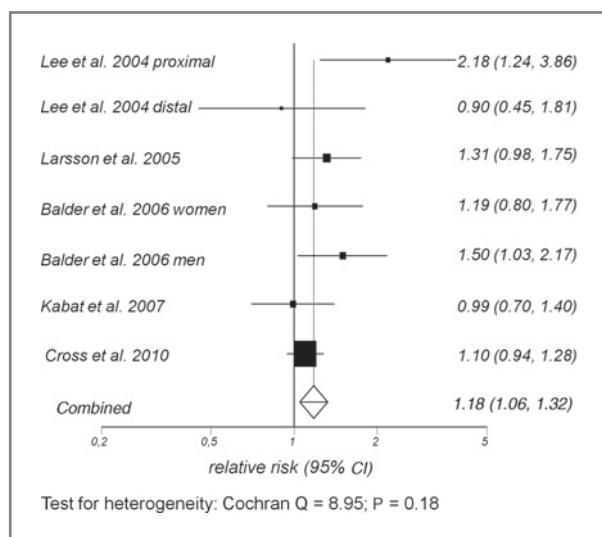


Figure 2. Relative risks of colon cancer in prospective cohort studies, comparing the highest with the lowest category of heme iron consumption. Studies are ordered by year of publication. Squares represent study-specific RR and the size of squares is proportional to the statistical weight that each contributed to the summary estimate of relative risk (percentage weight of each study: Lee *et al.*, 2004, proximal: 6.6%; Lee *et al.*, 2004, distal: 4.6%; Larsson *et al.*, 2005: 18%; Balder *et al.*, 2006, women: 11.7%; Balder *et al.*, 2006, men: 12.86%; Kabat *et al.*, 2007, 14.2%; Cross *et al.*, 2010: 32%). Horizontal lines represent 95% CI. The diamond represents the summary estimate of the relative risk of all studies included in the meta-analysis.

association between heme iron and colon cancer. This makes sense, as calcium inhibits heme-induced cytotoxicity, colonic epithelial hyperproliferation, and promotion of chemically induced carcinogenesis in animal models (20–22).

Two studies we excluded from the meta-analysis found similar results. An ecological study found a direct correlation between the dietary iron index and colon and rectal cancer (23). Ferrucci and colleagues observed a positive, but not significant, association between heme iron in diet and colorectal adenoma.

The present meta-analysis is the first to examine the relation between heme iron and colon cancer. But this study also has its limitations; first it includes only 5 cohort studies, and the way heme intake was measured differs in each study. Lee and colleagues and Larsson and colleagues calculated heme iron content in the diet by applying a factor of 0.4 to the total iron content of all meat items which essentially is reporting an overall red meat effect (16, 18). Balder and colleagues multiplied the heme iron content of each meat item by the mean daily intake of the relevant food item, estimated from the Dutch Food Composition Database (17), but the 2 methods yielded similar results (15). Cross and colleagues developed a new heme iron database based on measured values in conjunction with a detailed meat cooking questionnaire (19).

In conclusion, this meta-analysis showed a significant and consistent but modest increase in the risk of colon cancer associated with high heme iron intake. This study should be pursued by future prospective cohort studies, but this epidemiological result is in line with experimental *in vivo* results detailed in the following text.

Experimental evidence of colorectal cancer promotion by heme iron

Sawa and colleagues showed that dietary hemoglobin produces lipid peroxyl radicals and increases the incidence of nitrosomethylurea-induced colon cancer in rats fed polyunsaturated fat (24). Sesink and colleagues studied the effect of hemin-supplemented diet in non-initiated rats. Dietary hemin increases fat peroxidation and cytotoxic activity of fecal water, and epithelial proliferation by 70% (13). In hemin, the iron atom is stabilized by a freely exchangeable chloride. Pierre and colleagues also showed that hemin and hemoglobin increase the number of azoxymethane-induced aberrant crypt foci, which are putative preneoplastic lesions, in the colon of rats (21). In contrast with hemin, dietary hemoglobin does not increase the cytotoxicity of fecal water, and it is less potent than hemin in promoting colon carcinogenesis. Hemoglobin may be a suitable substitute for myoglobin in nutritional experiments with animal model, and a model agent for studies on the cytotoxicity of red meat (21).

Pierre and colleagues also fed 3 types of meat with different heme content (chicken, beef, and blood sausage) to rats treated with azoxymethane and fed a low-calcium diet (25). This study was the first to show that dietary meat can promote colon carcinogenesis, and that the effect depends on the heme concentration. The results of this study of meat contrast with those of several earlier studies, where red-meat based high-calcium diets failed to promote colon carcinogenesis, indicating probable protection by calcium (26). Subsequently, Pierre and colleagues tested the hypothesis, suggested by epidemiology, that nitrosyl heme in processed meat was more toxic than native heme in fresh meat (27). Cured meat can indeed promote colon carcinogenesis in rats (27). Dietary hemin, but not hemoglobin, could be used as a model agent to mimic the effects of processed meat in rats (27). In a recent study, Pierre and colleagues demonstrated that the nitrosylation of heme was a key event in the promoting effect of processed meat in rats (28).

Analysis of the results of experimental studies of rats with chemically-induced colon cancer (21, 22, 25, 29), showed that the global standardized effect size for number of aberrant crypt foci per colon was 1.73 (95% CI: 1.33–2.14) in rats given dietary heme iron in hemoglobin or beef meat, compared with control rats. The logistic regression approach showed a significant correlation between the number of aberrant crypts per colon and the concentration of heme in the diet ($P = 0.02$; see Methods and Figure in Supplementary Data). This experimental evidence that heme iron promotes carcinogenesis in rats is consistent

with epidemiological evidence. Heme promotion may explain why the intake of red and processed meat is associated with a risk of CRC.

Possible mechanisms of heme toxicity in the gastrointestinal tract

The mechanisms implicated in the promotion of colorectal cancer by heme are poorly understood. The mechanistic hypotheses are based on the catalytic effect of heme iron on (i) the formation of NOC and (ii) the formation of lipid oxidation endproducts.

Heme iron catalyzes N-nitrosation

NOC are formed by N-nitrosation of amines and amides, produced primarily by bacterial decarboxylation of amino acids in the presence of a nitrosating agent (30). There was no *a priori* reason to think that nitrosation would require heme iron. The structure of nitrosamine is shown in Figure 1. NOC can be detected by thermal energy analysis following the release of nitric oxide from biological samples. This analytical procedure comprises nitrosyl iron and S-nitrosothiols in addition to nitrosamines and nitrosamides, which are collectively referred to as apparent total *N*-nitroso compounds (ATNC; ref. 31).

Animal and human studies. Bacon-fed rats had a fecal concentration of ATNC 10 to 20 times higher than control rats (32). In addition, mice fed a diet of hot-dogs (18%), had 4 to 5 times more ATNC, and mice fed a beef diet had 2 to 3 times more ATNC in their feces than controls fed no meat (33, 34).

Human volunteers given a high red meat diet excreted much more ATNC in their stools than controls given no or little red meat, or only white meat (31, 35, 36). The fecal concentration of ATNC was 60 times higher in volunteers given cured red meat than in volunteers given a vegetarian diet (37). Heme iron, and not inorganic iron or meat proteins, may be responsible for the nitrosation observed in the gut of volunteers fed red meat (38).

Nature of ATNC. A red meat diet increased nitrosyl iron and nitrosothiols in ileal outputs and in stools of volunteers, compared with a vegetarian diet, suggesting that these compounds contribute significantly to ATNC (39, 40). Nitrosothiols are rapidly formed from nitrite and thiol groups at low pH in the stomach and can be precursors for the formation of nitrosyl heme and NOC in the gut (39). The strong correlation between fecal nitrosyl iron and fecal heme suggests that nitrosyl heme is the main source of nitrosyl iron (39). Moreover, ATNC precursors from hot dogs were partially purified and separated by HPLC (41). One fraction was identified as 1-deoxy-N-1-glucosyl glycine by mass spectrometry, and the nitrosated fraction was shown to be mutagenic by the Ames test (41).

Carcinogenicity of nitrosated compounds. The carcinogenicity of ATNC formed in the gut after eating heme from red or processed meat is unknown. Parnaud and colleagues

found no initiation or promotion of preneoplastic lesions by ATNC in the colon of rats fed a bacon-based diet (32). Kunhle and colleagues speculated that nitrosyl iron compounds and nitrosothiols may contribute to the tumorigenic potential of the diet (39). By contrast, in a commentary on Kunhle's article, Hogg speculated that the sequestration of the "nitrosating potential" of the diet as nitrosothiol or as nitrosyl iron may be a protective mechanism that would limit the formation of DNA alkylating agents (42).

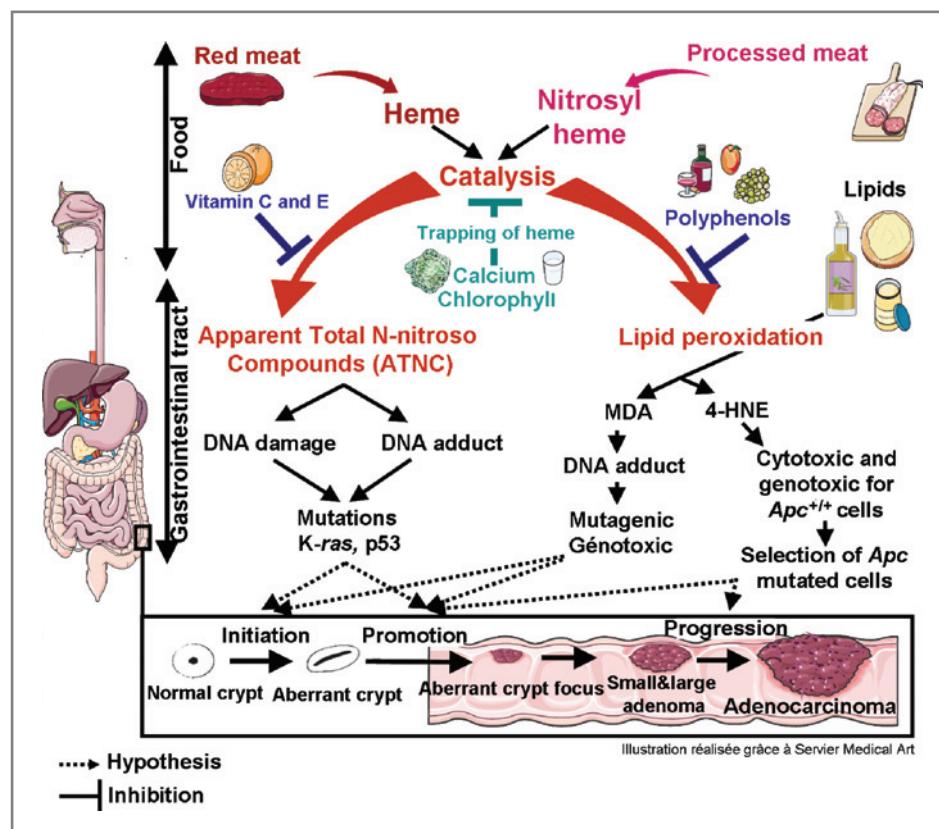
However, several arguments suggest that ATNC may be important genotoxins. First, most NOC, such as nitrosamines, nitrosamides, and nitrosoguanidines, can yield alkylating agents during metabolism, and cause DNA damage. For instance *N*-methyl-*N*-nitrosurea intrarectally perfused induced G → A transitions in *K-ras* in 30% of rat colon carcinoma (43). In addition, nitrosated glycine derivatives reacted with DNA to give rise to promutagenic and toxic adducts including O⁶-methylguanine and O⁶-carboxymethylguanine (44). O⁶-Carboxymethylguanine adducts were found in stool exfoliated colonocytes from volunteers eating red meat, with a correlation between the level of adducts and of fecal ATNC, suggesting that ATNC are genotoxic (45). Moreover, potassium diazoacetate, a stable form of nitrosated glycine, was shown to induce mutations in the *p53* gene in a functional yeast assay (46). The patterns of mutations were similar to the patterns observed in human colon tumors. This supports the hypotheses that nitrosation of compounds related to glycine contributes to *p53* mutations in humans, and that O⁶-carboxymethylguanine adducts in exfoliated colorectal cells are related to CRC (46).

Heme iron catalyzes the oxidation of polyunsaturated fats

The polyunsaturated fatty acid residues of phospholipids are extremely sensitive to oxidation. Lipid peroxidation is initiated by free-radical attack of membrane lipids and is catalyzed by heme with the following reaction: LOOH (lipid hydroperoxide) + Fe-ligand (heme) → LOFe ligands → LO[•] (lipid alkoxy radical) + 'OFe ligands (heme oxiradical; ref. 47). The initial products of unsaturated fatty acid oxidation are lipid hydroperoxides, but they are relatively short lived. They are either reduced by glutathione peroxidase to unreactive fatty acid alcohols or they react with metals to produce a variety of reactive compounds such as epoxides and aldehydes. The major aldehyde products of lipid peroxidation are malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE; ref. 48). These dietary lipid oxidation end products are risk factors for several human diseases (for review, see refs. 49, 50).

Malondialdehyde. MDA is formed by oxidation of polyunsaturated fatty acids with 2 or more double bonds. MDA-induced DNA damage is mutagenic in bacterial, mammalian, and human cells (51–53). MDA reacts with DNA to form adducts with deoxyguanosine, deoxyadenosine, and deoxycytidine (for review, see ref. 54). The major

Figure 3. Catalytic effects of heme on the formation of ATNC and lipid peroxidation, and their inhibition. Consequences for the development of CRC. Heme catalyzes the formation of ATNC and lipid peroxidation endproducts, which partially explains the promoting effect of red and processed meat on CRC. The catalytic effects of heme can be inhibited by trapping the heme (calcium, chlorophyll). The endogenous formation of ATNC is inhibited by vitamins C and E, and it appears that polyphenols can inhibit lipid peroxidation.



DNA adduct formed by reaction of MDA with DNA is 1,N²-malondialdehyde-deoxyguanosine (M₁dG). M₁dG was detected in colorectal biopsies from normal mucosa of 162 participants in the United Kingdom FlexiScope Sigmoidoscopy Screening Trial and the EPIC study (55). The level of this adduct was modulated by dietary and lifestyle habits, and there is a higher M₁dG levels in subjects with adenoma compared with adenoma-free subjects ($P < 0.005$; ref. 55).

4-Hydroxynonenal. In contrast with MDA, 4-HNE is weakly mutagenic but appears to be the main toxic product of lipid peroxidation (Fig. 1). 4-HNE has powerful effects on signal transduction pathways and some of its effects appear to be independent of DNA damage (48). Indeed, 4-HNE present in fecal water can induce apoptosis and necrosis of human colon carcinoma cells through caspase 3 activation (56). Mutations in the *adenomatous polyposis coli* (*Apc*) gene on the chromosome 5q21 locus are considered to be one of the earliest events in the initiation of CRC (57). Moreover, *Apc* mutation was shown to reduce the level of caspases 3, 7, and 9 in mouse colonocytes, leading to resistance to apoptosis (58). An intestinal cell line derived from C57BL/6J mice (*Apc*^{+/+}) and Min mice (*Apc* Min^{+/}) retained the heterozygous *Apc* genotype and the disordered actin cytoskeleton network for the *Apc* Min^{+/} cell line (59, 60). By

exposing this cell line to fecal water of heme-fed rats or to 4-HNE, Pierre and colleagues showed that apoptosis was suppressed in *Apc* Min^{+/} cells (61). The heterozygote *Apc* mutation is thus a strong selective advantage for colonic cells exposed to a lipoperoxidation-related genotoxic environment such as excess heme iron or 4-HNE (61).

In summary, heme catalyzes the formation of ATNC and of lipid oxidation end products, which may explain the promoting effects of red and processed meat on CRC. However, the procarcinogenic effect of heme can be inhibited by several molecules. First, calcium salts and chlorophyll can precipitate heme molecules and inhibit the cytotoxic and hyperproliferative effect of heme in the rat epithelium (17, 20–22, 62, 63). Moreover, the endogenous formation of ATNC is inhibited by vitamins C and E, and lipoperoxidation is inhibited by several polyphenols such as quercetin, α -tocopherol, or red wine polyphenols (64–68). The catalytic effects of heme and its inhibition are summarized in Figure 3.

Conclusion

CRC is the leading cause of cancer death among nonsmokers in affluent countries, and its prevention is thus a major goal for public health. Epidemiological studies

demonstrate a modest but significant and consistent relation between red meat and processed meat intake and CRC risk. The dietary recommendations are to reduce red meat intake and to avoid processed meat intake (1). However, meat is an important source of proteins, providing all essential amino acids, and it is an excellent source of iron and zinc. Iron deficiency is the most widespread nutritional disorder in the world, especially among children and premenopausal women, and results in iron deficiency anemia (1). Knowledge of the mechanism of CRC promotion by meat may allow an alternative prevention strategy to be developed: inhibiting red and processed meat toxicity instead of stopping meat intake. Among the hypotheses explaining the association between meat intake and the risk of CRC, the effect of heme iron is supported by both epidemiological (Fig. 2) and experimental evidence (Supplementary Fig. S1). Several mechanisms may explain the effect of heme on CRC, and the 2 major hypotheses are: (i) heme catalyzes the endogenous formation of ATNC; and (ii) heme catalyzes the peroxidation of dietary fats (Fig. 3). Calcium salts, chlorophyll, vitamin C, and several polyphenols may

reduce these deleterious effects of heme. Specific recommendations might be made, for example, "eat a yogurt after your steak." Moreover, vitamins or polyphenols could be added during the curing process. Ascorbic acid is already added during the processing of processed meats specifically to inhibit the formation of volatile NOC in the meat (69). We expect that this will reduce the risk of CRC without losing the benefit and the pleasure of eating meat.

Disclosure of Potential Conflicts of Interests

No potential conflicts of interests were disclosed.

Acknowledgments

We thank Luc Dauchet (INSERM, CHU Rouen, France) for his advice concerning the meta-analysis, and Daphne Goodfellow who carefully read the manuscript and corrected typographical and grammatical errors.

Received May 12, 2010; revised November 22, 2010; accepted December 1, 2010; published OnlineFirst January 5, 2011.

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Cancer Prevention Research

Heme Iron from Meat and Risk of Colorectal Cancer: A Meta-analysis and a Review of the Mechanisms Involved

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Cancer Prev Res 2011;4:177-184. Published OnlineFirst January 5, 2011.

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ARTICLES

Dietary Fat Reduction and Breast Cancer Outcome: Interim Efficacy Results From the Women's Intervention Nutrition Study

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Background: Preclinical and observational studies suggest a relationship between dietary fat intake and breast cancer, but the association remains controversial. We carried out a randomized, prospective, multicenter clinical trial to test the effect of a dietary intervention designed to reduce fat intake in women with resected, early-stage breast cancer receiving conventional cancer management. **Methods:** A total of 2437 women were randomly assigned between February 1994 and January 2001 in a ratio of 40 : 60 to dietary intervention ($n = 975$) or control ($n = 1462$) groups. An interim analysis was performed after a median follow-up of 60 months when funding for the intervention ceased. Mean differences between dietary intervention and control groups in nutrient intakes and anthropometric variables were compared with *t* tests. Relapse-free survival was examined using Kaplan-Meier analysis, stratified log-rank tests, and Cox proportional hazards models. Statistical tests were two-sided. **Results:** Dietary fat intake was lower in the intervention than in the control group (fat grams/day at 12 months, 33.3 [95% confidence interval {CI} = 32.2 to 34.5] versus 51.3 [95% CI = 50.0 to 52.7], respectively; $P < .001$), corresponding to a statistically significant ($P = .005$), 6-pound lower mean body weight in the intervention group. A total of 277 relapse events (local, regional, distant, or ipsilateral breast cancer recurrence or new contralateral breast cancer) have been reported in 96 of 975 (9.8%) women in the dietary group and 181 of 1462 (12.4%) women in the control group. The hazard ratio of relapse events in the intervention group compared with the control group was 0.76 (95% CI = 0.60 to 0.98, $P = .077$ for stratified log rank and $P = .034$ for adjusted Cox model analysis). Exploratory analyses suggested a differential effect of the dietary intervention based on hormonal receptor status. **Conclusions:** A lifestyle intervention reducing dietary fat intake, with modest influence on body weight, may improve relapse-free survival of breast cancer patients receiving conventional cancer management. Longer, ongoing nonintervention follow-up will address original protocol design plans, which called for 3 years of follow-up after completion of recruitment. [J Natl Cancer Inst 2006;98:1767–76]

The question of the influence of dietary fat on breast cancer has been controversial. Whereas preclinical and human

ecologic studies have suggested an association of higher dietary fat intake with breast cancer risk (1,2), cohort studies have reported less consistent effects (3–5). Similarly, observational studies of dietary fat influence on breast cancer recurrence have had mixed results (6,7), with some suggesting that higher fat intake is associated with higher risk of recurrence, especially in postmenopausal women (8–10). The varying associations may be due to the modest range of fat intake seen and the difficulty in accurately measuring fat intake with current methods (11,12).

Feasibility trials have demonstrated that dietary fat reduction can be achieved within the context of standard multimodality breast cancer management (13,14). The Women's Intervention Nutrition Study (WINS) was subsequently designed to test the hypothesis that a dietary intervention targeting fat intake

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See "Notes" following "References."

DOI: 10.1093/jnci/djj494

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reduction would prolong relapse-free survival in women with resected breast cancer.

PATIENTS AND METHODS

Study Overview

In this phase III multicenter randomized trial, the effect on relapse of a dietary intervention designed to reduce fat intake was compared with that of a control condition with minimal dietary counseling in women with early-stage, resected breast cancer receiving conventional cancer management. Patients in this study were accrued in approximately 7 years, between February 1994 and January 2001. This report represents an interim analysis after a median of 60 months of follow-up. The WINS trial protocol is available online as supplementary data at <http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol98/issue24>.

Study Population

Women were recruited from 39 clinical centers. Eligibility criteria included histologically confirmed, resected, unilateral invasive breast carcinoma; lymph node evaluation; age between 48 and 79 years; life expectancy of at least 10 years excluding the cancer diagnosis; acceptable adjuvant systemic therapy (see below); at least 20% of calories obtained from fat; medically able to accept either randomization assignment; and trial entry within 365 days of surgery. Exclusion criteria included inflammatory carcinoma; chest wall or skin involved; tumor size less than 1 cm with negative nodes; tumor size greater than 5 cm with positive nodes; 10 or more nodes positive; preoperative chemotherapy; or any previous neoplasm other than carcinoma *in situ* of the cervix or basal cell skin carcinoma. Eligibility was confirmed by central review of medical records and pathology reports. The Institutional Review Board of each participating institution approved the study protocol. All patients gave written informed consent.

Standard Breast Cancer Management

Standard breast cancer management was protocol defined, including adequate surgery and radiotherapy. Estrogen receptor (ER) and progesterone receptor (PgR) status (positive or negative) was defined by local laboratory standards. Women with ER-positive tumors received tamoxifen (20 mg per day) for 5 years. Chemotherapy with a protocol-approved regimen (adriamycin plus cyclophosphamide; cyclophosphamide plus methotrexate plus 5-fluorouracil in two schedules; 5-fluorouracil plus adriamycin plus cyclophosphamide in two schedules [5-fluorouracil–adriamycin–cyclophosphamide or cyclophosphamide–adriamycin–fluorouracil]; or adriamycin–cyclophosphamide followed by paclitaxel) was required for women with ER-negative tumors and optional for those with ER-positive tumors. As an eligibility criterion, chemotherapy had to be initiated within 4 months after diagnosis for women given chemotherapy, and tamoxifen had to be initiated within 6 months after diagnosis for those given only tamoxifen. Recommended drug dosages and schedules paralleled those in use by cooperative group trials at the time (15–17).

Randomization

Adaptive randomization was carried out at the Statistical Coordinating Unit of the WINS study using a random stratified permuted block design. The trial was designed with an unbalanced randomization (60% control subjects, 40% dietary intervention subjects) to facilitate resource allocation to the dietary intervention. Women were initially stratified according to lymph node status (negative or positive) and systemic adjuvant therapy received (tamoxifen alone, tamoxifen plus chemotherapy, or chemotherapy alone). An additional stratum for sentinel node evaluation (yes or no) was introduced in 1999 to reflect clinical practice trends.

Dietary Intervention and Study Assessments

The goal of the dietary intervention was to reduce percentage of calories from fat to 15% while maintaining nutritional adequacy. Feasibility studies had indicated that this goal would result in a sustained reduction in fat intake to approximately 20% of calories (14), which was the basis for the sample size calculation. Women in the dietary intervention group were given an individual fat gram goal and counseled by registered dieticians who implemented a previously developed low-fat eating plan (14–18). Study dieticians were trained centrally on diet intervention and dietary and anthropometric data collection. Training continued with annual workshops incorporating training on motivational interviewing and with monthly conference calls.

The low-fat eating plan, which was based on nutritional and behavioral science principles (18), incorporated social cognitive theory and included self-monitoring (fat gram counting and recording), goal setting, modeling, social support, and relapse prevention and management. Individual fat gram goals were based on energy intake needed to maintain weight, and no counseling on weight reduction was provided. The low-fat eating plan was initiated during eight biweekly individual, in-person counseling sessions, each lasting approximately 1 hour. Subsequent dietitian contacts (visits or calls) occurred every 3 months, with available, optional monthly dietary group sessions. Women in the dietary intervention group were instructed to keep a written record of their fat gram intake daily throughout the trial using a previously developed “keeping score” book (18). Control subjects had one baseline dietitian visit and contacts with a dietitian every 3 months subsequently. They received written information on general dietary guidelines and were counseled on nutritional adequacy for vitamin and mineral intake only.

Questionnaires administered at baseline were used to collect information on demographic characteristics; medical, reproductive, and family history; personal habits such as smoking and alcohol use; prior use of menopausal hormone therapy and oral contraceptives; and current use of medications and dietary supplements. Weight and height were measured at baseline and annually using standardized techniques on calibrated scales/stadiometer (19). Body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters) squared.

Unannounced telephone calls were used to estimate dietary intakes. Trained interviewers who were blinded to randomization status collected dietary information; the information was entered into the Nutrition Data System for Research interactive software as previously described (18). A multilayered quality control process was used to maintain nutrition data quality (20). Data on

dietary intakes from three follow-up calls (including one on a weekend day) were collected over 2 weeks for eligibility determination regarding dietary fat intake. Two additional follow-up calls (including one on a weekend day) were made annually to collect updated data on dietary intakes for all women.

Recurrence information was solicited during the every-3-month dietician contacts. Recurrences were confirmed initially by medical record and pathology report review by the clinical director (RTC) and subsequently by the WINS Pathology Committee, both of whom were blinded to random assignment.

Outcome Definitions

The primary study endpoint was relapse-free survival, defined as the time from random assignment to breast cancer recurrence at any site. Relapse events included local, regional, and distant recurrence; ipsilateral breast recurrence after lumpectomy; and contralateral breast cancer. Overall survival, defined as the time from randomization to death from any cause, was a secondary endpoint. For comparability to other adjuvant trials (15,21–24), disease-free survival and recurrence-free survival were also examined. Disease-free survival events included any secondary invasive cancers, excluding basal and squamous skin cancers, and death without breast cancer recurrence. Recurrence-free survival events included local, regional, distant, or ipsilateral breast recurrence after lumpectomy but excluded contralateral breast cancer. Breast cancer size and nodal status were used to calculate tumor size–node–metastasis stage (25) at baseline.

Termination of the Dietary Intervention

WINS was supported by a multicenter RO1 grant from the National Cancer Institute, with continued funding dependent on ongoing peer review. Funding for the clinical centers and the active dietary intervention ended in May 2004, even though the protocol-defined follow-up period had not been completed and even though secular trends in therapy had reduced recurrence events compared with the number of events projected at the time the study was designed. The WINS External Advisory Committee reviewed results of the last (November 2003) interim analysis (which incorporated nutrient data through August 31, 2003, and all available efficacy data through October 31, 2003), after a median follow-up of 60 months. Based on the change in trial status, both the WINS External Advisory Committee and the WINS Executive Committee supported reporting the available results but recognized the need for additional follow-up. These results were presented at the American Society of Clinical Oncology annual meeting (26) on May 17, 2005, and were provided to study participants by mail. This interim efficacy report provides details of that dataset. The protocol design called for 3 years of follow-up after completion of recruitment through January 2004. Currently ongoing nonintervention follow-up will address the original design plans.

Statistical Analysis

Sample size was based on a model that generated power estimates via simulation. Using published information from cooperative group trials involving tamoxifen (27,28), we calculated a total sample size of 2502 under assumptions of 6 years of accrual, 3 years of follow-up after completion of accrual, and a

7.5% increase in relapse-free survival with a drop-in (defined as control group women with intake <20% of calories from fat at any interval while on study) rate of 10% and a drop-out rate of 30% for 84% power at a two-sided alpha level of .05.

The primary efficacy analysis was a stratified log-rank test. Exploratory Cox proportional hazards models for relapse-free survival were investigated, as were various Cox models for prognostic factors in addition to those used in the stratified randomization. The final Cox model included randomized group, stratification factors (ER status [positive versus negative] and tumor size [<2 versus ≥ 2 cm]), and surgery type (mastectomy versus lumpectomy) based on their established influences on breast cancer outcomes. The cumulative incidence method was applied for Cox model analyses. Model assessment suggested a reasonable data fit. The assumption of proportionality for Cox models was verified by graphical and numerical methods of Lin et al. (29). The *P* value for the Kolmogorov-type supremum test (for group) based on 1000 simulations was 0.5170, supporting the proportional hazards assumptions.

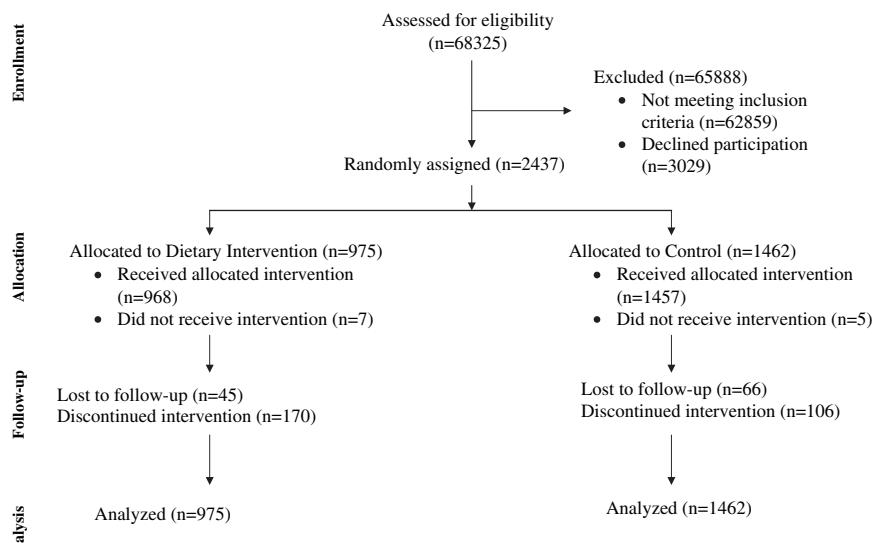
Analyses included all randomly assigned patients, following the intent-to-treat paradigm. All *P* values were derived from a two-sided test for significance. The 95% confidence interval (CI) for the hazard ratio (HR) was obtained for each term in the final Cox model, with particular attention to the interaction between treatment and other factors in the model. The *P* values and confidence intervals are exploratory. Five protocol-planned interim analyses were carried out between February 2000 and November 2003. The Haybittle–Peto approach to the α -spending function was used to account for interim analyses (30). The Kaplan–Meier method was used to calculate probability estimates for relapse-free and overall survival. In exploratory analyses, which were not defined prospectively, dietary effects on relapse-free survival in subgroups based on BMI, hormone receptor status, and nodal status were examined using the Cox model. Tests for interaction used Cox models and a likelihood ratio test. Differences in baseline variables between and within groups were analyzed using *t* tests or paired *t* tests or the appropriate tests with categorical variables. The *t* tests were performed to compare differences in the nutrient intakes and anthropometric variables, and corresponding 95% confidence intervals are reported.

RESULTS

A total of 2437 apparently eligible women were randomly assigned, 975 to the dietary intervention group and 1462 to the control group (Fig. 1). Thirty-four women (12 in the dietary group and 22 in the control group) were subsequently found to be ineligible, most commonly based on a longer interval from diagnosis and/or use of other chemotherapy ($n = 10$), pagetoid nipple involvement ($n = 9$), or size or margin issues ($n = 5$). However, all randomly assigned patients are included in the presented analyses.

The characteristics of the participants were well balanced across the two groups (Table 1). The characteristics of the breast cancers of study participants and the therapy given for these cancers by treatment group are provided in Table 2. Breast cancer characteristics were closely comparable in the two groups. Breast cancer therapy was also closely comparable in the two groups, except for the type of primary surgery. More women in the dietary intervention compared to control group had mastectomy rather than breast-conserving therapy (*P* = .004).

Fig. 1. CONSORT trial flow diagram. Detailed reasons for not completing study were as follows. Not receiving intervention, intervention group: lost interest in study ($n=4$), personal–family problem ($n=2$), did not like low-fat eating plan ($n=1$). Not receiving intervention, control group: lost interest in study ($n=2$), did not like control group allocation ($n=3$). Lost to follow-up, intervention group: unable to contact participant ($n=24$), not interested in study ($n=3$), personal–family problem ($n=2$), did not like low-fat eating plan ($n=3$), medical problem ($n=3$), time commitment ($n=2$), 5 years was enough ($n=2$), moved ($n=1$), refused to be contacted ($n=4$), unknown ($n=1$). Lost to follow-up, control group: unable to contact participant ($n=39$), not interested in study ($n=5$), personal–family problem ($n=2$), did not like control group ($n=2$), medical problem ($n=2$), time commitment ($n=2$), 5 years was enough ($n=2$), moved ($n=2$), refused to be contacted ($n=6$), unknown ($n=4$). Discontinued study, intervention group: unable to contact the participant ($n=49$), not interested in study ($n=15$), personal–family problem ($n=26$), did not like low-fat eating plan ($n=21$), medical problem ($n=12$), time commitment ($n=10$), 5 years was enough ($n=23$), moved ($n=7$), refused to be contacted ($n=3$), unknown ($n=4$). Discontinued study, control group: unable to contact the participant ($n=51$), not interested in study ($n=7$), personal–family problem ($n=6$), did not like control group ($n=1$), medical problem ($n=3$), time commitment ($n=5$), 5 years was enough ($n=15$), moved ($n=3$), refused to be contacted ($n=8$), unknown ($n=7$).



As of October 31, 2003, 95% of the women in the dietary intervention group and 94% of those in the control group were being followed as part of the study or had experienced a study event, with 84% of women in the dietary intervention group and 89% of those in the control group contacted within 12 months of October 31, 2003 (Fig. 1). The median interval between last contact and the analysis closeout date was 2.6 months for women in the dietary intervention group and 3.1 months for those in the control group.

Adherence to Dietary Intervention

The dietary intervention adherence results are based on telephone follow-up calls. The reported dietary intake differences by randomization group over time are outlined in Table 3. In all, 80% of women provided dietary data for at least three time periods after baseline. After 1 year, mean daily fat gram intake was slightly reduced in the control group (from 56.3 g at baseline to 51.3 g at 12 months, mean difference = -5.09 g, 95% CI = -6.5 to -3.7, $P<.0001$) but was reduced to a statistically significantly greater extent in the dietary intervention group (from 57.3 g at baseline to 33.3 g at 12 months, mean difference = -24.4 g, 95% CI = -26.1 to -22.6, $P<.0001$; $P<.001$ comparing the mean difference between groups). The difference in fat gram intake was maintained through 5 years (difference in fat grams per day in dietary versus control groups of -18.0 g [95% CI = -19.9 to -16.1] at 12 months and -19.0 g [95% CI = -22.1 to -16.0] at 60 months [both $P<.0001$]). Similar differences were seen for all fat categories and in percentage of calories from fat (Table 3). In addition, energy intake was somewhat lower and fiber intake slightly higher in intervention group participants (Table 3). Nutrient adequacy was maintained in both groups, with the exception of calcium, vitamin D, and vitamin E, for which intakes were somewhat below recommended levels at baseline and throughout the trial in both groups, a situation that was addressed with counseling

and supplement use. No adverse events were associated with the dietary intervention.

Body weight was not an intervention target. However, there was a modest but statistically significant ($P = .005$) weight difference of about 6 pounds between groups, with dietary intervention women weighing less through 5 years of observation. Changes in BMI in both groups reflect the weight changes observed (Table 3).

Efficacy

After a median of 60 months, 277 relapse-free survival events and 389 disease-free survival events were reported (Table 4). Recurrence events were confirmed by central review in 99.5% of cases. For relapse-free survival—the primary endpoint—the hazard ratio of an event in the dietary intervention compared with the control group was 0.76 (95% CI = 0.60 to 0.98) (Fig. 2, $P = .077$ for stratified log rank and $P = .034$ for adjusted Cox model analysis). For recurrence-free survival (i.e., excluding contralateral breast cancers), the HR was 0.71 (95% CI = 0.53 to 0.94; stratified log rank $P = .050$). For disease-free survival, the HR was 0.81 (95% CI = 0.65 to 0.99; stratified log rank $P = .078$). There was no difference in overall survival comparing women receiving the dietary intervention with control group women (HR = 0.89; 95% CI = 0.65 to 1.21; stratified log rank $P = .56$). Based on the effects on the primary endpoint, 38 women would need to adopt a lifestyle intervention reducing dietary fat intake to prevent one additional breast cancer recurrence.

Dietary intervention effects were examined based on BMI, hormone receptor, and nodal status in subgroup analyses by using adjusted Cox model. A total of eight subgroup analyses were performed, and none of the interactions tested were statistically significant. However, the dietary intervention had a greater effect on relapse-free survival in women with ER-negative

Table 1. Characteristics of participants at baseline by intervention group*

Characteristic	Dietary intervention (N = 975)	Control (N = 1462)
Age (y), mean (95% CI)	58.6 (44.4 to 72.8)	58.5 (43.6 to 73.4)
Race or ethnic group, No. (%)		
White	826 (84.7)	1235 (84.5)
Black	52 (5.3)	75 (5.1)
Hispanics	37 (3.8)	58 (4.0)
Asian or Pacific Islander	58 (4.0)	86 (5.9)
American Indian	1 (0.1)	2 (0.1)
Unknown	1 (0.1)	6 (0.3)
Education, No. (%)		
Less than high school	21 (2.6)	39 (2.7)
More than high school	326 (33.6)	452 (31.0)
College degree	232 (23.9)	394 (27.1)
Graduate school	236 (24.4)	334 (23.0)
Unknown	160	243
Current alcohol use, No. (%)		
None	292 (30.1)	452 (31.1)
Some	677 (69.9)	1002 (68.9)
Unknown	6	8
Smoking status, No. (%)		
Never	483 (49.9)	708 (48.7)
Past	428 (44.2)	641 (44.1)
Current	57 (5.9)	105 (7.2)
Unknown	7	8
BMI (kg/m^2), No. (%)		
<26	440 (46.0)	664 (46.6)
26–29	256 (26.8)	383 (26.9)
≥30	261 (27.3)	377 (26.5)
Unknown	18	38
Waist circumference (cm)		
Mean (SD)	87.36 (14.2)	87.12 (14.0)
≤88, No. (%)	562 (59.7)	819 (57.9)
>88, No. (%)	379 (40.3)	596 (42.1)
Unknown	34	47
Diabetes, No. (%)		
Yes	28 (5.4)	40 (4.8)
No	512 (94.6)	787 (95.2)
Unknown	435	635
Daily dietary intake†		
Kcal, mean (SD)	1667.3 (500.6)	1659 (417.0)
Fat (g/d), mean (SD)	57.62 (24.4)	56.33 (23.2)
Fat, % of caloric intake (SD)	29.61 (7.1)	29.60 (6.7)
First degree family history of breast cancer, No. (%)		
No	722 (74.2)	1083 (74.2)
Yes	251 (25.8)	377 (25.8)
Unknown	2	2
Prior bilateral oophorectomy, No. (%)		
Yes	153 (15.9)	208 (14.4)
No	801 (83.1)	1229 (84.9)
Unknown	21	25
Prior menopausal hormone therapy, No. (%)		
None	332 (34.3)	516 (35.5)
Yes	633 (65.3)	931 (64.0)
Unknown	10	15

*With the exception of the race or ethnic group category, percentages are given relative to participants with known values for each characteristic. Differences in baseline variables between groups were analyzed using *t* tests, paired *t* tests, or the appropriate tests with categorical variables. None of the comparisons between intervention groups were statistically significant at the two-sided $P < .05$ level. CI = confidence interval; BMI = body mass index; SD = standard deviation.

†Information on dietary intake was available for all 975 intervention group participants and 1461 control women.

cancer (HR = 0.58; 95% CI = 0.37 to 0.91) than in women with ER-positive disease (HR = 0.85; 95% CI = 0.63 to 1.14); interaction test, $P = .15$ (Fig. 3). Findings by PgR status were similar (Table 5).

Table 2. Characteristics of breast cancers and breast cancer therapy by intervention group*

Characteristic	Dietary intervention (N = 975)	Control (N = 1462)
Histologic type, No. (%)		
Infiltrating ductal (only)	842 (86.4)	1277 (87.4)
Infiltrating lobular (+/– other)	98 (10.1)	125 (8.6)
Other	35 (3.6)	60 (4.0)
Tumor size (cm)		
Mean (SD)	1.93 (0.9)	1.89 (0.9)
<0.5, No. (%)	2 (0.2)	7 (0.5)
>0.5–1, No. (%)	108 (11.1)	153 (10.5)
>1–2, No. (%)	573 (58.8)	871 (59.6)
>2–5, No. (%)	284 (29.1)	422 (28.9)
>5, No. (%)	8 (0.8)	9 (0.6)
Nodal Status		
Mean No. positive (SD)	2.02 (1.5)	2.02 (1.6)
Negative, No. (%)	708 (73.1)	1060 (72.9)
1–3 positive nodes, No. (%)	230 (23.8)	338 (23.3)
>3 positive nodes, No. (%)	30 (3.1)	56 (3.8)
Unknown	7	8
Stage		
I	531 (54.5)	797 (54.5)
II A	312 (32.0)	467 (31.9)
II B	102 (10.5)	140 (9.6)
III A	30 (3.1)	58 (4.0)
ER status, No. (%)		
Positive	770 (79.0)	1189 (81.3)
Negative	205 (21.0)	273 (18.7)
PgR status, No. (%)		
Positive	641 (69.6)	960 (69.4)
Negative	268 (29.1)	414 (29.9)
Borderline	12 (1.3)	9 (0.7)
Unknown	54	79
Type of surgery, No. (%)		
Mastectomy	343 (35.5)†	434 (29.9)
Breast conserving	624 (64.5)	1018 (70.1)
Unknown	8	10
Radiation therapy, No. (%)		
Yes	659 (68.7)	1019 (70.5)
No	300 (31.3)	427 (29.5)
Unknown	16	16
Type of nodal evaluation, No. (%)		
Axillary dissection/sampling, No. (%)	918 (94.8)	1361 (93.6)
Sentinel node procedure, No. (%)	50 (5.2)	93 (6.4)
No lymph node dissection	7	8
Systemic therapy, No. (%)		
Tamoxifen alone	465 (47.7)	693 (47.4)
Tamoxifen plus chemotherapy	375 (38.5)	555 (38.0)
Chemotherapy alone	135 (13.9)	214 (14.6)
Chemotherapy regimens, No. (%)‡		
Adriamycin–cyclophosphamide, No. (%)	169 (33.5)	243 (31.9)
Cyclophosphamide–methotrexate–5-fluorouracil	269 (53.5)	410 (53.7)
5-Fluorouracil–adriamycin–cyclophosphamide/–cyclophosphamide–adriamycin–5-fluorouracil	35 (7.0)	53 (7.0)
Adriamycin–cyclophosphamide → paclitaxel	32 (6.3)	57 (7.5)

*Percentages are given relative to participants with known values for each characteristic. Differences in breast cancer characteristics and breast cancer therapy were analyzed using *t* tests, paired *t* tests, or the appropriate test with categorical variables. All *P* values were two-sided. ER = estrogen receptor; PgR = progesterone receptor; SD = standard deviation.

†Statistically significant difference in the frequency of mastectomy versus breast-conserving surgery in dietary intervention versus control subjects, $P = .004$.

‡Data on chemotherapy regimens were available for 505 of the 510 women in the dietary intervention group who had chemotherapy and 763 of the 769 women in the control group who had chemotherapy.

Table 3. Mean values (with 95% confidence intervals) of nutrient intakes and anthropometric data at baseline and subsequently, by intervention group*

Variable	Baseline		At year 1		Dietary intervention minus control at year 1		Dietary intervention minus control at year 5	
	Dietary intervention	Control	Dietary intervention	Control	Dietary intervention minus control at year 1	Dietary intervention minus control at year 5	Dietary intervention minus control at year 5	
Daily dietary intake†								
Total fat (g)	57.3 (55.7 to 58.8)	56.3 (55.1 to 57.5)	33.3 (32.2 to 34.5)	51.3 (50.0 to 52.7)	-18.0 (-19.9 to -16.1), <i>P</i> <.0001	-19.7 (-21.9 to -17.5), <i>P</i> <.0001	-19.0 (-22.1 to -16.0), <i>P</i> <.0001	
Fat (% of calories)	29.6 (29.2 to 30.1)	29.6 (29.3 to 29.9)	20.3 (19.8 to 20.9)	29.2 (28.8 to 29.6)	-8.9 (-9.6 to -8.2), <i>P</i> <.0001	-9.0 (-9.8 to -8.1), <i>P</i> <.0001	-8.0 (-9.1 to -6.9), <i>P</i> <.0001	
Saturated fat (g)	18.7 (18.1 to 19.3)	18.5 (18.1 to 19.0)	10.4 (10.0 to 10.9)	16.6 (16.1 to 17.1)	-6.2 (-6.9 to -5.5), <i>P</i> <.0001	-6.7 (-7.5 to -5.9), <i>P</i> <.0001	-6.6 (-7.8 to -5.5), <i>P</i> <.0001	
Polyunsaturated fat (g)	12.2 (11.8 to 12.5)	11.9 (11.6 to 12.2)	7.3 (7.0 to 7.6)	10.8 (10.4 to 11.1)	-3.5 (-3.9 to -3.0), <i>P</i> <.0001	-3.9 (-4.4 to -3.7), <i>P</i> <.0001	-3.7 (-4.4 to -2.9), <i>P</i> <.0001	
Monounsaturated fat (g)	21.6 (21.0 to 22.3)	21.3 (20.8 to 21.8)	12.3 (11.8 to 12.7)	19.6 (19.1 to 20.2)	-7.3 (-8.2 to -6.6), <i>P</i> <.0001	-7.9 (-8.8 to -7.0), <i>P</i> <.0001	-7.6 (-8.8 to -6.3), <i>P</i> <.0001	
Energy (kcal)	1687 (1656 to 1719)	1660 (1635 to 1684)	1460 (1433 to 1487)	1531 (1506 to 1556)	-71 (-1.9 to -33), <i>P</i> =.0002	-142 (-184 to -100), <i>P</i> <.0001	-167 (-223 to -111), <i>P</i> <.0001	
Fiber (g/d)	18.4 (18.0 to 18.9)	18.0 (17.6 to 18.3)	19.5 (18.9 to 20.1)	17.3 (16.9 to 17.7)	2.2 (1.6 to 2.9), <i>P</i> <.0001	1.2 (0.4 to 2.0), <i>P</i> =.0035	2.4 (0.4 to 2.4), <i>P</i> =.0045	
Anthropomorphic factors								
BMI (kg/m^2)‡	27.6 (27.2 to 28.0)	27.5 (27.2 to 27.8)	26.8 (26.4 to 27.2)	27.6 (27.3 to 27.9)	-0.8 (-1.3 to -0.3) <i>P</i> <.0001	-0.77 (-1.3 to -0.2) <i>P</i> <.0001	-1.1 (-1.9 to -0.4) <i>P</i> <.0001	
Weight (kg)§	72.7 (71.7 to 73.7)	72.6 (71.8 to 73.4)	70.6 (69.6 to 71.6)	72.8 (72.0 to 73.7)	-2.3 (-3.6 to -1.0) <i>P</i> <.0001	-1.8 (-3.1 to -0.2) <i>P</i> <.0001	-2.7 (-4.5 to -0.9) <i>P</i> <.0001	

*BMI = body mass index. Differences in breast cancer characteristics and breast cancer therapy were analyzed using *t* tests, paired *t* tests, or the appropriate test with categorical variables. All statistical tests were two-sided.

†Information on dietary intake was available for 975 and 1461 of women in the dietary intervention group and the control group, respectively, at baseline; for 840 and 1328 women, respectively, at year 1; for 654 and 1077 women, respectively, at year 3; and for 380 and 648 women, respectively, at year 5.

‡Information on BMI was available for 957 and 1424 of women in the dietary intervention group and the control group, respectively, at baseline; for 755 and 1230 women, respectively, at year 1; for 600 and 981 women, respectively, at year 3; and for 313 and 534 women, respectively, at year 5.

§Information on weight was available for all 975 and 1462 women in the dietary intervention group and the control group, respectively, at baseline; for 854 and 1310 women, respectively, at year 1; for 698 and 1044 women, respectively, at year 3; and for 386 and 998 women, respectively, at year 5.

Table 4. Endpoints, including breast cancer recurrences, by intervention group

	Dietary intervention (n = 975)	Control (n = 1462)
Events		
Recurrence		
Local recurrence, No. (%)	3 (0.3)	8 (0.6)
Regional recurrence, No. (%)	6 (0.6)	12 (0.8)
Distant recurrence (except opposite breast), No. (%)	52 (5.3)	93 (6.4)
Ipsilateral breast recurrence in patients with lumpectomy, No. (%)	11 (1.1)	31 (2.1)
New breast cancer in the contralateral breast, No. (%)	24 (2.5)	37 (2.5)
Relapse-free survival events, total No. (%)*	96 (9.8)	181 (12.4)
Second primary cancer (excludes opposite breast cancer), No. (%)	28 (2.9)	50 (3.4)
Death (without breast cancer recurrence), No. (%)	15 (1.5)	19 (1.3)
Disease-free survival events, total No. (%)†	139 (14.3)	250 (17.1)

*Relapse-free survival events include local, regional, and distant recurrence, ipsilateral breast recurrence after lumpectomy, and contralateral breast cancer.

†Disease-free survival events include those for relapse-free survival and also include any secondary invasive cancer, excluding basal and squamous skin cancer, and death without breast cancer recurrence.

DISCUSSION

WINS is, to our knowledge, the first large-scale randomized trial to test whether a dietary intervention can improve the clinical outcome of women with breast cancer. The WINS results indicate that a lifestyle intervention designed to reduce dietary fat intake can be successfully implemented in women with early-stage, resected breast cancer receiving conventional cancer management in a multicenter clinical trial setting. After approximately 5 years of follow-up, women in the dietary intervention group had a 24% lower risk of relapse than those in the control group (HR = 0.76; 95% CI = 0.60 to 0.98). Although these results are suggestive of benefit for the dietary intervention, given the level of statistical significance ($P = .034$ for adjusted Cox model analysis), the higher relapse-free survival seen in the dietary group could also be a result of chance.

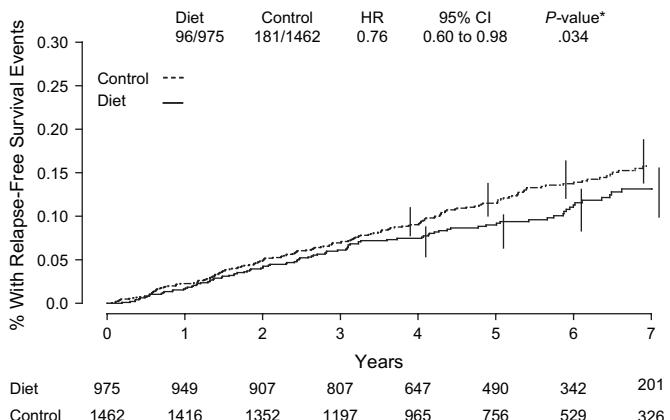


Fig. 2. Kaplan-Meier estimates of relapse-free survival. Number of events/number of patients in the dietary intervention and control groups are indicated. Hazard ratio (HR) and 95% confidence interval (CI) were calculated from adjusted Cox proportional hazard model comparisons of control to dietary intervention groups through the 60-month median follow-up period. P value is two-sided. Numbers of patients at risk are indicated below the graph.

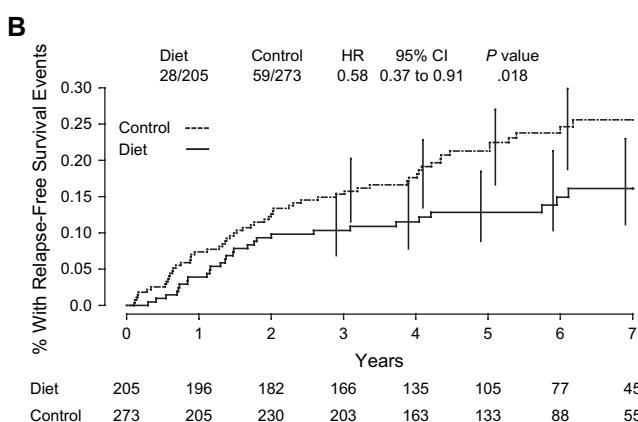
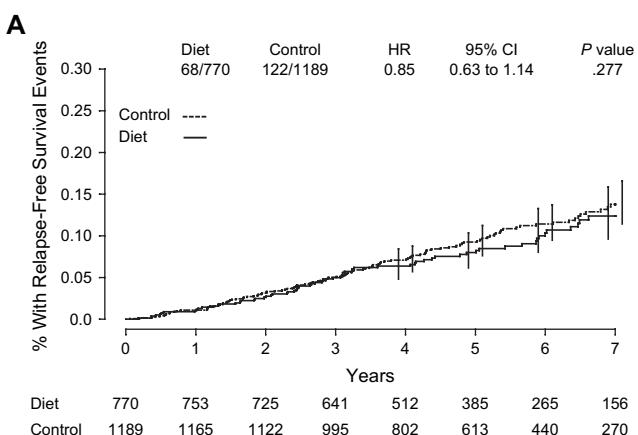


Fig. 3. Kaplan-Meier estimates of relapse-free survival. (A) Estrogen receptor-positive subjects. (B) Estrogen receptor-negative subjects. Number of events/number of patients in the dietary intervention and control groups are indicated. Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated from adjusted Cox proportional hazard model comparisons of control to dietary intervention groups through the 60-month median follow-up period. P values are two-sided. Numbers of patients at risk are indicated below the graph.

Secondary analyses suggested a stronger effect for dietary fat reduction on breast cancer recurrence in women with hormone receptor-negative cancers compared with women whose cancers were hormone receptor positive; however, the interaction between dietary intervention and hormone receptor status was not statistically significant. These findings are consistent with trends suggestive of a differential dietary influence by hormone receptor status on breast cancer incidence recently described in the Women's Health Initiative randomized primary prevention trial and in a Nurse's Health Study cohort (31,32). A differential influence of dietary fat on breast cancer recurrence based on hormone receptor status will require confirmation. However, some of the most active adjuvant breast cancer interventions, including aromatase inhibitors (21–24) and trastuzumab (33–35), are effective only in biologically defined subgroups, and interactions have been observed between ER status and adjuvant chemotherapy effect (36). Thus, it would not be surprising to find that a lifestyle intervention has an effect on only some categories of breast cancer.

Because ER-positive cancers are under estrogen influence, a predominant influence of diet on ER-negative cancers would implicate factors other than estrogen (37,38) as mediators. Other potential mechanisms include reduced insulin levels (39,40), reduced insulin resistance (41), reduced insulin-like growth

Table 5. Relapse-free survival, by baseline characteristics and intervention group*

Variable	Relapse events/total N		HR (95% CI)
	Dietary intervention	Control	
BMI†			
<25	33/371	55/563	0.83 (0.54 to 1.27)
25–30	33/325	62/484	0.77 (0.51 to 1.18)
>30	29/261	61/377	0.66 (0.42 to 1.04)
Axillary lymph nodes‡			
Positive	40/258	72/392	0.83 (0.57 to 1.21)
Negative	56/710	109/1062	0.77 (0.56 to 1.07)
ER§			
Positive	68/770	122/1189	0.85 (0.63 to 1.14)
Negative	28/205	59/273	0.58 (0.37 to 0.91)
PgR			
Positive	55/641	97/960	0.83 (0.59 to 1.15)
Negative	28/268	75/414	0.54 (0.35 to 0.83)
Receptor subgroups			
ER+, PgR+	49/598	90/921	0.83 (0.58 to 1.17)
ER+, PgR-	12/121	27/199	0.73 (0.37 to 1.46)
ER-, PgR+	6/43	7/39	0.57 (0.17 to 1.87)
ER-, PgR-	16/147	48/215	0.44 (0.25 to 0.77)

*HR = hazard ratio; CI = confidence interval; BMI = body mass index; ER = estrogen receptor; PgR = progesterone receptor.

†BMI = weight in kg/height in m². Adjusted for nodal status (positive or negative), systemic adjuvant therapy (chemotherapy alone, tamoxifen alone, or chemotherapy plus tamoxifen), ER status (positive or negative), tumor size (<2 or ≥2 cm), and mastectomy (yes or no); excludes 56 women without baseline body weight measurements.

‡Adjusted for systemic adjuvant therapy, ER status, tumor size, and mastectomy; excludes 15 women with no axillary node dissection.

§Adjusted for systemic adjuvant therapy, nodal status, tumor size, and mastectomy.

||Excludes 154 patients with no PgR information or who were classified as borderline PgR by their local laboratory; adjusted for systemic adjuvant therapy, nodal status, tumor size, and mastectomy.

factor 1 (42,43), or reduced inflammation markers—all factors that may be influenced by dietary fat decrease and/or weight loss (44,45). Planned analyses of serial fasting blood samples in the two groups will address these issues.

The most appropriate endpoint for breast cancer adjuvant trials is controversial, and even the definitions of these endpoints are inconsistent (46). Endpoints from recent selected adjuvant therapy trials are compared to the relapse-free survival endpoint of the WINS trial in Table 6. Collectively, these reports define the endpoint of “disease-free survival” in four different ways (21–24,27). We defined relapse-free survival, the prospectively identified primary study endpoint in WINS, in the same way that Goss et al. (22) recently defined a disease-free survival endpoint.

To facilitate comparison of our study with others, we included death without breast cancer recurrence and second primary cancers as events in additional analyses; all results led to similar conclusions regarding the effects of the dietary intervention. Establishment of a common terminology for breast cancer adjuvant trial endpoints should be a future priority of the research community.

This study has several potential limitations. One is the imbalance of surgical treatments between the groups: 5.6% more women in the control group than in the intervention group had breast-conserving therapy. As a result, 55 more women in the control group with such surgery were at risk for an ipsilateral recurrence. However, considering all 1018 control group women with breast-conserving surgery, only 2.1% experienced ipsilateral recurrence. Consequently, only about one additional in-breast recurrence would be anticipated based on the surgical distribution. Thus, the modest imbalance in surgical management is unlikely to explain the difference in clinical outcome that we observed. In addition, in a large National Surgical Adjuvant Breast and Bowel Project Cancer randomized trial comparing mastectomy with lumpectomy plus radiation, women with mastectomy had a similar rate of local and regional recurrences after approximately 5 years of follow-up, even when ipsilateral breast recurrences were included (45 such recurrences were seen in the mastectomy group compared with 43 in the lumpectomy plus radiation group) (47). Moreover, our adjustment for surgery type (mastectomy versus lumpectomy) in the Cox proportional hazards models should have controlled for the effect of the differences in surgical management.

A second limitation of the study is its reliance on self-report of dietary intake because no validated “gold standard” exists for assessing dietary fat intake (48). However, body weight was statistically significantly lower in the dietary group, providing biologic plausibility that a dietary change did occur in women on the low-fat eating plan. Although the dietary intervention focused on reducing fat intake, intake of other nutrients changed, as did body weight. Thus, it is possible that weight change (49,50) and/or dietary factors other than fat intake influenced the breast cancer outcome. Planned future analyses will examine time trends and associations of changes in body weight and other dietary factors with breast cancer recurrence.

Study strengths include the randomized study design, closely comparable anticancer systemic therapies provided to women in the two randomization groups, and the degree of dietary adherence achieved by study participants. The reduction in

Table 6. Primary study endpoints in selected adjuvant therapy trials in early breast cancer*

Endpoint: as defined in each trial	Group/trial†	Local-regional recurrence	Distant recurrence	Ipsilateral breast tumor recurrence‡	Contralateral breast cancer	Death without recurrence	New primary cancer
Relapse-free survival	WINS	X	X	X	X	O	O
Disease-free survival	MA-17	X	X	X	X	O	O
Disease-free survival	IES	X	X	X	X	X	O
Recurrence-free survival	ATAC	X	X	X	X	X	O
Disease-free survival	NSABP	X	X	O	X	X	X
Disease-free survival	BIG 1-98	X	X	X	X	X	X

*An “X” indicates the endpoint was included in the definition, an “O” indicates the endpoint was not included in the definition.

†WINS = Women’s Intervention Nutrition Study; MA-17 = Mammary Study-17 (22); IES = International Exemestane Study (23); ATAC = Anastrozole, Tamoxifen Alone, and Combined (21); NSABP = National Surgical Adjuvant Breast and Bowel Project (15,27); BIG 1-98 = Breast International Group (24).

‡Among patients with lumpectomy.

dietary fat intake seen in the WINS trial is similar to or greater than that reported in other trials evaluating breast cancer and diet associations either for primary prevention (31,51) or recurrence (52).

Replication in clinical practice of this dietary intervention will likely require on-going counseling with a dietician trained in these techniques. The WINS low-fat eating plan was intensive, individualized, and delivered using a standardized protocol by registered dieticians who had received centralized training which included motivational interviewing techniques.

In summary, an interim efficacy analysis suggests that a lifestyle intervention reducing dietary fat intake with modest body weight loss may improve the relapse-free survival of postmenopausal breast cancer patients. Ongoing follow-up will address original protocol design plans calling for 3 years of follow-up after completion of recruitment.

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NOTES

The following investigators were members of the WINS: Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center—R. T. Chlebowski, L. Lillington, M. Jackson; Institute for Cancer Prevention—E. L. Wynder (deceased), D. W. Nixon, B. Winters, K. Hoy, J. Richie; Beth Israel Deaconess Medical Center—G. L. Blackburn, T. Copeland; University of California Los Angeles—R. Elashoff, T. Johnson, W. Liu, M. N. Brooks; John Wayne Cancer Institute—A. Giuliano; Cedars-Sinai Medical Center—P. McAndrew; University of California Irvine Clinical Cancer Center—J. Butler; University of Hawaii—M. Goodman; Kaiser Permanente Medical Group, Oakland—B. Caan; Nutrition Information Consultants—K. Storch; North Shore University Hospital—V. Vinciguerra; Saint Barnabas Medical Center—R. Michaelson; Evanston Hospital, Kellogg Cancer Care Center—D. Merkel; Memorial-Sloan Kettering Cancer Center—C. Hudis; Lombardi Cancer Research Center—C. Issacs; Rhode Island Hospital—M. Winkler; Wayne State University—M. Simon; University of Arizona—C. Thomson; Fred Hutchinson Cancer Research Center—A. Kristal; Kaiser Permanente Center for Health Research, Portland—K. Karanja; Ohio State University—W. Farrar; Medical University of South Carolina—R. Hall; Duke University—W. Demark-Wahnefried; Bennett Cancer Center Hematology and Oncology—S. Del Prete; Geisinger Medical Center—A. Bernath; Park Nicollet Institute Oncology Research—A. Shapiro; Medical College of Wisconsin—G. Schechtman; Christus Spohn Breast Care Program—E. Salloum; Good Samaritan Medical Center—E. McKeen; University of Miami—G. Shor-Posner; Midwestern Regional Medical Center—P. Vashi; University of Iowa—L. Snetselaar; Bay State Medical Center—G. Markari-Judson.

The following nutritionists delivered the WINS dietary intervention: N. Adamowicz, L. Ahrens, S. Anderson, M. Beddome, R. Beller, K. Bierl, D. Brake, A. Campa, M. Chrabszewski, C. Coy, S. Dahlk, F. Ebel, N. Falla, E. Falk, S. Anikstein Feldman, J. Ferrares, V. Fortunato, J. Galicinao, A. Gallagher, P. Gregory, S. Heilman, W. Hirsch, M. Horan, P. Jensen, L. Kelleher, K. Lombardi, M. Lubin, A. M. Maggi, M. Malone, S. Marshall, V. Marsoobian, D. Moran, K. Mulligan, E. Nardi, L. Nylin, J. T. Papoutsakis, J. Pleuss, K. Radokovich, J. Reddan, C. Rheingrubler, C. Richardson, J. Schenk, K. Schwab, S. Shannon, L. Shepard, S. Sloan, M. Sokil, D. Strong, A. Snyder, M. Vides, B. Whilden, C. Willeford, D. Wilson, L. Wolf, R. Zinaman.

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The following investigators were members of the WINS External Advisory Committee: Sunnybrook Health Science Center—K. Pritchard, Chair; Mt Sinai Hospital—P. Goodwin; Tufts University—J. Dwyer; University of Hawaii—L. Kolonel; Harvard University—W. Willett; and Cornell University—B. Turnbull.

The following investigators were members of the Pathology Committee: R. Chlebowski, Chair, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center; M. Sanchez, Englewood Hospital; and T. Ahmed, New York Medical College.

This study was primarily funded by the National Cancer Institute, National Institutes of Health, Department of Health and Human Services. Funding for supplemental projects was provided by the Breast Cancer Research Foundation and the American Institute for Cancer Research. This study was supported by an investigator-initiated RO1 grant. The WINS investigators (with input from the independent WINS External Advisory Committee) were solely responsible for the design; data collection, analysis, and interpretation; writing of the manuscript; and decision to submit for publication.

Manuscript received March 27, 2006; revised October 4, 2006; accepted October 24, 2006.

Effect of processed and red meat on endogenous nitrosation and DNA damage

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Haem in red meat (RM) stimulates the endogenous production of mutagenic nitroso compounds (NOC). Processed (nitrite-preserved red) meat additionally contains high concentrations of preformed NOC. In two studies, of a fresh RM versus a vegetarian (VEG) diet (six males and six females) and of a nitrite-preserved red meat (PM) versus a VEG diet (5 males and 11 females), we investigated whether processing of meat might increase colorectal cancer risk by stimulating nitrosation and DNA damage. Meat diets contained 420 g (males) or 366 g (females) meat/day. Faecal homogenates from day 10 onwards were analysed for haem and NOC and associated supernatants for genotoxicity. Means are adjusted for differences in male to female ratios between studies. Faecal NOC concentrations on VEG diets were low (2.6 and 3.5 mmol/g) but significantly higher on meat diets (PM 175 ± 19 nmol/g versus RM 185 ± 22 nmol/g; $P = 0.75$). The RM diet resulted in a larger proportion of nitrosyl iron (RM 78% versus PM 54%; $P < 0.0001$) and less nitrosothiols (RM 12% versus PM 19%; $P < 0.01$) and other NOC (RM 10% versus PM 27%; $P < 0.0001$). There was no statistically significant difference in DNA breaks induced by faecal water (FW) following PM and RM diets ($P = 0.80$). However, PM resulted in higher levels of oxidized pyrimidines ($P < 0.05$). Surprisingly, VEG diets resulted in significantly more FW-induced DNA strand breaks than the meat diets ($P < 0.05$), which needs to be clarified in further studies. Meats cured with nitrite have the same effect as fresh RM on endogenous nitrosation but show increased FW-induced oxidative DNA damage.

Introduction

Red and processed (nitrite-preserved red) meat (PM) increase the risk of colorectal cancer with PM showing higher risk estimates per gram of intake than red meat (RM) (1–3). Heterocyclic amines—known carcinogens (4)—are formed during cooking of meat at high temperatures, but this is not specific for red and PM (5).

RM intake shows a dose-response relation with the endogenous formation of nitroso compounds (NOC), whereas there is no such relation for white meat (6,7). This is probably due to the abundant presence of haem in RM (8,9), which can readily become nitrosylated and act as a nitrosating agent (10). Recently, we confirmed that nitrosyl iron (FeNO) is the main contributor to high RM diet-induced

Abbreviations: CI, confidence interval; EDTA, ethylenediaminetetraacetic acid; EndoIII, endonuclease III; FW, faecal water; FeNO, nitrosyl iron; FPG, formamidopyrimidine; MTT, mean transit time; NOC, nitroso compounds; PM, processed (nitrite-preserved red) meat; RM, red meat; RSNO, nitrosothiols; VEG, vegetarian.

endogenous formation of NOC (11). In addition, nitrosothiols (RSNO) are rapidly formed from nitrite and thiol groups at low pH in the stomach and can be precursors for the endogenous formation of nitrosyl haem and other NOC, such as *N*- and *O*-NOC, in the small and large bowel (11,12).

Many NOC are known carcinogens and the alkylation of DNA can induce G to A transitions in genes mutated in colorectal cancer such as *ras* (13). The NOC-specific DNA adduct O^6 -carboxymethyl-2'-deoxy-guanosine increases in exfoliated colonic cells from volunteers fed a high RM diet (14). O^6 -carboxymethyl-2'-deoxy-guanosine is not repaired by O^6 -alkylguanine transferase in *in vitro* assays and may at least partly explain the link between meat consumption and colorectal cancer.

PM is a mixed category of meats that are preserved by a variety of mechanical, chemical or enzymatic procedures. Different preservatives, such as nitrite and nitrate in ham and sulphur dioxide in sausages, may be used. Minced products such as hamburgers may or may not be classified as PM. In most populations on which existing epidemiological findings are based, PM mainly consists of processed RMs such as beef and pork (3,15).

Supplements of nitrate have been shown to increase faecal NOC levels (16), but it is not clear whether preservation by this method would result in an increased endogenous NOC production, and hence explain some of the higher colorectal cancer risk with processed as compared with RM. In two studies, of a fresh RM (beef and pork) versus a vegetarian (VEG) diet—referred to as the RM study—and of a nitrite-preserved red meat (bacon, ham, luncheon meat and corned beef) versus a VEG diet—referred to as the PM study—we investigated whether this form of processing might increase colorectal cancer risk by stimulating nitrosation and DNA damage.

Methods

Subjects

Healthy males and females from Cambridgeshire were recruited through local advertisements. Participants had to be between 20 and 85 years of age, non-smokers, free from diabetes and bowel disease, not taking medication affecting the gut for at least 3 months prior to the study, not pregnant and not participating in another biochemical intervention study at the same time. Prior to the study, subjects were examined by a medical practitioner.

All subjects received verbal and written information and signed a written consent form. The studies were approved by the Cambridge Local Research Ethics Committee.

Sixteen volunteers were included in the PM study (5 males and 11 females); 12 volunteers participated in the RM study (six males and six females), which has been described previously (14).

Study design

The studies had a randomized crossover design of a high meat versus a VEG period: PM versus VEG in the PM study and RM versus VEG in the RM study. Each dietary period lasted at least 14 days. Subjects lived in the volunteer suite of the MRC Dunn Human Nutrition Unit, where all food was provided and carefully controlled and all specimens could be collected and processed immediately. Subjects followed their normal routine but were only allowed to consume foods and drinks prepared by the diet technicians. Body weights were monitored to ensure a constant weight throughout. Faecal samples were collected and weighed daily and radio opaque marker capsules were taken throughout to check compliance and for the measurement of transit time (17). After subjects had consumed each diet for 10 days, stools were collected on dry ice for analysis of NOC, haem and genotoxicity.

Study diets

All diets were provided as similar menus on a 3 day rotating schedule. Male PM diets contained 420 g PM per day [given as 100 g bacon (days 1 and 2) or pork luncheon meat (day 3) at lunch and 320 g corned beef (days 1 and 2) or gammon (day 3) at dinner] and female PM diets contained 366 g PM per day [given as 60 g bacon or pork luncheon meat at lunch and 306 g corned beef or gammon at dinner]. RM diets contained the same amount of meat, but lunch was roast beef (days 1, 2 and 3) and dinner beef mince (days 1 and 3) or pork (day 2). Over the course of the study, each volunteer consumed the same amount of each type of meat.

Energy and macronutrient composition of the diets (Appendix) were calculated using Data Into Nutrients for Epidemiological Research (DINER) (18). Fat content of the diets was kept constant by exchanging protein for carbohydrates. Energy requirements were estimated from body weight and physical activity using standard equations for basal metabolic rate and estimates of physical activity level (19). The energy intake of each participant was matched to estimated energy requirement with 1 MJ standardized increments (chocolate bar, shortbread or a combination of white bread, butter and marmalade) added to 8 MJ/day (female) or 10 MJ/day (males) basal diets.

Meat was not overcooked to minimize the formation of heterocyclic amines. Purified water was given throughout for drinking and used for cooking and low-nitrate vegetables were used to keep nitrate intake at constant low levels. Tea, coffee and an aspartame-based sweetener were provided in the suite and consumed freely, but subjects were asked to keep their intake constant during the study.

Dietary and faecal NOC and haem

Duplicates for each daily diet were prepared. All foods consumed on 1 day were prepared as normal, added together and diluted 1:2 in ultrapure water. The mixture was homogenized with a food processor, snap frozen on dry ice and stored at -20°C until analysis. Results are presented per gram of diet after correction for dilution. For analysis of the separate nitrite-preserved red meats consumed in the PM study, meats were cooked as normal and homogenized and frozen separately as described above. Results are presented per gram of cooked weight after correction for dilution.

For stool analysis, ~40 g of frozen stool was thawed, diluted 1:5 in ultrapure water and homogenized for 20 min in a stomacher (Colworth 3500, Seward Medical, London, UK). The faecal homogenates were snap frozen on dry ice and stored at -20°C until analysis. Results are presented per gram of faeces after correction for dilution.

NOC were analysed using a modification of the method previously used (11), using an Ecomedics CLD 88 Exhalizer (Ecomedics, Duernten, Switzerland). Approximately 100 µl of faecal homogenates or 500 µl of homogenized diet or meat were weighed and incubated briefly with 100 µl of an aqueous solution of *N*-ethylmaleimide (50 mM) and diethylene triamine pentaacetic acid (100 µM) to protect RSNO. Thereafter, 500 µl of a 5% (wt/vol) sulfamic acid solution was added to remove nitrite and samples were injected into a purge vessel kept at 60°C and filled with a standard tri-iodide reagent (20) (38 mg I₂ was added to a solution of 108 mg KI in 1 ml water; to this mixture, 13.5 ml glacial acetic acid was added) to determine total NOC. To determine mercury(II) stable compounds, 100 µl 10 mM aqueous HgCl₂ was added prior to analysis; to determine mercury(II) and ferricyanide stable compounds, 100 µl each of 10 mM aqueous HgCl₂ and 10 mM aqueous K₃Fe(CN)₆ solution were added prior to analysis. RSNO were determined as the difference between total NOC and mercury(II) stable NOC; FeNO was determined as difference between mercury(II) stable NOC and mercury(II) and K₃Fe(CN)₆ stable compounds. Other NOC were determined as mercury(II) and K₃Fe(CN)₆ stable compounds.

Haem was analysed using the HemoQuant assay (8) as described previously (11). Briefly, 400 µl hot oxalic acid reagent (3 M oxalic acid, 0.1 M FeSO₄, 60 mM uric acid and 60 mM mannitol) was added to ~500 mg dietary or faecal homogenate, mixed and incubated for 30 min at 100°C. After cooling, 1 ml 3 M KAc and 3 ml acetate/acetic acid (10/1 vol/vol) were added. To 2 ml of the organic phase, 0.8 ml *n*-butanol and 6 ml 3 M KAc in 1 M KOH was added, mixed and 1 ml of the organic phase subsequently extracted with 4 ml phosphoric acid/acetic acid (2 M phosphoric acid:glacial acetic acid, 9:1 vol/vol). Fluorescence (excitation, 402 nm; fluorescence, 600 nm) of the aqueous phase was determined using a Spectramax Gemini XS fluorimeter (Molecular Devices, Sunnyvale, CA). To quantify samples, dietary and faecal homogenates were spiked with haemoglobin to obtain a calibration curve. Results are expressed as mmol/d for diets or nmol/g for faeces.

Faecal water genotoxicity with the comet assay

Faecal water (FW) was prepared by centrifuging ~25 g of thawed faecal homogenate at 50 000g for 2 h at 4°C. The clear supernatant was aliquoted, snap frozen on dry ice and stored at -80°C until analysis.

Caco2 cells (European Collection of Cell Cultures, Salisbury, UK) were cultured as monolayers in Eagle's Minimum Essential Medium containing 10% foetal bovine serum, 2 mM L-glutamine, 1% non-essential amino acids and 100 U/l penicillin-streptomycin (all Sigma-Aldrich, Gillingham, UK). Cells were harvested after a 3 min incubation at 37°C with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich) and resuspended in non-supplemented Eagle's Minimum Essential Medium. Three hundred microlites of cell suspension (~12 × 10⁴ cells) was incubated with 300 µl FW (50%) for 30 min at 37°C. A quality control was included in each experiment. Samples were measured in duplicate in separate experiments.

Cells were centrifuged at 200g for 3 min at 4°C, the supernatant was discarded and cells were resuspended in 420 µl 1% low melting point agarose made in phosphate-buffered saline. Cells from one treatment were divided as roughly equal drops of ~70 µl over three microscope slides (two gels per slide), a 22 × 22 mm coverslip was applied and gels were allowed to set at 4°C. Coverslips were removed and cells were lysed in 2.5 M NaCl; 0.1 M Na₂EDTA; 10 mM Tris-HCl, pH 10 and 1% Triton X-100 for 1 h at 4°C. Microscope slides had been precoated with 1% normal melting point agarose and left to dry before use.

Slides were rinsed in enzyme buffer (40 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 0.1 M KCl, 0.5 mM EDTA and 0.2 mg/ml bovine serum albumin, pH 8) three times for 5 min each at 4°C. Per treatment, one slide was then incubated with enzyme buffer alone, one with endonuclease III (EndoIII) in buffer and one with formamidopyrimidine (FPG) in buffer for 30 min at 37°C. EndoIII recognizes oxidized pyrimidines and FPG recognizes altered purines, creating additional DNA strand breaks at these sites.

Subsequently, the DNA was allowed to unwind in electrophoresis solution (0.3 M NaOH and 1 mM Na₂EDTA, pH 13) for 40 min at 4°C before electrophoresis at 0.9 V/cm, 300 mA for 30 min at 4°C. Slides were neutralized in phosphate-buffered saline and rinsed in ultrapure water for 10 min each at 4°C and left to dry overnight at room temperature.

Gels were stained with 4',6-diamidino-2-phenylindole and viewed by fluorescence microscopy. Comets were classified with visual scoring (21); 100 randomly selected comets were classified into five classes (0–4) according to the extent of tail DNA to give an overall score of between 0 and 400 arbitrary units per treatment.

Statistical analysis

Faecal nitrite values and transit times were ¹⁰log transformed to normalize the distribution and are presented as geometric means and antilogged 95% confidence intervals (CIs). Other results are presented as mean ± SE. Meat versus VEG results were compared within each study using a paired Student's *t*-test (two tailed). Statistical differences between PM and RM diets (between studies) were analysed by analysis of covariance with type of meat (PM or RM) as independent variable and gender as dichotomous covariate to adjust for differences in male to female ratios between the two studies. Where PM and RM results are compared (between studies), results are presented as adjusted mean ± SE. Where meat and VEG results are compared (within studies), results are presented as unadjusted means.

Associations between continuous variables were tested using Pearson's correlation coefficient (*r*). *P* < 0.05 was considered statistically significant. SPSS 16 for Macintosh (SPSS, Chicago, IL, 2008) was used for the analysis.

Results

Dietary NOC and haem

Only male diets were analysed as they contained the largest amount of meat. PM diets contained 58 ± 15 mmol/d total NOC, composed of 24 ± 8 mmol/d RSNO, 20 ± 6 mmol/d FeNO and 14 ± 2 mmol/d other NOC and 17 ± 10 µmol/d nitrite. The NOC composition of the processed meats, i.e. bacon, corned beef, gammon and pork luncheon meat, is shown in Table I. RM and VEG diets contained negligible amounts of NOC and nitrite.

Table I. NOC composition (nmol/g cooked weight) of the nitrite-preserved red meats consumed in the PM study

	Total NOC	RSNO	FeNO	Other NOC
Bacon	428	357	45	26
Corned beef	110	26	42	42
Gammon	84	15	50	19
Pork luncheon meat	58	17	27	14

PM diets contained similar amounts of haem (86 mmol/d) to RM diets (110 mmol/d). The haem content of the VEG diets was negligible.

Faecal NOC and haem

Faecal levels of NOC on VEG diets were very low but increased significantly on both PM and RM diets (Table II). There was no statistically significant difference in total NOC concentrations on the PM and RM diets (adjusted means PM 175 ± 19 nmol/g versus RM 185 ± 22 nmol/g; $P = 0.75$). FeNO was the main contributor to total NOC on both meat diets although the RM diet resulted in a larger proportion of FeNO (adjusted means RM 78% versus PM 54%; $P < 0.0001$) and less RSNO (adjusted means RM 12% versus PM 19%; $P < 0.01$) and other NOC (adjusted means RM 10% versus PM 27%; $P < 0.0001$) compared with PM. Similar results were obtained when absolute concentrations were used.

NOC concentrations in FW used in the comet assay were $\sim 1.7\%$ of the concentration in homogenates before correction for dilution (PM 0.65 nmol/g and RM 0.41 nmol/g).

There was no statistically significant difference between adjusted mean faecal nitrite concentrations on the PM diet (19 nmol/g, 95% CI 11–35) and those on the RM diet (28 nmol/g, 95% CI 14–56, $P = 0.41$). Concentrations on both meat diets were significantly higher than after VEG diets (3 nmol/g, 95% CI 2–6, $P < 0.0001$). There was no statistically significant correlation between concentrations of faecal nitrite and total NOC.

Faecal haem was significantly higher when the meat diets were consumed as compared with the VEG diets ($P < 0.0001$, Table II) although to a lesser extent on the PM diet compared with the RM diet (adjusted means PM 326 ± 69 nmol/g versus RM 1033 ± 84 nmol/g, $n = 11$; $P < 0.0001$). Haem concentrations were positively related with FeNO concentrations on the RM diet ($r = 0.63$, $P < 0.05$; $n = 11$) and marginally on the PM diet ($r = 0.45$, $P = 0.08$). Haem concentrations were positively related to RSNO on the RM diet ($r = 0.64$, $P < 0.05$; $n = 11$) but not on the PM diet ($r = 0.15$, $P = 0.58$).

FW genotoxicity with the comet assay

There was no statistically significant difference in DNA breaks induced in Caco2 cells by FW from both PM and RM diets (adjusted means PM 167 ± 12 versus RM 163 ± 13 ; $P = 0.80$). However, PM resulted in higher levels of EndoIII-sensitive sites (adjusted means PM 24 ± 5 versus RM 9 ± 5 ; $P < 0.05$) and a larger variation in FPG-sensitive sites compared with RM (Figure 1A). Surprisingly, FW from both dietary studies induced significantly more DNA strand breaks on the VEG diets compared with the meat diets ($P < 0.05$; Table III).

In the comet assay, FPG detects oxidatively damaged purines but also seems to have a high sensitivity towards N-7 alkylation of guanine (ring opened) (22). There was no statistically significant correlation between FPG-sensitive sites and NOC concentrations in faecal homogenates on both meat diets, but NOC concentrations in FW—despite being relatively low—trended towards a positive association with FPG-sensitive sites on the PM ($r = 0.55$, $P = 0.06$; $n = 12$, three samples not analysed, one sample with no peak in assay) but not on the RM diet ($r = 0.25$, $P = 0.49$; $n = 10$, two samples no peak in assay) (Figure 1A). There were no significant associations with DNA strand breaks and EndoIII-sensitive sites (Figure 1B). No NOC could be detected in FW on the VEG diets. Nitrite concentrations in FW were $\sim 18\%$ of those in faecal homogenates (PM study only, RM not analysed); there were no statistically significant associations with DNA strand breaks or enzyme-sensitive sites.

Mean transit time

Mean transit time (MTT) on the PM diet (adjusted geometric mean 44 h, 95% CI 35–55; $n = 15$) was significantly lower than on the RM diet (adjusted geometric mean 70 h, 95% CI 55–90; $P < 0.01$). MTT during both VEG periods were similar (VEG in PM study 44 h, 95% CI 35–55; $n = 15$ versus VEG in RM study 55 h, 95% CI 43–70; $P = 0.18$) and not significantly different from their respective meat periods (PM study $P = 0.80$ and RM study $P = 0.07$).

MTT was positively related with total NOC concentrations ($r = 0.63$, $P = 0.01$; $n = 15$), FeNO ($r = 0.60$, $P = 0.02$; $n = 15$) and other NOC ($r = 0.74$, $P < 0.01$; $n = 15$) but not with RSNO ($r = 0.13$, $P = 0.64$; $n = 15$) on the PM diet. In contrast, there were no statistically significant correlations between MTT and NOC concentrations on the RM diet ($P > 0.25$).

Daily faecal weight, calculated as the mean faecal weight over the last 4 days of the diet, was significantly higher on the VEG diet compared with the meat diet in both studies (PM 167.5 ± 11.9 g/d versus VEG 223.8 ± 24.1 g/d; $P < 0.05$ and RM 148.1 ± 19.5 g/d versus VEG 308.3 ± 41.4 g/d; $P < 0.0001$). There were no statistically significant differences between studies ($P > 0.12$). Daily faecal weight was not statistically significantly associated with MTT on any of the diets or for both studies in total ($P > 0.09$).

Discussion

Recently, we implicated haem as a facilitator of the endogenous formation of NOC from dietary nitrite or nitrate and nitrite from

Table II. Faecal NOC concentrations (nmol/g) (a) PM diet versus VEG diet ($n = 16$); (b) RM diet versus VEG diet ($n = 12$) (unadjusted means, comparison within studies)

	Intake	Excretion		Intake	Excretion	
	mmol/d	nmol/g	mmol/d	mmol/d	nmol/g	mmol/d
(a)	PM			VEG		
Haem	86	329 ± 41	0.05 ± 0.007	a	$61 \pm 5^*$	$0.01 \pm 0.001^*$
Total NOC	58	181 ± 20	28.6 ± 2.9	a	$2.6 \pm 0.3^*$	$0.6 \pm 0.06^*$
RSNO	24	33 ± 4	5.2 ± 0.6	a	$0.2 \pm 0.1^*$	$0.05 \pm 0.02^*$
FeNO	20	95 ± 9	15.3 ± 1.6	a	$2.0 \pm 0.3^*$	$0.4 \pm 0.05^*$
Other NOC	14	53 ± 8	8.1 ± 1.2	a	$0.5 \pm 0.1^*$	$0.1 \pm 0.02^*$
(b)	RM			VEG		
Haem ($n = 11$)	110	1028 ± 109	0.13 ± 0.02	a	$63 \pm 11^*$	$0.02 \pm 0.005^*$
Total NOC	a	177 ± 26	21.7 ± 2.2	a	$3.5 \pm 0.7^*$	$1.0 \pm 0.2^*$
RSNO	a	19 ± 4	2.3 ± 0.3	a	$0.4 \pm 0.1^*$	$0.1 \pm 0.02^*$
FeNO	a	140 ± 20	17.1 ± 1.9	a	$1.8 \pm 0.3^*$	$0.5 \pm 0.1^*$
Other NOC	a	18 ± 4	2.2 ± 0.5	a	$1.2 \pm 0.4^{**}$	$0.3 \pm 0.08^{**}$

Only male diets analysed. Excretion measured in faecal homogenates and adjusted for dilution. P values for paired Students' t -test of meat versus VEG,

* $P < 0.0001$, ** $P = 0.001$, *** $P < 0.01$.

^aNegligible amounts.

PM, processed (nitrite-preserved red) meat.

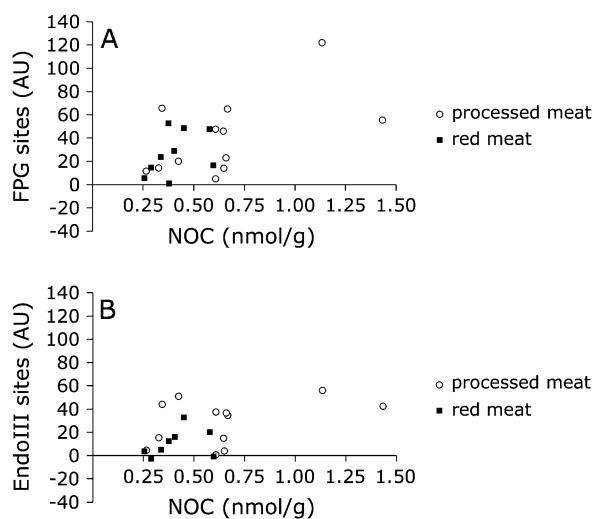


Fig. 1. FW-induced FPG-sensitive sites (A) and EndoIII-sensitive sites (B) in Caco2 cells measured with the comet assay versus total NOC concentrations in FW. Processed meat diet ($n = 13$, open circles) and RM diet ($n = 9$, filled squares).

Table III. Level of FW-induced DNA strand breaks, EndoIII- and FPG-sensitive sites in Caco2 cells measured with the comet assay (a) PM diet versus VEG diet ($n = 16$); (b) RM diet versus VEG diet ($n = 12$) (unadjusted means, comparison within studies)

	Mean	SE	Mean	SE	Mean	SE
(a)	Control slides		PM		VEG	
Strand breaks (AU)	113	3	164	13	206*	17
EndoIII sites (AU)	8	2	24	5	26	6
FPG sites (AU)	25	5	39	8	42	7
(b)	Control slides		RM		VEG	
Strand breaks (AU)	113	3	167	13	196*	15
EndoIII sites (AU)	8	2	9	4	15	6
FPG sites (AU)	25	5	24	6	28	4

AU, arbitrary units; PM, processed (nitrite-preserved red) meat. P value for paired Students' t -test of meat versus VEG,

* $P < 0.05$. Control slides were incubated with phosphate-buffered saline and represent the level of background DNA strand breaks.

inducible and endogenous nitric oxide synthases (9,11,23). Similar to our previous studies, faecal NOC levels were low (3–4 nmol/g) on diets containing no meat and a negligible amount of haem. On nitrite-preserved (PM) and fresh RM diets containing similar amounts of haem (86–110 mmol/d), faecal NOC levels increase significantly to ~180 nmol/g.

Dietary NOC in the PM diet (58 mmol/d) was in the same order of magnitude or greater as excretion, whereas NOC content of the RM diet was negligible but resulted in similar levels of excretion in faeces as on the PM diet (adjusted means PM 28.4 ± 2.6 mmol/d and RM 22.0 ± 3.0 mmol/d). However, on a RM diet, faecal FeNO represent a greater proportion of total NOC compared with the PM diet and concentrations are positively associated with haem concentrations. In addition, on both meat diets, concentrations of FeNO were significantly higher than those of RSNO, which confirms the important contribution of haem to endogenous NOC production (9).

Mirvish *et al.* (24) studied mice fed hot dogs with or without NaNO₂ and concluded that faecal NOC excretion represents mainly excretion of ingested NOC and of NOC formed by nitrosation of precursor NOC by nitrite. In contrast, our results suggest that faecal NOC are unlikely to be simply derived from the diet and are mainly formed endogenously. However, the preformed NOC present in our study diets are not to be confused with the precursor NOC analysed in

faeces by Mirvish *et al.* (24), which were defined as NOC that were formed after *in vitro* nitrosation of faecal supernatant.

The endogenous formation of NOC is likely to begin with the formation of RSNO in the stomach, as acidic conditions facilitate the formation of these compounds (11). These compounds can then promote the formation of other NOC, in particular nitrosyl haem, in the anaerobic and reducing environment of the gastrointestinal tract. Considering the importance of NOC production along the gastrointestinal tract, the association between MTT and faecal NOC concentrations was investigated in the meat diets. The statistically significant association between MTT and faecal NOC concentrations on the PM diet—in particular for FeNO compounds—supports the hypothesis that these compounds are formed in the anaerobic conditions of the small and large intestines. However, the lack of association following the RM diet with a significantly longer transit times suggests a threshold effect.

The alkaline comet assay detects DNA strand breaks and alkali-labile sites, i.e. apurinic or apyrimidinic sites or baseless sugars. These can result from a variety of damage and might also represent intermediates in the repair process. Including DNA glycosylase enzymes results in excision of oxidatively damaged nucleobases from the DNA strand, which leaves additional strand breaks (21). Despite the different composition of faecal NOC on PM and RM diets, there was no clear effect on FW-induced DNA strand breaks, but PM resulted in significantly more FW-induced EndoIII-sensitive sites. However, due to the very low concentrations of total NOC in the FW, we were not able to analyse NOC composition and hence cannot relate NOC composition of the FW and the faecal homogenates. We have currently no explanation for the unexpected higher level of DNA strand breaks on the VEG diets as compared with the high meat diets, which is intriguing given a recent study showing a higher colorectal cancer incidence in vegetarians than in meat eaters (25). Nevertheless, further studies are underway exchanging red or PM with white meat or fish to eliminate possible interfering plant constituents, e.g. polyphenols. Dietary analysis of total phenols using the Folin-Ciolalteu method showed similar levels of phenols in our meat and VEG study diets (data not shown), but this test does not distinguish between different kinds of polyphenolic compounds. However, we have not been able as yet to obtain reliable estimates for FW levels with this method.

The aqueous fraction of faeces, prepared by either direct ultracentrifugation, dilution in phosphate-buffered saline or ultrapure water or reconstitution of freeze-dried faeces, has been shown to be genotoxic in several colonic cell lines (26–30). Previous studies, the most recent ones using nuclear magnetic resonance profiling, have identified a number of compounds in FW including short chain fatty acids, organic acids, phenolic compounds and amino acids. Inter- and intra-individual differences are related to variation in concentrations rather than composition, which in turn seem to be related to diet (31,32). However, still little is known about the nature of FW, which may explain inconsistent results when investigating the effect of diet on DNA damage using the comet assay (26,27,29).

Colonic cells have been shown to be susceptible to NOC-induced damage. Potassium diazoacetate, a stable nitrosated derivative of glycine, shows a dose-response effect on genotoxicity in Caco2 cells, human lymphocytes and rat colonocytes (33) and *N*-nitrosomorpholine causes a dose-dependent increase in DNA strand breaks in Caco2 cells, all of which originated from alkali-labile sites (34). No oxidative damage was observed, although pretreatment with vitamins E and C reduced the formation of strand breaks (34). Our results showed the presence of altered purines and pyrimidines induced by FW, with higher levels of oxidized pyrimidines on the PM diet compared with the RM diet. Despite the NOC levels in FW being very low compared with the faecal homogenates, they tended to be positively related to altered purines on the PM diet indicating that they are related to oxidative damage.

Nitrite intake from PM was 0.8 mg/d (17 µmol/d) in our study. Intake of NaNO₂ by PM consumption in the UK, Spain and Germany reported in the European Prospective Study of Cancer and Nutrition

was 0.1–3.3 mg/d for women and 0.8–5.4 mg/d for men (adjusted for energy intake, age, weekday and season), which would be somehow lower when nitrite alone is calculated (15). The link between dietary nitrite and cancers of the gastrointestinal tract is inconclusive (35,36), which may be because most nitrite is absorbed early and the majority does not reach the colon (37). There was no association between FW nitrite and FW-induced DNA damage on our PM diet.

In conclusion, meats cured with nitrite have the same effect as fresh RM on endogenous nitrosation but show increased FW-induced oxidative DNA damage, which could be a result of pro-oxidative compounds derived from both dietary factors or endogenous immune response. The higher level of DNA strand breaks on the VEG diets is an intriguing finding that needs to be clarified in further studies.

Acknowledgements

We thank Valerie Church, Hilary Slack and Judith Wills for preparing the study diets and taking care of the volunteers and Marleen Lentjes for help in using DINER.

Conflict of Interest Statement: None declared.

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Received March 24, 2009; revised May 8, 2009; accepted May 19, 2009

Appendix. Nutrient composition of the diets^{a,b}

	PM			VEG		
	All (n = 16)	Female (n = 11)	Male (n = 5)	All (n = 16)	Female (n = 11)	Male (n = 5)
Energy intake (MJ)	9	8	11	9	8	12
Protein (g/d)	145 (27)	137 (29)	163 (25)	77 (14)	73 (15)	85 (12)
Fat (g/d)	80 (33)	71 (32)	101 (33)	79 (31)	66 (29)	107 (34)
SFA (g/d)	30 (12)	27 (12)	37 (12)	33 (12)	24 (11)	51 (16)
MUFA (g/d)	27 (11)	24 (11)	33 (11)	23 (9)	20 (9)	32 (10)
PUFA (g/d)	16 (6)	13 (6)	22 (7)	14 (6)	15 (7)	11 (4)
Carbohydrates (g/d)	230 (43)	200 (42)	296 (45)	321 (59)	289 (59)	389 (57)
Fibre (g/d)	19	16	23	30	28	34
Calcium (mg/d)	811	762	918	1187	1115	1348
Iron (mg/d)	18	17	22	14	13	17
Folate (µg/d)	189	168	236	244	229	277
Vitamin C (mg/d)	74	73	77	112	100	138
RM						
	All (n = 12)	Female (n = 6)	Male (n = 6)	All (n = 12)	Female (n = 6)	Male (n = 6)
Energy intake (MJ)	10	9	11	9	8	10
Protein (g/d)	147 (25)	133 (25)	161 (25)	77 (14)	73 (15)	80 (13)
Fat (g/d)	80 (29)	70 (29)	90 (30)	81 (31)	68 (29)	95 (34)
SFA (g/d)	34 (12)	26 (11)	41 (14)	33 (13)	24 (10)	42 (15)
MUFA (g/d)	27 (10)	24 (10)	30 (10)	25 (10)	21 (9)	29 (10)
PUFA (g/d)	10 (4)	13 (5)	7 (2)	14 (5)	16 (7)	12 (4)
Carbohydrates (g/d)	293 (49)	265 (50)	321 (49)	323 (58)	296 (59)	351 (57)
Fibre (g/d)	12	11	13	30	28	33
Calcium (mg/d)	895	875	915	1093	1058	1128
Iron (mg/d)	16	15	18	16	15	18
Folate (µg/d)	318	274	361	291	267	315
Vitamin C (mg/d)	93	90	95	86	71	100

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PM, processed (nitrite-preserved red) meat; PUFA, polyunsaturated fatty acids.

^aMean nutrient intake.

^bValues between parentheses represent %energy.

Diet and breast cancer prognosis: making sense of the Women's Healthy Eating and Living and Women's Intervention Nutrition Study trials

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Current Opinion in Obstetrics and Gynecology
2009, 21:86–91

Purpose of review

To clarify the role of dietary pattern on prognosis in breast cancer survivors.

Recent findings

Observational trials show mixed results that do not strongly support an independent role for dietary pattern in prognosis. Women's Intervention Nutrition Study and Women's Healthy Eating and Living (WHEL) are two large randomized controlled trials that address this question. The interventions from both studies achieved significant reductions in energy from fat, and the WHEL Study achieved large increases in vegetables, fruit and fiber. Women's Intervention Nutrition Study examined postmenopausal women only and reported a not-quite-significant improved prognosis for women in the intervention group, with the benefit focused on ipsilateral localized recurrences, but little improvement in the more important distal recurrences. This review considers only WHEL postmenopausal women to aid a direct comparison with Women's Intervention Nutrition Study. The WHEL Study reported a convincing lack of association between diet and prognosis. However, a secondary analysis suggests that the dietary intervention reduced distal recurrences among the subgroup without hot flashes at baseline.

Summary

There is no convincing evidence that changing dietary pattern following breast cancer diagnosis will improve prognosis for most women with early stage breast cancer. However, it would appear to be important for some subgroups. Further investigation of mechanisms for such selective action is needed.

Keywords

breast cancer prognosis, dietary pattern, randomized trials

Curr Opin Obstet Gynecol 21:86–91
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1040-872X

Introduction

Breast cancer is one of the most common cancers among women in developed countries, and significant advances in early detection and treatment have led to high 10-year survival rates and large prevalent survivor populations. Clinical outcome among women diagnosed with apparently similar cancers varies considerably, and does not appear to be explained by treatment differences. Laboratory studies have identified multiple bioactive constituents in foods that appear to either promote carcinogenesis or protect against it. As a result, there is considerable interest in the question of whether dietary pattern can influence a woman's prognosis following breast cancer.

Prospective observational studies, or cohort studies, measure the dietary component or pattern and then monitor prognosis over time. These association studies

do not provide sufficient evidence to conclude that changing dietary pattern would alter prognosis. The 'gold standard' studies are randomized trials in which some participants are assigned to make a major dietary change. Several prospective observational studies have investigated the link between dietary pattern and breast cancer prognosis, and to date, two randomized clinical trials have tested this association.

Observational studies

Most dietary studies, however, are compromised by measurement errors in the commonly used self-reported dietary patterns [1], and there are few proven biomarkers of exposure to validate self-report. The extent of these measurement errors has been highlighted by two separate studies that have demonstrated that different self-report methods change the study outcome [2,3]. Another

study showed an effect with a biomarker that was not seen with self-reported intake [4,5].

Reviews of the observational studies of diet and breast cancer prognosis have identified 14 studies since 1990 [6,7,8*,9–21]. However, many of these studies measured prebreast cancer dietary pattern shortly following diagnosis with breast cancer. As diets commonly change following diagnosis [22], dietary recalls over extended periods that include diagnosis have additional measurement problems and are excluded from this review.

In 1994, the Canadian National Breast Screening Study reported 5-year survival data from the subsample of 678 breast cancer cases that had completed a diet history prior to diagnosis [23]. In this study, lower saturated fat intake (but not intake of total fat or oleic acid) was associated with survival, and women in the highest versus lowest quartiles of intake of β carotene and vitamin C from food sources appeared protected.

The Nurses' Health Study is a well-known long-term investigation of a cohort of female registered nurses. Holmes *et al.* [15] reported on a subsample ($n=1982$) who were diagnosed with breast cancer from 1976 to 1990 and completed a dietary assessment in the postdiagnosis period and followed through 1994. Consumption of fruit, red meat or grain-based products was not associated with either all-cause mortality or breast carcinoma death. However, among women with metastatic cancer, consumption of vegetables, carotenoids and fiber had lower mortality rates. Kroenke *et al.* [17] reported on the 2619 nurses who were diagnosed from 1982 to 1998 and completed a dietary questionnaire at least 1 year after diagnosis. Over a median follow-up of 9 years, 9% of participants died from breast cancer. Using factor analysis, researchers identified two dietary patterns: one in which vegetables, fruit, whole grains, low fat dairy, poultry, fish and fiber consumption increased across quintiles (prudent dietary pattern), and the other in which consumption of refined grains, red meat, processed meat, high-fat dairy, saturated fat and dessert increased across quintiles (western dietary pattern). Neither dietary pattern was related to breast cancer mortality. However, the prudent dietary pattern reduced and Western dietary pattern increased deaths from other causes. Thus, the no-association result for dietary fat consumption was consistent across these two overlapping cohorts, but the conclusions for a mortality association with the consumption of vegetables, fruit and fiber were not.

The Women's Healthy Eating and Living (WHEL) Study reported observational data from women randomized to the comparison group. Dietary assessment occurred an average of 2 years after diagnosis, and dietary patterns were validated with a plasma carotenoid con-

centration, the accepted biomarker of vegetable and fruit intake. The first report [19] focused on 205 women who had a breast cancer event prior to June 2004 (average of 5 years follow-up), and identified those in the lowest baseline tertile (compared to all others) of circulating carotenoid concentrations as having an approximate 40% increased risk. The second report [24**] focused on overall mortality and included follow-up through December 2005. No association was found for energy from fat. Vegetable–fruit consumption and physical activity were weakly associated by themselves, but there was an interaction between them with the combination of vegetable–fruit intake (at least 5 vegetables and fruits/day), and physical activity (equivalent to walking briskly for 30 min, 6 days a week) reduced the risk of dying from breast cancer by half, regardless of weight, although fewer obese women were physically active with a healthy dietary pattern (16 versus 30%). The effect was stronger in women who had hormone receptor-positive cancers.

Comparing Women's Healthy Eating and Living and Women's Intervention Nutrition Study randomized trials

Both the Women's Intervention Nutrition Study (WINS) and the WHEL Study randomized women who had been diagnosed with early stage breast cancer in the United States during the period from 1990 to 1999, before the widespread use of aromatase inhibitors. Both studies excluded stage I patients with <1 cm tumors, but there were three important differences in the eligibility criteria. The first was age: WINS enrolled postmenopausal women aged 48–79 years at diagnosis, whereas WHEL enrolled women aged 18–70 years. However, to compare studies, it is possible to categorize WHEL participants by menopausal status (Table 1). Both the WHEL ($n=2448$) and the WINS ($n=2437$) studies had almost equivalently-sized populations of postmenopausal women.

The second difference was the time between diagnosis and enrollment. WINS enrolled women within 1 year of diagnosis, whereas the WHEL Study enrolled women within 4 years of diagnosis (Table 1). Thus, the WHEL Study undersampled the population who would recur within 4 years of diagnosis and is really a study of breast cancer events between 2 and 10 years from diagnosis [25]. WINS, on the contrary, is focused much more on breast cancer events within the first 5 years from diagnosis. The third difference was that WINS excluded women with worse prognoses (based on tumor size and nodes), whereas WHEL postmenopausal women had more advanced cancer characteristics at diagnosis. Over half of WINS participants were stage I compared with just over a third of the WHEL sample, and 25% of WHEL women were stage IIB or IIIA, compared with only 10% of WINS. In both studies, about one-fifth of postmenopausal women had estrogen receptor-negative tumors at diagnosis.

Table 1 Cancer characteristics of Women's Intervention Nutrition Study and Women's Healthy Eating and Living Study populations

	WINS Study		WHEL Study			
	Postmenopausal		Postmenopausal		Premenopausal and perimenopausal	
	Intervention N=975	Comparison N=1462	Intervention N=1228	Comparison N=1220	Intervention N=309	Comparison N=331
Time since diagnosis						
<1 year	100%	100%	18.6	17.1	40.1	42.6
1–2 years	—	—	33.1	33.4	26.2	30.2
2–3 years	—	—	26.2	26.2	17.2	15.7
3–4 years	—	—	22.1	23.2	16.5	11.5
Stage of initial cancer						
I	54.5%	54.5%	37.2	37.9	41.4	43.5
IIA	32.0%	31.9%	37.8	37.9	35.0	36.9
IIB	10.5%	9.6%	20.0	19.2	19.1	14.8
IIIA			5.1	5.1	4.5	4.8
Estrogen receptor status						
Positive, %	79.0%	81.3%	77.0	75.5	71.2	65.9
Negative, %	21.0%	18.7%	21.7	24.3	27.8	32.0

N, sample size.

Despite the above differences in cancer characteristics, both studies recruited breast cancer survivors from similar populations. Socio-demographic characteristics of WINS and WHEL participants were markedly similar (Table 2) in terms of age (55 versus 58 years), race (85% were Caucasian) and education (almost 50% college graduates). Approximately 27% of postmenopausal participants in both studies were obese, and the mean energy intake from fat was 29%.

These WINS and WHEL comparisons include WHEL participants who were premenopausal and perimenopausal. These women were younger, more highly educated and less likely to be obese (Table 2). They were also more likely to be recruited in the first year and more likely to be estrogen receptor negative (Table 1).

The effect of the Women's Intervention Nutrition Study intervention

Both the WINS and the WHEL studies used sets of four 24 h recalls to assess current dietary pattern at multiple points in time. The WINS intervention focused on reducing fat intake to 15% of energy. At 1 year, the intervention reported a 9% between-group difference in dietary fat

intake [26] (Table 3). Although it appeared that this between-group difference was maintained through 5 years, the low response rate for dietary assessments makes this result questionable (year 3 response approximately 70%; year 5 response approximately 40%). In reporting a completers-only analysis, the authors assumed that diets of the large proportion of nonrespondents were similar to the diets of the respondents – a very questionable assumption. Using a more conservative assumption (that nonrespondents didn't lower their fat intake), an estimate of the between-group difference is approximately 6% at year 3 and 3.5% at year 5. WINS also reported that the intervention group lost weight, resulting in a between-group difference of 2.3 kg in year 1 (conservative assumption = approximately 1.6 kg) to 2.7 kg in year 5 (conservative assumption = approximately 1.1 kg).

The effect of the Women's Healthy Eating and Living Study intervention

The WHEL Study intervention encouraged women to adopt a daily dietary pattern including five vegetable servings, 16 oz of vegetable juice, three fruit servings, 30 g of fiber and 20% energy from fat. Unlike WINS, there was only a small decline in completion rates for

Table 2 Comparison of Women's Intervention Nutrition Study and Women's Healthy Eating and Living Study populations

	WINS Study		WHEL Study			
	Postmenopausal		Postmenopausal		Premenopausal and perimenopausal	
	Intervention N=975	Comparison N=1462	Intervention N=1228	Comparison N=1220	Intervention N=309	Comparison N=331
Mean age, years (95% CI)	58.6 (44.4–72.8)	58.5 (43.6–73.4)	55 (40–70)	55 (40–70)	43 (32–54)	42 (30–54)
Caucasian, %	84.7	84.5	86.2	85.8	79.9	84.9
College graduate, %	48.3	50.1	54.7	49.8	58.6	64.4
Obese (BMI >30), %	27.3	26.5	28.1	27.1	21.0	18.7
Dietary energy from fat, %	29.6	29.6	28.4	28.6	29.0	29.0

CI, confidence interval; N, sample size.

Table 3 Change achieved by Women's Intervention Nutrition Study intervention

	Baseline	Year 1	Year 3	Year 5
N (RR%)				
Intervention (I)	975 (100)	840 (86)	654 (67)	380 (39)
Comparison (C)	1461 (100)	1328 (91)	1077 (74)	648 (44)
	I C	I-C Difference		
Energy from fat (%)	29.6	29.6	-8.9	-9.0 (adj ~6.0)
Weight (kg)	72.7	72.6	-2.3	-1.8

adj, adjusted for large non-response; C, comparison; I, intervention; N, sample size; RR, response rate. Adapted with permission from [26].

dietary assessment and 85% were assessed at year 6 (Table 4). The study has presented dietary change data in two ways: using a completers-only analysis [24**] as well as with the conservative assumption that nonresponders did not change their dietary pattern [27].

To compare dietary change with WINS, we present the completers-only data in Table 4 [24**]. The intervention was associated with a between-group difference of 4.7 vegetable–fruit servings/day at 1 year that decreased to 3 servings/day by 6 years. The between-group difference for fiber consumption was 8 g/day at year 1, decreasing to 5 g/day at year 6. The between-group difference in energy from fat was 5.7% at 1 year, decreasing to 3.5% at 6 years, similar to the conservative estimate of the WINS intervention effect. Body weight changed little over 6 years. The conservative analysis concluded that, at 4 years, the relative between-group differences were 65% for vegetables (including juice), 25% for fruit, 30% for fiber and 13% for energy from fat.

Study outcomes: Women's Intervention Nutrition Study versus Women's Healthy Eating and Living Study

The WINS and the WHEL studies have reported conflicting results. WINS assessed the summary variable 'any breast cancer event' for their analysis and the 2.6% between-group difference was borderline significant in the planned stratified log rank test ($P=0.077$), although statistically significant in the multivariate Cox model ($P=0.034$) [26]. Further, an exploratory analysis suggested that the between-group differences in breast cancer events might be confined to the 20% of the sample with initial tumors that were estrogen receptor negative; however, the interaction between dietary intervention and hormone receptor status was not statistically significant.

The WHEL Study [24**] reported no between-group differences in either any breast cancer event [adjusted Hazard Ratio (HR_{adj}) = 0.63] or in overall mortality ($HR_{adj} = 0.43$). Additionally, for breast cancer events, the likelihood ratio tests for baseline dietary pattern by study group interaction were not significant for vegetable–fruit, fiber or energy from fat. However, for overall mortality, the likelihood ratio test was significant for quartiles of energy from fat ($P=0.04$), although the effect seemed to be only in the second quartile of baseline energy from fat, and the intervention group difference was not in a protective direction. Further, hormone receptor status did not differ between groups ($P=0.85$).

Given the considerable similarities between these randomized trials, the marked difference in these findings requires further investigation. Table 5 presents details of the different study outcomes for postmenopausal women. As expected, from the initial cancer characteristics (Table 1) and the longer follow-up period, WHEL reported more study events than WINS (Table 5) and particularly more distal recurrences that Tang [28] has argued are the most important study outcomes for patients with a preexisting breast cancer diagnosis. The size of the between-group difference in these distal recurrences was not that different for both of these studies (WINS = -1.1, WHEL = -0.9).

The difference in the studies occurred in the between-group differences in the proportion of women who had local recurrences and new primary breast cancer events (WINS = -1.3%, WHEL = +0.5%), and, in particular, the WINS group effect was among women who had a lumpectomy for their initial cancer and had an ipsilateral breast cancer recurrence during the follow-up period

Table 4 Change achieved by Women's Healthy Eating and Living Study intervention

	Baseline	Year 1	Year 3	Year 5
N (RR%)				
Intervention (I)	1537 (100)	1463 (95)	1355 (88)	1308 (85)
Comparison (C)	1551 (100)	1484 (96)	1363 (88)	1313 (85)
	I C	I-C Difference		
Vegetable–fruit (serving/day)	7.4	7.2	+4.7	+3.5
Fiber (g/day)	21.1	21.2	+8.0	+5.9
Energy from fat (%)	28.5	28.7	-5.7	-4.3
Weight (kg)	73.5	73.3	-0.8	+0.1

C, comparison; I, intervention; N, sample size; RR, response rate. Adapted with permission from [24**].

Table 5 Comparison of cancer outcomes in Women's Intervention Nutrition Study and Women's Healthy Eating and Living Study populations

	WINS Study			WHEL Study		
	Postmenopausal			Postmenopausal		
	Comparison N=1462	Intervention N=975	Difference	Comparison N=1220	Intervention N=1228	Difference
Study events						
Local or new primary	76 (5.2)	38 (3.9)	-1.3	45 (3.7)	51 (4.2)	+0.5
Regional	12 (0.8)	6 (0.6)	-0.2	8 (0.7)	7 (0.6)	-0.1
Distal	93 (6.4)	52 (5.3)	-1.1	142 (11.6)	131 (10.7)	-0.9
Any breast cancer event	181 (12.4)	96 (9.8)	-2.6	195 (16.0)	189 (15.4)	-0.6
Additional primary cancer	50 (3.4)	28 (2.9)	-0.5	40 (4.1)	52 (4.3)	+0.1
Death without breast cancer	19 (1.3)	15 (1.5)	+0.2	21 (1.7)	25 (2.0)	+0.3
Any additional cancer or death	250 (17.1%)	139 (14.3%)	-2.8	266 (21.8)	266 (21.7)	-0.1
Cancer-free survival	1212 (82.9%)	836 (85.7%)		954 (78.2)	962 (78.3)	

N, sample size.

(comparison = 2.1%; intervention = 1.1%). It is possible that the lack of a WHEL effect in this subpopulation of women reflected the fact that WHEL underestimated events in the first few years from diagnosis. Another plausible explanation is that this finding is an artifact, particularly as the main hypothesis was only marginally statistically significant.

Women's Healthy Eating and Living Study secondary analysis

The initial WHEL Study protocol [25] postulated that diet could affect prognosis by reducing circulating estrogen concentrations. The study's nested case-control analysis showed that women with higher circulating estradiol concentrations at baseline were less likely to report hot flashes at that time and more likely to have a secondary cancer event during the follow-up period [29*]. In two separate studies, early stage breast cancer survivors who did not report hot flashes shortly after treatment were approximately 30% more likely to have additional breast cancer events [30*,31]. Given that the WHEL Study intervention has been shown to reduce circulating estrogen concentrations [32], the study undertook a secondary analysis to see whether an intervention effect may have been limited to this group of women who did not report hot flashes at baseline [33**].

About one-third of WHEL participants ($n=900$) were in the subgroup who did not report hot flashes at baseline (no hot flash subgroup) [24**,33**]. The intervention participants in this no hot flash subgroup had a similarly large change in dietary pattern (vegetables, fruit, fiber and energy from fat) over the course of the study to that seen in the overall intervention group. Among the no hot flash subgroup, 76.4% of the comparison group and 83.9% of the intervention group remained breast cancer free ($P=0.002$). This effect was almost entirely restricted to the distal recurrence group (comparison = 15.9%, intervention = 9.4%), and the approximately 60% lower event rate in the intervention group was not explained by other

variables in a series of sensitivity analyses [33**]. This effect did not differ significantly by hormone receptor status ($P=0.63$).

Conclusion

The evidence from prospective observational studies is mixed particularly for vegetable-fruit-fiber consumption, though more consistently negative for fat consumption. Two randomized trials have been reported: WINS focused on the dietary fat hypothesis, whereas WHEL focused on a plant-based dietary pattern that included a reduction in dietary fat. WHEL reported that the comprehensive dietary change had no effect on prognosis, whereas WINS found a marginal positive effect from changing fat.

This review compares postmenopausal participants from these studies, focusing on what might explain the different results. Both studies indicate that dietary pattern is not a strong 'across the board' predictor of prognosis. Each study suggests that the dietary pattern may be effective within a subgroup of people. Further investigation of subgroups is required, particularly focused on population evidence of the mechanism by which these dietary patterns may impact prognosis.

Acknowledgements

The author wishes to thank the WHEL Study Group for providing partitioned study data for use in this study. The author would also like to thank Sheila Kealey and Christine Hayes for assistance in manuscript preparation.

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Papers of particular interest, published within the annual period of review, have been highlighted as:

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- of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 108).

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Low-Fat Dietary Pattern and Risk of Invasive Breast Cancer

Ross L. Prentice, PhD; et al., *Journal of the American Medical Association*, February 8, 2006

The hypothesis that a low-fat dietary pattern can reduce breast cancer risk has existed for decades. We report the principal results of the Women's Health Initiative (WHI) Dietary Modification Trial, the first large-scale randomized trial to test whether adopting a low-fat dietary pattern in the middle to later decades of life reduces the risk for breast cancer.

The WHI began in 1992 and included a full-scale randomized controlled trial with a dietary modification intervention consisting of consumption of a reduced amount of fat (20% of energy) and of an increased amount of vegetables and fruit and grains (at least 5 servings daily and grains to at least 6 servings daily).

This dietary modification intervention trial is the first to directly assess the health benefits and risks of promoting a low-fat dietary pattern. Women were randomly assigned to the dietary modification intervention group or the comparison group. Comparison group participants were not asked to make dietary changes.

The relatively intensive dietary intervention implemented in the WHI resulted in a significant and sustained reduction in fat intake and an increase in vegetable and fruit intake.

Dietary fat intake was significantly lower in the dietary modification intervention group compared with the comparison group. The difference between groups in change from baseline for percentage of energy from fat varied from 10.7% at year 1 to 8.1% at year 6. Vegetable and fruit consumption was higher in the intervention group by at least 1 serving per day and a smaller, more transient difference was found for grain consumption.

The number of women who developed invasive breast cancer (annualized incidence rate) over the 8.1-year average follow-up period was 655 (0.42%) in the intervention group and 1072 (0.45%) in the comparison group.

Secondary analyses suggest a lower hazard ratio among adherent women, provide greater evidence of risk reduction among women having a high-fat diet at baseline, and suggest a dietary effect that varies by hormone receptor characteristics of the tumor.

We concluded that among postmenopausal

women, a low-fat dietary pattern did not result in a statistically significant reduction in invasive breast cancer risk over an 8.1-year average follow-up period. However, the nonsignificant trends observed suggesting reduced risk associated with a low-fat dietary pattern indicate that longer, planned, nonintervention follow-up may yield a more definitive comparison.

A total of 48,835 postmenopausal women, aged 50 to 79 years, without prior breast cancer, including 18.6% of minority race/ethnicity, were enrolled [in a] randomized, controlled, primary prevention trial conducted at 40 US clinical centers from 1993 to 2005." MB

Full text of this article may be ordered online. Per copy price is \$15 prepaid. Web site: <http://jama.ama-assn.org/cgi/content/abstract/295/6/629>

US Health Policy in the Aftermath of Hurricane Katrina

Sara Rosenbaum, JD, *Journal of the American Medical Association*, January 25, 2006

Hurricane Katrina exposed a health care system incapable of withstanding the long-term impact of a major disaster. Through destruction and permanent displacement, Katrina illuminated the fundamental weaknesses inherent in the national approach to health care financing, as well as the extent to which these weaknesses can threaten recovery.

The consequences for low-income populations have been particularly severe. To rebuild the region means rebuilding health care services, since accessible and affordable health care is essential to basic population health and safety. However, the rebuilding task faces particularly great challenges; even if capital can be found, the population is so pervasively uninsured that its ability to sustain reclaimed facilities is open to question.

As the region struggles to recover, and as temporary dislocation becomes permanent relocation for many, health insurance coverage most likely will continue to be seriously lacking. Employee health benefits—the 'mainstream' system of coverage—were never strong to begin with, and the population, along with regional public and private health care providers, including physicians, face a heightened risk of noncoverage. The emerging picture by mid-September was one of devastated state economies and community health infrastructures, long-term joblessness and deepening poverty

The NEW ENGLAND JOURNAL of MEDICINE

ESTABLISHED IN 1812

APRIL 24, 2003

VOL. 348 NO. 17

Overweight, Obesity, and Mortality from Cancer in a Prospectively Studied Cohort of U.S. Adults

Eugenia E. Calle, Ph.D., Carmen Rodriguez, M.D., M.P.H., Kimberly Walker-Thurmond, B.A., and Michael J. Thun, M.D.

ABSTRACT

BACKGROUND

The influence of excess body weight on the risk of death from cancer has not been fully characterized.

METHODS

In a prospectively studied population of more than 900,000 U.S. adults (404,576 men and 495,477 women) who were free of cancer at enrollment in 1982, there were 57,145 deaths from cancer during 16 years of follow-up. We examined the relation in men and women between the body-mass index in 1982 and the risk of death from all cancers and from cancers at individual sites, while controlling for other risk factors in multivariate proportional-hazards models. We calculated the proportion of all deaths from cancer that was attributable to overweight and obesity in the U.S. population on the basis of risk estimates from the current study and national estimates of the prevalence of overweight and obesity in the U.S. adult population.

RESULTS

The heaviest members of this cohort (those with a body-mass index [the weight in kilograms divided by the square of the height in meters] of at least 40) had death rates from all cancers combined that were 52 percent higher (for men) and 62 percent higher (for women) than the rates in men and women of normal weight. For men, the relative risk of death was 1.52 (95 percent confidence interval, 1.13 to 2.05); for women, the relative risk was 1.62 (95 percent confidence interval, 1.40 to 1.87). In both men and women, body-mass index was also significantly associated with higher rates of death due to cancer of the esophagus, colon and rectum, liver, gallbladder, pancreas, and kidney; the same was true for death due to non-Hodgkin's lymphoma and multiple myeloma. Significant trends of increasing risk with higher body-mass-index values were observed for death from cancers of the stomach and prostate in men and for death from cancers of the breast, uterus, cervix, and ovary in women. On the basis of associations observed in this study, we estimate that current patterns of overweight and obesity in the United States could account for 14 percent of all deaths from cancer in men and 20 percent of those in women.

CONCLUSIONS

Increased body weight was associated with increased death rates for all cancers combined and for cancers at multiple specific sites.

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N Engl J Med 2003;348:1625-38.

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THE RELATIONS BETWEEN EXCESS BODY weight and mortality, not only from all causes but also from cardiovascular disease, are well established.¹⁻⁶ Although we have known for some time that excess weight is also an important factor in death from cancer,⁷ our knowledge of the magnitude of the relation, both for all cancers and for cancers at individual sites, and the public health effect of excess weight in terms of total mortality from cancer is limited.

Previous studies have consistently shown associations between adiposity and increased risk of cancers of the endometrium, kidney, gallbladder (in women), breast (in postmenopausal women), and colon (particularly in men).⁸⁻¹² Adenocarcinoma of the esophagus has been linked to obesity.^{11,13,14} Data on cancers of the pancreas, prostate, liver, cervix, and ovary and on hematopoietic cancers are scarce or inconsistent.^{7-11,15-17} The lack of consistency may be attributable to the limited number of studies (especially those with prospective cohorts), the limited range and variable categorization of overweight and obesity among studies, bias introduced by reverse causality with respect to smoking-related cancers, and possibly real differences between the effects of overweight and obesity on the incidence of cancer and on the rates of death from some cancers.^{18,19}

We conducted a prospective investigation in a large cohort of U.S. men and women to determine the relations between body-mass index (the weight in kilograms divided by the square of the height in meters) and the risk of death from cancer at specific sites. This cohort has been used previously to examine the association of body-mass index and death from any cause.⁵

METHODS

STUDY POPULATION

The men and women in this study were selected from the 1,184,617 participants in the Cancer Prevention Study II, a prospective mortality study begun by the American Cancer Society in 1982.^{20,21} The participants were identified and enrolled by more than 77,000 volunteers in all 50 states, the District of Columbia, and Puerto Rico. Families were enrolled if at least one household member was 45 years of age or older and all enrolled members were 30 years of age or older. The average age of the participants at enrollment was 57 years. In 1982 they completed a confidential mailed questionnaire that in-

cluded personal identifiers and elicited information on demographic characteristics, personal and family history of cancer and other diseases, and various behavioral, environmental, occupational, and dietary exposures.

Over 99 percent of deaths that occurred from the month of enrollment until September 1988 were ascertained by means of personal inquiries made by volunteers in September 1984, 1986, and 1988.²² Approximately 93 percent of all deaths occurring after September 1988 were ascertained by linkage with the National Death Index.²² By December 31, 1998, 24.0 percent of the participants had died, 75.8 percent were still living, and 0.2 percent were dropped from follow-up on September 1, 1988, because of insufficient data for linkage with the National Death Index. Death certificates or multiple cause-of-death codes were obtained for 98.8 percent of all known deaths.

In the base-line questionnaire, the participants were asked their current weight, weight one year previously, and height (without shoes). We excluded from the analysis participants whose values for height or weight were missing, whose weight one year before the interview was unknown, or who had lost more than 10 lb (4.5 kg) in the previous year; 65,436 men and 91,282 women were excluded for these reasons. We also excluded participants with below-normal weight according to World Health Organization guidelines²³ as indicated by a body-mass index of less than 18.5 (3393 men and 15,769 women). In addition, we excluded participants who had cancer (other than nonmelanoma skin cancer) at base line (20,839 men and 47,120 women) and those with missing information on race or smoking history (14,086 men and 26,639 women). The eligible participants for the current analysis included 404,576 men and 495,477 women. After 16 years of follow-up, there were a total of 32,303 deaths from cancer in men and 24,842 deaths from cancer in women in this population.

From the final population of 900,053 participants, we defined a subgroup of those who had never smoked (107,030 men and 276,564 women). Within this subgroup, there were a total of 5314 deaths from cancer among men and 11,648 among women. This subgroup provided us with an opportunity to evaluate whether the association between body-mass index and mortality was subject to residual confounding by smoking status for smoking-related cancers.

BODY-MASS INDEX

The body-mass index, a measure of adiposity, was categorized as follows: 18.5 to 24.9, 25.0 to 29.9, 30.0 to 34.9, 35.0 to 39.9, and 40.0 or more. These categories correspond to those proposed by the World Health Organization²³ for "normal range," "grade 1 overweight," "grade 2 overweight" (30.0 to 39.9), and "grade 3 overweight," respectively. For many analyses, especially for cancers in specific sites and among participants who had never smoked, the upper categories of body-mass index were combined, because of the small numbers. In our analyses and discussion, we refer to the range of 25.0 to 29.9 as corresponding to "overweight" and to values of 30.0 or more as corresponding to "obesity."

In all primary analyses, the body-mass index category of 18.5 to 24.9 ("normal range") was considered the reference group. We also conducted analyses in which we divided this group into two categories of 18.5 to 22.9 and 23.0 to 24.9 and considered the lower category to be the reference group.

MORTALITY END POINTS

The end points in our analyses were deaths from all cancers (codes 140.0 to 195.8 and 199.0 to 208.9 of the International Classification of Diseases, Ninth Revision [ICD-9])²⁴ and from cancers at selected sites. Specific cancers accounting for at least 150 deaths in men and 150 deaths in women included esophageal cancer (ICD-9 codes 150.0 to 150.9), stomach cancer (151.0 to 151.9), colorectal cancer (153.0 to 154.8), liver cancer (155.0 to 155.2), gallbladder cancer (156.0 to 156.9), pancreatic cancer (157.0 to 157.9), lung cancer (162.0 to 162.9), melanoma (172.0 to 172.9), breast cancer in women (174.0 to 174.9), cancer of the corpus and uterus, not otherwise specified (179 and 182.0 to 182.8), cervical cancer (180.0 to 180.9), ovarian cancer (183.0 to 183.9), prostate cancer (185), bladder cancer (188.0 to 188.9), kidney cancer (189.0 to 189.9), brain cancer (191.0 to 191.9), non-Hodgkin's lymphoma (202.0 to 202.9), multiple myeloma (203.0 to 203.8), and leukemia (204.0 to 208.9). All other specific cancers that contributed to total deaths from cancer but that caused fewer than 150 deaths or were coded as unspecified (199.0 to 199.1) were analyzed as a separate category of "other" cancers. Approximately 11 percent of cancers in both men and women fell into the "other" category. Of these, 45 percent had a specific (coded) site and caused fewer than 150 deaths and 55 percent had a site

that was coded as unspecified. Data regarding cancer subsites or histologic findings were not available.

INFORMATION ON COVARIATES

Potential confounders included in data analyses were age (in single years), race (white, black, or other), smoking status (never smoked, formerly smoked, or currently smokes, with three categories of cigarettes smoked per day: 0 to 19, 20, and more than 20), education (less than high school, high-school graduate, some college, or college graduate), physical activity (none, slight, moderate, or heavy), alcohol use (none, less than one drink per day, one drink per day, or two or more drinks per day), marital status (not married or married), current aspirin use (use or nonuse), a crude index of fat consumption (estimated grams per week for 20 food items, with the participants divided into three roughly equal groups),²⁵ and vegetable consumption (the frequency per week of consumption of nine vegetables — not including potatoes — with participants divided into three roughly equal groups), and status with respect to estrogen-replacement therapy in women (never used, currently used, or formerly used).

STATISTICAL ANALYSIS

Age-adjusted death rates were calculated for each category of body-mass index and were directly standardized to the age distribution of the entire male or female study population. Relative risks (the age-adjusted death rate in a specific body-mass-index category divided by the corresponding rate in the reference category [18.50 to 24.99]) were computed; the 95 percent confidence intervals used approximate variance formulas.^{26,27}

We also used Cox proportional-hazards modeling²⁸ to compute relative risks and to adjust for other potential risk factors reported at base line. The Cox models were stratified according to age at enrollment by the inclusion of age (in single years) in the strata statement of the Cox model. The relative risks we report are from the multivariate Cox models, unless otherwise noted. Tests of linear trend were performed by scoring the categories of body-mass index, entering the score as a continuous term in the regression model, and testing the significance of the term by the Wald chi-square test.²⁹

We present results for all cancers combined and for cancer at each site on the basis of analyses of the total populations of men and women. For most in-

Table 1. Mortality from Cancer According to Body-Mass Index among U.S. Men in the Cancer Prevention Study II, 1982 through 1998.*

Type of Cancer	Body-Mass Index†					P for Trend
	18.5–24.9	25.0–29.9	30.0–34.9	35.0–39.9	≥40.0	
All cancers						
No. of deaths	13,855	15,372	2683	350	43	
Death rate‡	578.30	546.21	636.30	738.69	841.62	
RR (95% CI)§	1.00	0.97 (0.94–0.99)	1.09 (1.05–1.14)	1.20 (1.08–1.34)	1.52 (1.13–2.05)	0.001
All cancers						
No. of deaths	13,855	15,372	2683	393¶		
Death rate‡	578.30	546.21	636.30	749.86¶		
RR (95% CI)§	1.00	0.97 (0.94–0.99)	1.09 (1.05–1.14)	1.23 (1.11–1.36)¶		0.002
Esophageal cancer						
No. of deaths	329	452	81	14		
Death rate‡	13.97	15.74	18.07	24.18		
RR (95% CI)§	1.00	1.15 (0.99–1.32)	1.28 (1.00–1.63)	1.63 (0.95–2.80)		0.008
Stomach cancer						
No. of deaths	388	455	84	18		
Death rate‡	16.24	16.09	20.34	33.99		
RR (95% CI)§	1.00	1.01 (0.88–1.16)	1.20 (0.94–1.52)	1.94 (1.21–3.13)		0.03
Colorectal cancer						
No. of deaths	1,292	1,811	337	54		
Death rate‡	53.51	64.43	79.50	101.25		
RR (95% CI)§	1.00	1.20 (1.12–1.30)	1.47 (1.30–1.66)	1.84 (1.39–2.41)		<0.001
Liver cancer						
No. of deaths	222	296	78	24		
Death rate‡	9.24	10.49	19.22	47.80		
RR (95% CI)§	1.00	1.13 (0.94–1.34)	1.90 (1.46–2.47)	4.52 (2.94–6.94)		<0.001
Gallbladder cancer						
No. of deaths	66	94	20			
Death rate‡	2.68	3.37	5.16			
RR (95% CI)§	1.00	1.34 (0.97–1.84)	1.76 (1.06–2.94)			0.02
Pancreatic cancer						
No. of deaths	740	961	182	25		
Death rate‡	31.07	33.98	42.20	48.80		
RR (95% CI)§	1.00	1.13 (1.03–1.25)	1.41 (1.19–1.66)	1.49 (0.99–2.22)		<0.001
Lung cancer						
No. of deaths	4,885	4,281	681	78		
Death rate‡	206.69	150.11	156.53	149.63		
RR (95% CI)§	1.00	0.78 (0.75–0.82)	0.79 (0.73–0.86)	0.67 (0.54–0.84)		<0.001
Melanoma						
No. of deaths	238	279	43			
Death rate‡	10.02	9.77	8.09			
RR (95% CI)§	1.00	0.95 (0.80–1.13)	0.85 (0.61–1.18)			0.32
Prostate cancer						
No. of deaths	1,681	1,971	311	41		
Death rate‡	67.36	73.02	83.00	87.35		
RR (95% CI)§	1.00	1.08 (1.01–1.15)	1.20 (1.06–1.36)	1.34 (0.98–1.83)		<0.001
Bladder cancer						
No. of deaths	375	421	76			
Death rate‡	15.19	15.47	16.69			
RR (95% CI)§	1.00	1.03 (0.89–1.18)	1.14 (0.88–1.46)			0.36
Kidney cancer						
No. of deaths	305	437	81	14		
Death rate‡	12.83	15.25	18.50	24.84		
RR (95% CI)§	1.00	1.18 (1.02–1.37)	1.36 (1.06–1.74)	1.70 (0.99–2.92)		0.002

Table 1. (Continued.)

Type of Cancer	Body-Mass Index [†]					P for Trend
	18.5–24.9	25.0–29.9	30.0–34.9	35.0–39.9	≥40.0	
Brain cancer						
No. of deaths	370	461	68			
Death rate [‡]	15.98	15.86	12.76			
RR (95% CI) [§]	1.00	0.98 (0.85–1.13)	0.79 (0.61–1.03)			0.14
Non-Hodgkin's lymphoma						
No. of deaths	518	672	147	18		
Death rate [‡]	21.51	24.04	35.25	33.22		
RR (95% CI) [§]	1.00	1.08 (0.96–1.21)	1.56 (1.29–1.87)	1.49 (0.93–2.39)		<0.001
Multiple myeloma						
No. of deaths	259	368	70	11		
Death rate [‡]	10.77	13.18	16.88	20.62		
RR (95% CI) [§]	1.00	1.18 (1.01–1.39)	1.44 (1.10–1.89)	1.71 (0.93–3.14)		0.002
Leukemia						
No. of deaths	546	720	128	20		
Death rate [‡]	22.51	25.60	30.40	40.52		
RR (95% CI) [§]	1.00	1.14 (1.02–1.28)	1.37 (1.13–1.67)	1.70 (1.08–2.66)		<0.001
All other cancers						
No. of deaths	1,641	1,693	320	52		
Death rate [‡]	68.72	59.81	73.29	101.88		
RR (95% CI) [§]	1.00	0.89 (0.83–0.95)	1.06 (0.94–1.20)	1.29 (0.98–1.71)		0.98

* Participants with any of the following features at study entry were excluded: missing data on height or current weight; unknown weight one year before entry; weight loss at least 10 lb (4.5 kg) in the previous year; body-mass index under 18.50; existing cancer (other than nonmelanoma skin cancer); unknown race or missing data; and missing data on smoking status. RR denotes relative risk, and CI confidence interval.

† The highest body-mass-index category examined varies for cancer at different sites; higher categories have been combined when necessary because of small numbers.

‡ The rate per 100,000 is given, standardized to the age distribution of men in the Cancer Prevention Study II.

§ The Cox proportional-hazards model was adjusted for age, education, smoking status and number of cigarettes smoked, physical activity, alcohol use, marital status, race, aspirin use, fat consumption, and vegetable consumption.

¶ This value is for the 35.0–39.9 and ≥40.0 groups combined and is provided to facilitate comparison with the types of cancer.

dividual cancer sites, the association of body-mass index and mortality was similar whether the analysis was based on the total population or on the population of those who had never smoked. However, for several cancers known to be related to smoking, the association between body-mass index and mortality was substantially different in the total population and the population of those who had never smoked. For these cancers (in men, all cancers, lung cancer, esophageal cancer, pancreatic cancer, and other cancers; in women, all cancers, lung cancer, esophageal cancer, and other cancers), the results from the population of those who had never smoked are also presented.

Because weight is a modifiable risk factor, we calculated the population attributable fraction (also termed population attributable risk, population attributable-risk proportion, and excess fraction),³⁰

an estimate of the proportion of all cancer deaths in the United States that might be avoided if the adult population maintained a body-mass index in the normal range. We used methods derived by Walter³¹ and presented by Kleinbaum et al.³² for a multiple-category exposure. In this analysis, calculations were based on the multivariate-adjusted relative risks for the total population in the Cancer Prevention Study II and for the population of those in that study who had never smoked and on prevalence estimates of overweight and obesity in U.S. men and women 50 to 69 years of age from the National Health and Nutrition Examination Survey for 1999–2000.³³ This calculation assumes that the relative-risk estimates associated with overweight and obesity that were observed in the current study were causal and are generalizable to the U.S. population.

Table 2. Mortality from Cancer According to Body-Mass Index among U.S. Women in the Cancer Prevention Study II, 1982 through 1998.*

Type of Cancer	Body-Mass Index†					P for Trend
	18.5–24.9	25.0–29.9	30.0–34.9	35.0–39.9	≥40.0	
All cancers						
No. of deaths	14,779	7107	2254	517	185	
Death rate‡	329.30	339.75	382.62	419.59	522.51	
RR (95% CI)§	1.00	1.08 (1.05–1.11)	1.23 (1.18–1.29)	1.32 (1.20–1.44)	1.62 (1.40–1.87)	<0.001
Esophageal cancer						
No. of deaths	112	56	21			
Death rate‡	2.56	2.68	2.90			
RR (95% CI)§	1.00	1.20 (0.86–1.66)	1.39 (0.86–2.25)			0.13
Stomach cancer						
No. of deaths	304	134	57	13		
Death rate‡	6.87	6.37	9.88	9.85		
RR (95% CI)§	1.00	0.89 (0.72–1.09)	1.30 (0.97–1.74)	1.08 (0.61–1.89)		0.46
Colorectal cancer						
No. of deaths	1,706	906	312	67	21	
Death rate‡	38.67	43.28	53.81	56.14	63.11	
RR (95% CI)§	1.00	1.10 (1.01–1.19)	1.33 (1.17–1.51)	1.36 (1.06–1.74)	1.46 (0.94–2.24)	<0.001
Liver cancer						
No. of deaths	200	96	37	12		
Death rate‡	4.53	4.54	6.34	7.52		
RR (95% CI)§	1.00	1.02 (0.80–1.31)	1.40 (0.97–2.00)	1.68 (0.93–3.05)		0.04
Gallbladder cancer						
No. of deaths	159	86	59			
Death rate‡	3.57	4.15	7.79			
RR (95% CI)§	1.00	1.12 (0.86–1.47)	2.13 (1.56–2.90)			<0.001
Pancreatic cancer						
No. of deaths	952	490	154	35	19	
Death rate‡	21.47	23.24	26.20	27.70	51.65	
RR (95% CI)§	1.00	1.11 (1.00–1.24)	1.28 (1.07–1.52)	1.41 (1.01–1.99)	2.76 (1.74–4.36)	<0.001
Lung cancer						
No. of deaths	3,693	1278	305	54	19	
Death rate‡	81.48	60.80	51.23	43.67	52.64	
RR (95% CI)§	1.00	0.88 (0.83–0.94)	0.82 (0.72–0.92)	0.66 (0.50–0.86)	0.81 (0.52–1.28)	<0.001
Melanoma						
No. of deaths	166	61	28			
Death rate‡	3.65	2.96	3.63			
RR (95% CI)§	1.00	0.85 (0.63–1.14)	1.10 (0.73–1.66)			0.95
Breast cancer¶						
No. of deaths	1,446	908	309	68	24	
Death rate‡	39.10	51.13	60.65	67.56	84.86	
RR (95% CI)§	1.00	1.34 (1.23–1.46)	1.63 (1.44–1.85)	1.70 (1.33–2.17)	2.12 (1.41–3.19)	<0.001
Cancer of the corpus and uterus, not otherwise specified						
No. of deaths	333	225	105	25	16	
Death rate‡	10.68	15.68	26.05	30.16	60.83	
RR (95% CI)§	1.00	1.50 (1.26–1.78)	2.53 (2.02–3.18)	2.77 (1.83–4.18)	6.25 (3.75–10.42)	<0.001
Cervical cancer						
No. of deaths	80	54	16	14		
Death rate‡	1.73	2.63	2.73	7.81		
RR (95% CI)§	1.00	1.38 (0.97–1.96)	1.23 (0.71–2.13)	3.20 (1.77–5.78)		0.001

Table 2. (Continued.)

Type of Cancer	Body-Mass Index [†]				P for Trend
	18.5–24.9	25.0–29.9	30.0–34.9	35.0–39.9	≥40.0
Ovarian cancer**					
No. of deaths	873	437	126	49	
Death rate‡	27.88	31.44	31.85	44.49	
RR (95% CI)§	1.00	1.15 (1.02–1.29)	1.16 (0.96–1.40)	1.51 (1.12–2.02)	0.001
Bladder cancer					
No. of deaths	180	83	34		
Death rate‡	4.21	3.93	4.82		
RR (95% CI)§	1.00	1.02 (0.78–1.33)	1.34 (0.91–1.95)		0.21
Kidney cancer					
No. of deaths	243	153	55	12	
Death rate‡	5.43	7.35	9.24	9.56	
RR (95% CI)§	1.00	1.33 (1.08–1.63)	1.66 (1.23–2.24)	1.70 (0.94–3.05)	4.75 (2.50–9.04)
Brain cancer					
No. of deaths	467	213	64	12	
Death rate‡	10.26	10.27	10.68	6.35	
RR (95% CI)§	1.00	1.02 (0.87–1.21)	1.10 (0.84–1.44)	0.74 (0.42–1.32)	0.96
Non-Hodgkin's lymphoma					
No. of deaths	576	327	88	38	
Death rate‡	13.02	15.48	14.99	24.09	
RR (95% CI)§	1.00	1.22 (1.06–1.40)	1.20 (0.95–1.51)	1.95 (1.39–2.72)	<0.001
Multiple myeloma					
No. of deaths	341	187	72	20	
Death rate‡	7.71	8.87	12.28	12.88	
RR (95% CI)§	1.00	1.12 (0.93–1.34)	1.47 (1.13–1.91)	1.44 (0.91–2.28)	0.004
Leukemia					
No. of deaths	574	282	83	18	
Death rate‡	13.05	13.53	14.17	12.72	
RR (95% CI)§	1.00	1.05 (0.91–1.21)	1.12 (0.89–1.42)	0.93 (0.58–1.49)	0.53
All other cancers					
No. of deaths	1,582	801	239	61	
Death rate‡	35.70	38.15	40.61	51.99	
RR (95% CI)§	1.00	1.11 (1.02–1.21)	1.20 (1.05–1.38)	1.47 (1.13–1.90)	1.83 (1.20–2.80)
					<0.001

* Participants with any of the following features at study entry were excluded: missing data on height or current weight; unknown weight one year before entry; weight loss of at least 10 lb (4.5 kg) in the previous year; body-mass index under 18.50; existing cancer (other than nonmelanoma skin cancer); unknown race or missing data; and missing data on smoking status. RR denotes relative risk, and CI confidence interval.

† The highest body-mass-index category examined varies for different cancer sites; upper categories have been combined when necessary because of small numbers.

‡ The rate per 100,000 is given, standardized to the age distribution of women in the Cancer Prevention Study II.

§ The Cox proportional-hazards model was adjusted for age, education, smoking status and number of cigarettes smoked, physical activity, alcohol use, marital status, race, aspirin use, estrogen-replacement therapy, fat consumption, and vegetable consumption.

¶ Women who were premenopausal or perimenopausal or whose menopausal status was unknown were excluded (147,583 women, with 871 deaths).

|| Women who had a hysterectomy were excluded (130,717 women, 25 deaths).

** Women who had either a hysterectomy or ovarian surgery were excluded (141,924 women, 389 deaths).

RESULTS

BODY-MASS INDEX AND MORTALITY FROM CANCER IN THE TOTAL POPULATION OF MEN AND WOMEN

The numbers of deaths among men were sufficient to permit only the death rates from all cancers to be

examined separately for the two highest body-mass-index categories of 35.0 to 39.9 and 40.0 or more. The relative risks of death for these categories, as compared with the group of men of normal weight (body-mass index, 18.5 to 24.9), were 1.20 (95 percent confidence interval, 1.08 to 1.34) and 1.52 (95 percent confidence interval, 1.13 to 2.05), respec-

tively (Table 1). We observed significant positive linear trends in death rates with increasing body-mass index for all cancers, esophageal cancer, stomach cancer, colorectal cancer, liver cancer, gallbladder cancer, pancreatic cancer, prostate cancer, kidney cancer, non-Hodgkin's lymphoma, multiple myeloma, and leukemia (Table 1). As compared with men of normal weight, men with a body-mass index of at least 35.0 had significantly elevated relative risks of death from cancer, which ranged from 1.23 (95 percent confidence interval, 1.11 to 1.36) for death from any cancer to 4.52 (95 percent confidence interval, 2.94 to 6.94) for death from liver cancer (Table 1). In the total population of men, a significant inverse association was observed between body-mass index and death from lung cancer. We did not find significant associations between body-mass index and death from brain cancer, bladder cancer, melanoma, or "other" cancers. Among men within the normal weight range, those with a body-mass index of 23.0 to 24.9 were not at higher risk for death from cancer than the leanest men (those with a body-mass index of 18.5 to 22.9), and the observed associations in men were not larger when a leaner group of men was used as the reference group (data not shown).

The results for the total population of women were similar. Women with a body-mass index of at least 40.0 had a relative risk of death from any cancer of 1.62 (95 percent confidence interval, 1.40 to 1.87), as compared with women of normal weight (Table 2). Significant positive linear trends in death rates were observed for colorectal cancer, liver cancer, gallbladder cancer, pancreatic cancer, breast cancer, cancer of the corpus and uterus, not otherwise specified, cervical cancer, ovarian cancer, kidney cancer, non-Hodgkin's lymphoma, multiple myeloma, and "other" cancers (Table 2). The highest relative risk we observed was for death from uterine cancer (relative risk, 6.25 for women with body-mass index of at least 40.0; 95 percent confidence interval, 3.75 to 10.42). As in men, a significant inverse association between body-mass index and death from lung cancer was seen in the total population, which included smokers. Significant associations with body-mass index were not observed for death from esophageal cancer, stomach cancer, melanoma, bladder cancer, brain cancer, or leukemia. Although the results for total cancer mortality in women were virtually unchanged when a leaner reference group was used (body-mass index, 18.5 to 22.9), there were significant differences within the

normal weight range for cancers of the gallbladder, breast, and corpus and uterus, resulting in larger elevations in risk for these cancers throughout the entire range of overweight and obesity as compared with the leanest reference group (the relative risk of death from gallbladder cancer for a body-mass index of at least 30.0 was 2.44 [95 percent confidence interval, 1.73 to 3.44]; the relative risks of death from breast and uterine cancers for a body-mass index of at least 40.0 were 2.32 [95 percent confidence interval, 1.54 to 3.50] and 6.87 [95 percent confidence interval, 4.09 to 11.55], respectively).

BODY-MASS INDEX AND MORTALITY FROM CANCER IN MEN AND WOMEN WHO HAD NEVER SMOKED

The association between body-mass index and death from several smoking-related cancers changed when the analysis was restricted to men who had never smoked. The positive associations with death from any cancer, esophageal cancer, pancreatic cancer, and "other" cancers were of greater magnitude among those who had never smoked than in the total population, and the apparent inverse association with death from lung cancer disappeared (Table 3).

As in men, the positive association between body-mass index and death from any cancer, esophageal cancer, and "other" cancers became stronger when the analysis was restricted to women who had never smoked, and the seemingly protective effect of high body-mass index on mortality from lung cancer was attenuated (Table 3). Among women who had never smoked, the relative risk of death from any cancer was 1.88 (95 percent confidence interval, 1.56 to 2.27) for those with a body-mass index of at least 40.0, as compared with women of normal weight.

The relative risks of cancers for which we found significant trends of increasing death rates with increasing body-mass index are summarized for the highest categories of body-mass index that we were able to examine in men (Fig. 1) and women (Fig. 2).

POPULATION ATTRIBUTABLE FRACTION

We estimated the proportion of all deaths from cancer in the U.S. population that are attributable to overweight and obesity to be from 4.2 percent to 14.2 percent among men and from 14.3 percent to 19.8 percent among women (Table 4). The lower estimates are based on relative risks for the entire population, whereas the higher estimates are based

Table 3. Mortality from Cancer According to Body-Mass Index among U.S. Men and Women in the Cancer Prevention Study II Who Had Never Smoked, 1982 through 1998.*

Type of Cancer	Body-Mass Index†				P for Trend	
	18.5–24.9	25.0–29.9	30.0–34.9	35.0–39.9		
Men						
All cancers						
No. of deaths	2119	2638	499	58		
Death rate‡	303.08	346.62	442.00	421.01		
RR (95% CI)§	1.00	1.11 (1.05–1.18)	1.38 (1.24–1.52)	1.31 (1.01–1.70)	<0.001	
Esophageal cancer						
No. of deaths	24	52	11			
Death rate‡	3.55	6.82	7.29			
RR (95% CI)§	1.00	1.76 (1.08–2.86)	1.91 (0.92–3.96)		0.04	
Pancreatic cancer						
No. of deaths	155	212	34	8		
Death rate‡	22.57	27.87	29.75	60.69		
RR (95% CI)§	1.00	1.24 (1.01–1.54)	1.34 (0.92–1.95)	2.61 (1.27–5.35)	0.005	
Lung cancer						
No. of deaths	156	179	30			
Death rate‡	22.72	23.51	23.45			
RR (95% CI)§	1.00	1.00 (0.80–1.24)	0.93 (0.63–1.39)		0.78	
All other cancers						
No. of deaths	239	290	81			
Death rate‡	34.65	37.99	62.18			
RR (95% CI)§	1.00	1.06 (0.89–1.26)	1.68 (1.30–2.18)		<0.001	
Women						
All cancers						
No. of deaths	6158	3763	1327	288	112	
Death rate¶	241.14	277.92	330.21	356.84	485.06	
RR (95% CI)§	1.00	1.14 (1.09–1.18)	1.33 (1.25–1.41)	1.40 (1.25–1.58)	1.88 (1.56–2.27)	
Esophageal cancer						
No. of deaths	29	23	14			
Death rate¶	1.08	1.62	2.82			
RR (95% CI)§	1.00	1.49 (0.85–2.59)	2.64 (1.36–5.12)		0.004	
Lung cancer						
No. of deaths	476	224	78	17		
Death rate¶	18.71	16.40	19.18	17.51		
RR (95% CI)§	1.00	0.85 (0.73–1.00)	0.99 (0.77–1.26)	0.81 (0.49–1.31)	0.21	
All other cancers						
No. of deaths	689	440	146	34	16	
Death rate¶	26.69	31.63	36.24	42.88	72.92	
RR (95% CI)§	1.00	1.17 (1.04–1.32)	1.30 (1.08–1.56)	1.54 (1.08–2.17)	2.51 (1.52–4.14)	
					<0.001	

* Participants with any of the following features at study entry were excluded: missing height or current weight; unknown weight one year before entry; weight loss of at least 10 lb (4.5 kg) in the previous year; body-mass index under 18.50; existing cancer (other than nonmelanoma skin cancer); and missing data on smoking status. RR denotes relative risk, and CI confidence interval.

† The highest body-mass-index category examined varies for different cancer sites; upper categories have been combined when necessary because of small numbers.

‡ The rate per 100,000 is given, standardized to the age distribution of men in the Cancer Prevention Study II.

§ The Cox proportional-hazards model was adjusted for age, education, physical activity, alcohol use, marital status, race, aspirin use, estrogen-replacement therapy (in women), fat consumption, and vegetable consumption.

¶ The rate per 100,000 is given, standardized to the age distribution of women in the Cancer Prevention Study II.

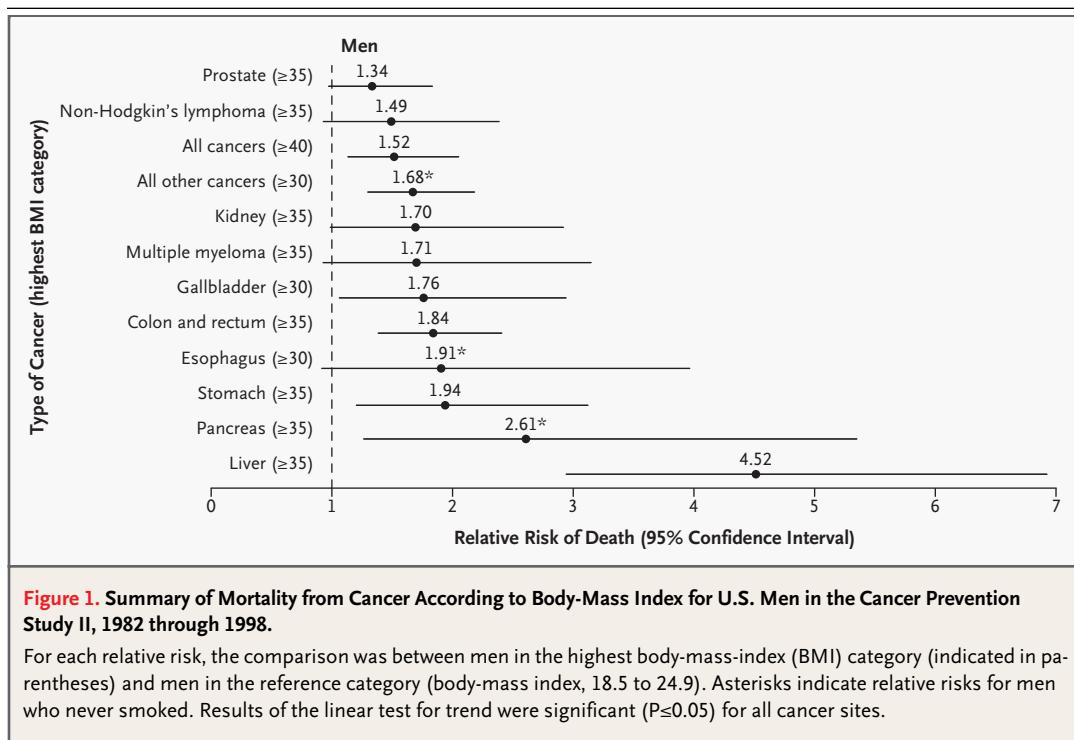


Figure 1. Summary of Mortality from Cancer According to Body-Mass Index for U.S. Men in the Cancer Prevention Study II, 1982 through 1998.

For each relative risk, the comparison was between men in the highest body-mass-index (BMI) category (indicated in parentheses) and men in the reference category (body-mass index, 18.5 to 24.9). Asterisks indicate relative risks for men who never smoked. Results of the linear test for trend were significant ($P \leq 0.05$) for all cancer sites.

on relative risks for those who never smoked. The estimates based on relative risks among men and women who never smoked (Table 4) do not describe the fraction of deaths attributable to overweight and obesity among this population only. Rather, they are estimates of the fraction of deaths attributable to overweight and obesity in the total U.S. population, on the assumption that the relative risks among those who never smoked offer the most valid estimates of the true effect of overweight and obesity on mortality from cancer.

DISCUSSION

The heaviest men and women (those with a body-mass index of at least 40.0) in the large cohort we studied prospectively had death rates from all cancers that were 52 percent and 62 percent higher, respectively, than the rates in men and women of normal weight. This finding is consistent with those of previous studies, but the magnitude of the effect is somewhat larger.^{7,16,17} The stronger associations we found probably reflect our ability to examine deaths from cancer across a wider range of overweight and obesity than has been possible previously. It is also likely that the stronger associations

seen in our study reflect a greater effect of body-mass index on mortality than on incidence of cancer at some sites.^{18,19} These risk estimates are probably conservative, since they are based on the total population, including current and former smokers. Among women who never smoked, the relative risk associated with a body-mass index of at least 40.0 was 88 percent; however, there were not enough deaths among men in this category for us to determine the relative risk.

The proportion of all deaths from cancer that is attributable to overweight and obesity in U.S. adults 50 years of age or older may be as high as 14 percent in men and 20 percent in women. These estimates are based on the relative risks in our study and the current patterns of overweight and obesity in the United States. Under the assumption that these relations are causal, the public health implications for the United States are profound: more than 90,000 deaths per year from cancer might be avoided if everyone in the adult population could maintain a body-mass index under 25.0 throughout life.

The International Agency for Research on Cancer (IARC) has concluded that there is sufficient evidence of a cancer-preventive effect of avoidance of weight gain for cancers of the colon, breast (in post-

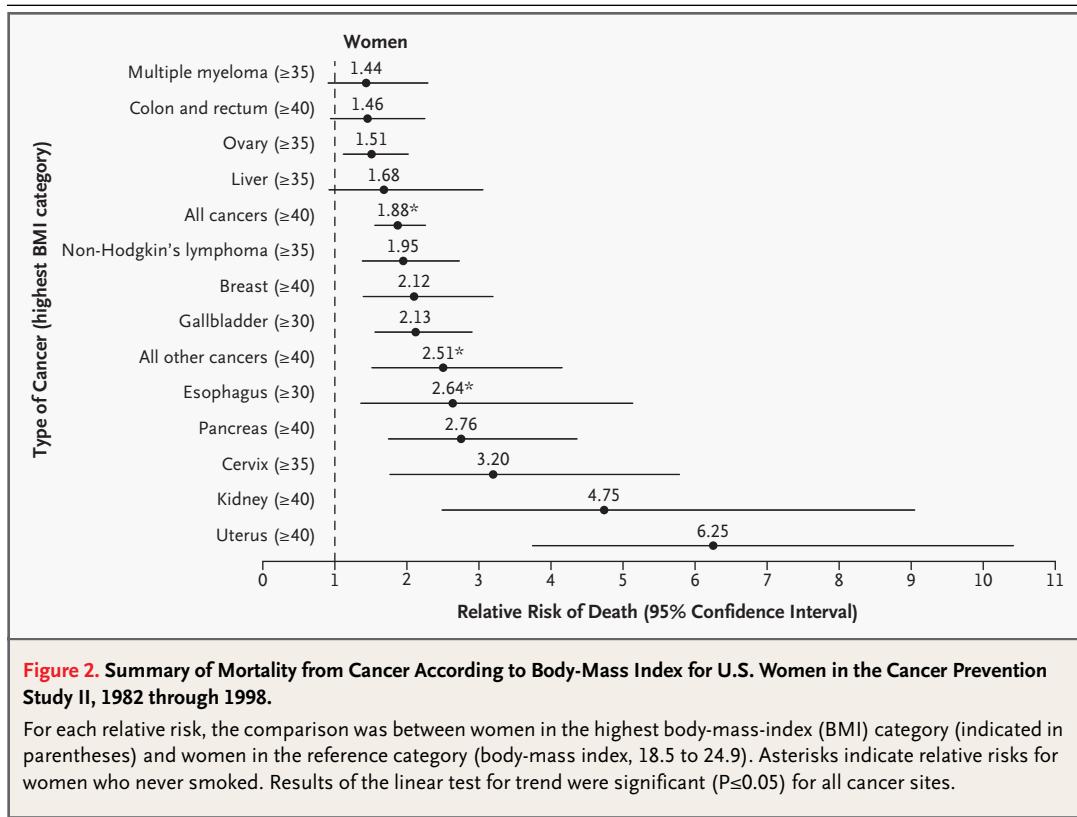


Figure 2. Summary of Mortality from Cancer According to Body-Mass Index for U.S. Women in the Cancer Prevention Study II, 1982 through 1998.

For each relative risk, the comparison was between women in the highest body-mass-index (BMI) category (indicated in parentheses) and women in the reference category (body-mass index, 18.5 to 24.9). Asterisks indicate relative risks for women who never smoked. Results of the linear test for trend were significant ($P \leq 0.05$) for all cancer sites.

menopausal women), endometrium, kidney (renal-cell carcinoma), and esophagus (adenocarcinoma).¹¹ Potential biologic mechanisms include increased levels of endogenous hormones (sex steroids, insulin, and insulin-like growth factor I) associated with overweight and obesity and the contribution of abdominal obesity to gastroesophageal reflux and esophageal adenocarcinoma.¹¹ Our study supports the conclusion of the IARC. Moderate relative risks (less than 2.0) associated with overweight and obesity both for colon cancer and for breast cancer in postmenopausal women have been documented consistently in case-control and cohort studies.^{8,34,35} Much higher relative risks have been observed for uterine cancer (2 to 10) and kidney cancer (1.5 to 4), and the increased risk of kidney cancer associated with excess weight is higher in women than in men in this and most previous studies.^{8,36,37} Increases by a factor of two to three in the risk of adenocarcinoma of the esophagus in association with high body-mass index have been reported,^{13,14} and the magnitude of this association has been found by other investigators to be greater in nonsmokers.¹³ Because we could not examine

esophageal cancer according to subsite, the stronger association observed in participants who had never smoked may be partly explained by the greater contribution of adenocarcinoma to all esophageal cancer in nonsmokers than in smokers.¹⁴

Recent studies of gallbladder cancer have consistently found elevated risks for women with a high body-mass index (by a factor of about two) but generally have involved too few cases for the association to be evaluated in men.^{7,16,17,38,39} Obesity may operate indirectly by increasing the risk of gallstones, which, in turn, increase the risk of gallbladder cancer.⁸

Studies suggest that high body-mass index is associated with approximately a doubling of the risk of pancreatic cancer in both men^{15,40,41} and women^{15,41} — a result similar to our findings. In contrast, there is no strong support for an association between body-mass index and prostate cancer.⁴²⁻⁴⁴ However, some data suggest a slight increase in the risk of advanced prostate cancer or death among patients with a high body-mass index.^{19,45,46} Positive associations of ovarian cancer with body-mass index have been found, with relative

Table 4. Estimated Population Attributable Fraction According to Body-Mass Index for Mortality from Cancer in U.S. Men and Women.*

Body-Mass Index	Men			Women		
	Prevalence of Exposure %	Relative Risk	Population Attributable Fraction %	Prevalence of Exposure %	Relative Risk	Population Attributable Fraction %
All subjects						
25.0–29.9	42.1	0.97	-1.2	28.8	1.08	2.0
30.0–34.9	21.0	1.09	1.8	22.5	1.23	4.5
35.0–39.9	9.2	1.20	1.8	10.7	1.32	3.0
≥40.0	3.6	1.52	1.9	7.9	1.62	4.9
Total population attributable fraction			4.2			14.3
Subjects who never smoked						
25.0–29.9	42.1	1.11	4.0	28.8	1.14	3.3
30.0–34.9	21.0	1.38	6.8	22.5	1.33	6.1
35.0–39.9†	12.8	1.31	3.4	10.7	1.40	3.5
≥40.0				7.9	1.88	7.0
Total population attributable fraction			14.2			19.8

* Data on prevalence of exposure among men are from the National Health and Nutrition Examination Survey (NHANES) (1999–2000) for U.S. men 50 to 69 years of age. Data on prevalence of exposure among women are from NHANES (1999–2000) for U.S. women 50 to 69 years of age. Data on relative risk are from the Cancer Prevention Study II (Table 1 for data for all men, Table 2 for data for all women, and Table 3 for data for men and women who never smoked). The population attributable fraction was calculated with the use of equation 9.6 in Kleinbaum et al.³²

† Values for men are applicable to men with a body-mass index of 35.0 or higher.

risks in the range of 1.5 to 2.0 for the highest body-mass-index categories studied^{7,47–49}; however, several studies have not shown an association.^{16,17,50,51}

Two studies that examined obesity and liver cancer found an excess risk in both men and women, with relative risks in the range of 2.0 to 4.0^{16,17}—a result similar to our findings. Our results and those of a prospective study in Sweden¹⁶ suggest that this excess risk is higher among men than among women. Obesity also increases the risk of adenocarcinoma of the gastric cardia,^{13,14,52} but the data are limited and inconsistent for noncardia cancers of the stomach.^{13,52} In an earlier American Cancer Society cohort, as in our study, mortality from stomach cancer was associated with body-mass index among men but not among women.⁷ This difference may reflect the greater contribution of the cardia to all cases of gastric cancer in men than in women. Our results for cervical cancer are also similar to those in the earlier American Cancer Society cohort,⁷ where-

as the increased risks observed in two cohorts of hospitalized patients with a diagnosis of obesity, as compared with the general population, were much smaller than those observed in our study.^{16,17} Data are scarce on the relation between hematopoietic cancers and body-mass index, and the findings have not been consistent.^{7,16,17,53}

Our results are based on data on mortality and reflect the combined influence of body-mass index both on the incidence of cancer and on survival, whereas most of the available literature on site-specific cancers is based on incidence data. Our results may be influenced by adiposity-related differences in the diagnosis or treatment of cancer, as well as by true biologic effects of adiposity on survival. For example, adiposity has been shown to be adversely associated with the incidence of breast cancer, survival among women with the disease,⁵⁴ and stage at diagnosis.^{55,56} These combined effects may explain why the association of body-mass index with mor-

tality from breast cancer in our study cohort is somewhat stronger than those in previous studies of incident breast cancer.¹⁸

Smoking profoundly alters the relation between body-mass index and many causes of death. We believe that public health recommendations regarding optimal body mass are most valid when they are based on data from studies of persons who have never smoked.^{5,57,58} For smoking-related cancers, the prospective effects of body-mass index on the risk of death among smokers cannot be separated from the prospective effects of smoking—namely, decreased body mass and increased risk of death. Previous analyses of the Cancer Prevention Study II cohort demonstrated that the apparent inverse association of body-mass index and mortality due to lung cancer was incrementally attenuated with increasingly complex statistical control for smoking in multivariate models, and it disappeared entirely when the analysis was restricted to those who had never smoked.⁵⁹ Thus, for smoking-related cancers, we believe that the estimates of relative risk and population attributable fraction presented for the total population (Tables 1, 2, and 4) are likely to be underestimates, whereas those presented for the population of those who never smoked (Tables 3 and 4) offer the most valid estimates of the true effect of overweight and obesity on mortality from these cancers.

We used self-reported weight and height at study entry to calculate the body-mass index, a widely used index of weight adjusted for height.^{60,61} Although

self-reported weight and height are highly correlated with measured weight and height,⁶²⁻⁶⁴ the small error that exists is generally systematic, with an overestimation of height and an underestimation of weight, especially at higher weights.⁶²⁻⁶⁴ Thus, our measure of body-mass index probably underestimated the true body-mass-index values among overweight persons. We had no direct measure of adiposity or of lean body mass and no measure of central adiposity, such as the waist-to-hip ratio. We also could not evaluate the effect of weight change or weight cycling throughout the follow-up period, and we could not estimate the extent of misclassification that weight change might introduce. The associations observed in this study were not changed in models that excluded deaths in the first two years of follow-up.

The large size of our cohort allowed us to investigate the effect of overweight and obesity on the occurrence of 57,000 deaths from cancer among 900,000 men and women who were free of cancer at base line. Overweight and obesity are associated with the risk of death from all cancers and with death from cancers at many specific sites. From our results, we estimate that 90,000 deaths due to cancer could be prevented each year in the United States if men and women could maintain normal weight. It is unlikely that this goal can be achieved without concerted effort and substantial investment on the part of policymakers, educators, clinicians, employers, and schools to promote physical activity and healthful dietary practices as a cultural norm.

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Common mechanisms of dysfunctional adipose tissue and obesity-related cancers

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Summary

The relation between cancer and metabolic disorders was recognized several decades ago, but the underlying mechanisms involved in cancer development and progression remain obscure. In the last years, many groups have been studying systemic adipose tissue markers in cancer patients. However, few consistent results were obtained. On the other hand, several studies revealed many aspects of adipose tissue physiology in obesity. Nowadays, it is recognized that excessive lipid uptake in adipocytes leads to hypertrophy and consequently to metabolic dysregulation, hypoxia, inflammation, impaired adipocytokine expression and angiogenesis, insulin resistance and macrophage recruitment. In obese patients, tumours commonly colocalize with excessive adipose tissue accumulation, and most of the features of hypertrophic adipose tissue are observed in cancer patients, namely breast and colon. This review aimed to summarize pathological adipose tissue alterations that may contribute to cancer aetiology and development. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords adipose tissue; cancer; obesity

Abbreviations ABCA1, ATP-binding cassette A1; ACC, acetyl-CoA carboxylase; acetyl-CoA, acetyl-coenzyme A; AMPK, AMP-activated kinase; bFGF, basic fibroblast growth factor; BMI, body mass index; BRCA1, breast cancer 1; cAMP, cyclic adenosine monophosphate; CPT-1, carnitine palmitoyl transferase 1; CSF-1, colony-stimulating factor 1; CRP, C-reactive protein; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ER, oestrogen receptor; FATP, fatty acid transport protein; FAS, fatty acid synthase; FFA, free fatty acid; GLUT, glucose transporter type; HbA_{1c}, glycated haemoglobin; HB-EGF, heparin-binding EGF-like growth factor; HGF, hepatocyte growth factor; HIF-1, hypoxia-inducible factor 1; HSL, hormone-sensitive lipase; HUVEC, human umbilical vein endothelial cells; IGF-1, insulin-like growth factor 1; IGFBP, insulin-like growth factor binding protein; IGFR, insulin-like growth factor receptor; IKK β , inhibitor of NF- κ B; IKK, IKK β kinase; IL-6, interleukin 6; IRS-1, insulin receptor substrate 1; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; LPL, lipoprotein lipase; MAPK, mitogen-activated protein kinase; Malonyl-CoA, malonyl-coenzyme A; MCP-1, monocyte chemoattractant protein 1; MCPBP, monocyte chemoattractant protein-induced protein; MMP, matrix metalloproteinase; MSC, mesenchymal stem cells; NF- κ B, nuclear factor kappa B; NO, nitric oxide; Ob-Rb, leptin receptor; PAI-1, plasminogen activator inhibitor 1; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PKA, protein kinase A; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; PUFA, polyunsaturated fatty acid; SOCS, suppressor of cytokine signalling; SREBP1c, sterol regulatory element-binding protein 1c; SR-1, scavenger receptor 1; STAT, signal transducer and activator of transcription; RANTES/CCL5, chemokine (C-C motif) ligand 5; ROS, reactive oxygen species; TAM, tumour-associated macrophage; TGF- β , transforming growth

Received: 10 August 2011

Revised: 7 June 2012

Accepted: 24 January 2013

factor beta; TNF- α , tumour necrosis factor alpha; TLR4, toll-like receptor 4; TZD, thiazolidinedione; uPA, urokinase plasminogen activator; VEC, vascular endothelial cell; VEGF, vascular endothelial growth factor

Epidemiology

The link between metabolic disorders and cancer was proposed more than 80 years ago and is still under intense investigation. The prevalence of obesity is rapidly increasing worldwide; 71% of US men and 62% of US women are overweight or obese. In Europe, metabolic syndrome affects 25–31% of individuals, and this will further increase because of the increasing prevalence of obesity in children and young adults [1–4]. Obesity increases the risk of multiple health conditions, including diabetes, hypertension and cancer. However, BMI does not distinguish adipose tissue from other tissues, nor does it inform fat distribution [1–4].

Epidemiological studies have shown that high BMI values were strongly associated with increased incidence of several types of cancer and also premalignant lesions [5,6]. However, colorectal cancer was shown to be more strongly associated with waist circumference and visceral adipose tissue [1,7]. This link is stronger in men because they have more visceral fat. However, when considering waist circumference (marker of visceral obesity), differences between sexes disappear [1,7–11]. Women and men with waist circumferences >99 and 101 cm, respectively, have 50% higher colon cancer risk [1,7,8,10]. In addition, infantile obesity was also demonstrated to increase colon cancer risk in adult life [9,12]. On the other hand, gastric bypass surgery was shown to decrease the risk of cancer mortality [13]. In postmenopausal women, obesity has been associated with higher incidence of breast cancer, reduced likelihood of survival and increased likelihood of recurrence [9].

Adipose tissue in obesity and cancer

Adipose tissue contains several cell types including adipocytes, preadipocytes, fibroblasts, endothelial cells and resident macrophages, which produce a broad range of cytokines, adipocytokines and growth factors, such as adiponectin, leptin, resistin, visfatin, VEGF, PAI-1, MCP-1 or TNF- α (Figures 1 and 2) [3,9,11,14,15]. Recent studies correlated pathological alterations with adipocyte hypertrophy, rather than hyperplasia. During adipocyte hypertrophy, activation of NF- κ B leads to inactivation of PPAR γ and insulin receptor. This conduces to decreased lipid and glucose uptake, adiponectin secretion and adipogenesis. In addition, increased expression of inflammatory, chemoattractant, growth and matrix remodelling factors is also observed (Figure 2) [1,9,11,15–17]. Consequently, FFAs accumulate in other tissues such as liver and muscle, causing hepatic and peripheral lipotoxicity and insulin resistance [18,19].

During this process, adipose tissue localization appears to be critical. As already discussed, visceral adiposity has been considered a major risk factor for metabolic syndrome and apparently for cancer.

It has been suggested that adipose tissue may support tumour cell growth. Adipose tissue-derived factors have been shown to influence the behaviour of tumour cells. Mature adipocytes promote proliferation of tumour cells in tridimensional cultures, and adipocyte-conditioned medium also promotes tumour cell invasion and proliferation [20]. Further, Carter and Church recently demonstrated that conditioned medium of adipocytes isolated from breast adipose tissue increased cell motility of both normal and malignant breast epithelial cells [21]. In mice, simultaneous (but not separate) injection of tumoral cells with adipose tissue results in tumour formation and metastasis [14]. In addition, Hirose and co-workers showed that *db/db* mice, which have hyperleptinaemia and hyperinsulinaemia, have an increased susceptibility to colon premalignant lesions induced by azoxymethane [22]. Authors from the same laboratory also showed that these effects were prevented by antioxidants [23,24]. Despite the fact that the model used on these studies has hyperleptinaemia, Aparicio and colleagues showed that leptin may reduce the development of premalignant lesions in the colon [25]. Thus, besides increasing tumour progression, obesity and diabetes apparently contribute to increased formation of premalignant lesions, but the role of the different players is still unknown.

The formation of hypoxic regions has a major effect on cell metabolism. Thus, it is expected that metabolic intermediates resulting from hypertrophy may somehow regulate angiogenesis. In the last decades, many studies have shown that inflammation is the link between them. Cells respond to metabolic alterations by mechanisms involved in innate immunity, activating inflammatory pathways [18,19]. Besides resulting in insulin resistance, this also contributes to angiogenesis. Inflammation and angiogenesis share common pathways, as most of cytokines and adipocytokines have pro-angiogenic effects, contributing to tissue irrigation and also to cancer progression [9,18–20]. Moreover, both adipose tissue and tumours recruit macrophages, whereas the tumour also recruits adipose stroma cells, showing a strong relation between adipose tissue and the tumour [8]. The integration of such mechanisms in adipose tissue and tumour physiology will be discussed in the following sections.

Metabolic dysregulation in adipose tissue and tumour cells

Dysregulation of lipid metabolism

Adipocyte hypertrophy results in the accumulation of secondary metabolites (such as long-chain acetyl-CoA or diacylglycerol) involved in the activation of several stress and inflammatory pathways, consequently leading to insulin resistance and triglyceride hydrolysis (Figure 2) [19,26–28]. Like adipocytes, macrophages have the ability to change

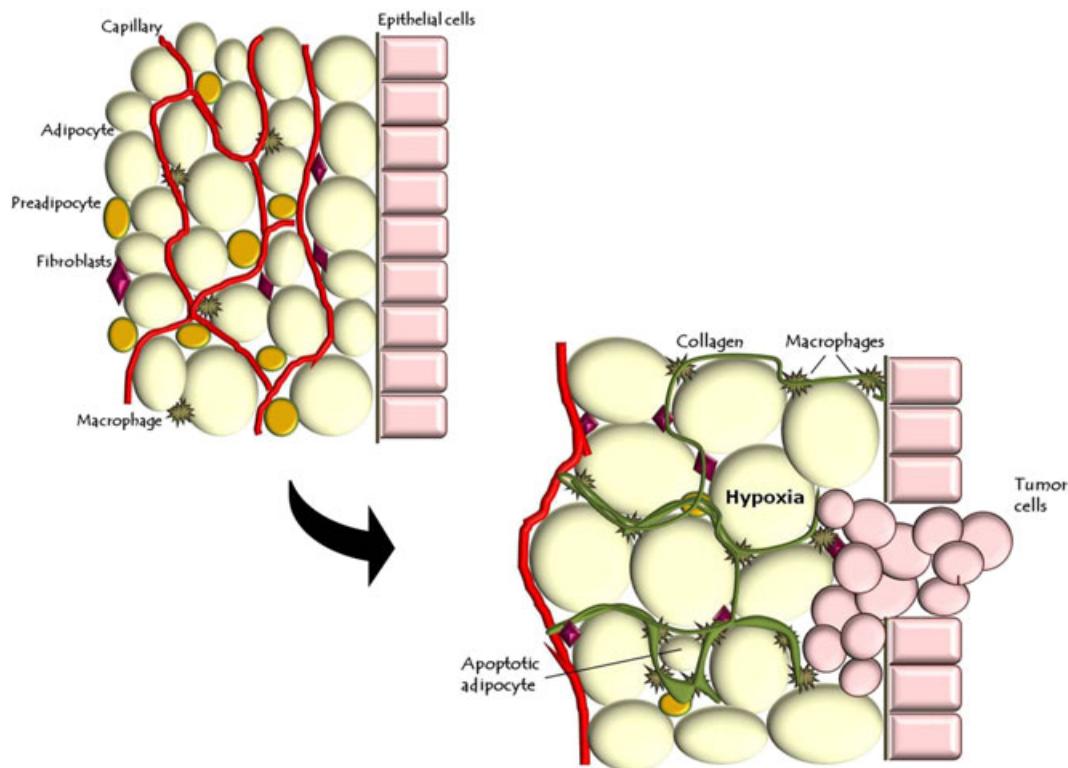


Figure 1. Schematic representation of adipose tissue localization in relation to tumours, common in breast or colon cancer. Adipocyte hypertrophy correlates with increased expression of inflammatory mediators and macrophage infiltration, common features of obesity-related cancer. Furthermore, macrophage infiltration contributes to collagen deposition and tumour cell migration to the blood

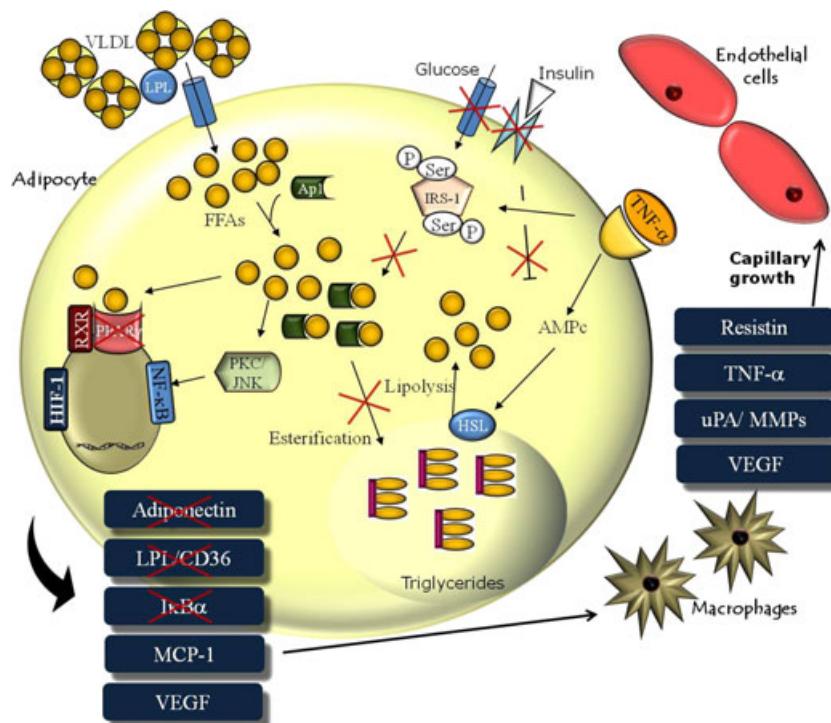


Figure 2. Metabolic, endocrine and inflammatory alterations in hypertrophic adipocytes. Hypertrophy is characterized by excessive cytoplasmatic accumulation of FFAs and hypoxia, leading to activation of inflammatory and stress pathways and inactivation of insulin signalling. These mechanisms involve increased TNF- α , MCP-1, VEGF, resistin and leptin and decreased adiponectin expression, contributing to increased macrophage infiltration. Tissue macrophages regulate matrix degradation and collagen deposition. These mechanisms constitute a hypoxia feedback mechanism to increase angiogenesis and inhibit continuous adipocyte growth, decreasing hypoxia. However, most of angiogenic and chemoattractant mediators and macrophages also regulate tumour growth

their phenotype and store large amounts of lipids, leading to foam cell formation, which are common in hypertrophic adipose tissue. These macrophages have an increased inflammatory potential, creating a positive inflammatory loop in adipose tissue [9]. Importantly, PPAR γ controls lipid storage in both adipocytes and macrophages but is inactivated by such inflammatory pathways (Figure 2). On the other hand, the amount of lipids that the adipocyte has to process and store depends on the ability of peripheral organs to oxidize fatty acids, which is mainly controlled by AMPK [1,9,11,29,30].

PPAR γ and diet regulation. PPAR γ is a nuclear receptor and transcription factor activated mostly by lipids, increasing the expression of several proteins involved in fatty acid uptake and storage [11,15,30] (Figure 2). Furthermore, PPAR γ directly inhibits nuclear translocation of the NF- κ B, thereby inhibiting the expression of cytokines and other inflammatory mediators [11,15,30,31]. Although most tumour cells express PPAR γ , it has not been yet established whether its expression is related to tumour differentiation and metastasis [1]. PPAR γ ligands, including TZDs, are known to inhibit proliferation of cancer cells, increasing nuclear levels of tumour suppressor BRCA1 [1,14]. Positive effects of PPAR γ activators on cancer progression may also be due to decreased ectopic accumulation of substrates in cytoplasm, namely glucose and lipids, and to decreased inflammation and insulin resistance.

As PPAR γ is a diet-regulated transcription factor, the involvement of dietary lipids in adipocyte homeostasis appears to be obvious. In fact, saturated FFAs can activate NF- κ B through TLR4 signalling, whereas PUFAs bind to and activate PPAR γ [30,31]. Despite PUFAs such as ω -3 and ω -6 activating PPAR γ , they were described to have distinct effects on cancer development and growth. ω -3 PUFAs inhibit cancer growth and cell motility, increasing PPAR γ activation in cell lines and enhancing cancer cell apoptosis in mice [11]. On the other hand, ω -6 PUFAs were positively correlated with tumour development in breast and were shown to promote tumour cell motility [11]. Thus, diet regulation of PPAR γ is important for tumour development, but the underlying mechanisms remain unknown.

AMP-activated kinase. AMPK, a major energy regulator activated by adiponectin, increases glycolysis and fatty acid oxidation and inhibits gluconeogenesis and fatty acid and cholesterol synthesis [29,32]. However, obese and type 2 diabetic individuals usually have decreased adiponectin levels, AMPK activity and fatty acid oxidation. AMPK inhibits SREBP1c and activates PPAR α . This results in increased expression of several enzymes involved in fatty acid oxidation and decreased expression of ACC and FAS, key enzymes of fatty acid synthesis [33]. Furthermore, AMPK directly inactivates ACC, thereby decreasing malonyl-CoA formation (inhibitor of CPT-1-mediated mitochondrial fatty acid uptake) from acetyl-CoA, resulting in higher rates of mitochondrial

fatty acid uptake and oxidation [9,29,32,34]. AMPK activates PI3K and Akt and induces GLUT-4 translocation to the cell membrane, thereby increasing glucose uptake [29,32,35]. Moreover, Abbot and colleagues recently showed that AMPK is necessary for Akt-mediated fatty acid uptake in the muscle [35].

Several authors suggested AMPK-induced inhibition of lipid synthesis as a good strategy to decrease lipid availability for cell membranes during tumour growth [34]. AMPK also activates p53, inhibiting the cell cycle [34]. Furthermore, AMPK inhibits aromatase expression in adipose tissue, contributing to decreased oestrogen levels and preventing the development of oestrogen-dependent tumours [34]. Thus, AMPK activation has been proposed as an effective strategy for cancer therapy, and positive results have been observed after metformin treatment, a known activator of AMPK [34].

Hypoxia

A reliable feature of hypertrophic adipose tissue is hypoxia (resulting from decreased blood supply), which is also conducive to the activation of inflammatory pathways, macrophage recruitment and dysregulation of adipocytokine secretion [9,36,37]. However, hypoxia is also common in tumours. HIF-1 is the key factor in cell response to hypoxia, stimulating inflammation and angiogenesis (discussed in the following sections) [38,39]. These mechanisms are likely to support tumour growth [9]. Furthermore, HIF-1 exerts significant effects on cellular metabolism. Cancer cells have a significant shift away from oxidative phosphorylation in mitochondria towards glycolysis, a state involved in the 'Warburg effect' [40–42]. Warburg firstly described that cancer cells have increased glucose uptake and ATP formation (see comprehensive revisions about this issue in [92–94]). As tumour is mostly hypoxic, HIF-1 increases the transcription of the cell-surface glucose transporter GLUT-1 and contributes to the expression of several glycolytic enzymes, including lactate dehydrogenase, and inhibits acetyl-CoA formation, which would be used in the oxidative phosphorylation [40,42]. In addition, because of the high rate of glucose consumption, malignant cells accumulate high amounts of pyruvate, which can be used to fuel the tricarboxylic acid cycle for biosynthetic processes, and even earlier by-products of glycolysis will enter the pentose phosphate pathway for the maintenance of DNA, RNA and fatty acids. Recently, our colleagues showed that methylglyoxal, a metabolite of glycolysis, increases HIF-1 degradation in hypoxic conditions [43]. However, cancer cells develop resistance mechanisms such as elevated expression of glyoxalases, which degrade methylglyoxal [44]. Glyoxalase expression is inhibited in most cells in hyperglycaemic conditions, possibly accounting for the different ability of normal and tumoral cells to adapt to hypoxic conditions [44,45]. The contribution of adipose tissue hypoxia to tumour development is unknown, but tumours are usually surrounded by adipose tissue; thus, it is likely that such an environment may contribute to a better tumour adaptation [46].

Cytokines and adipocytokines – metabolism, inflammation and angiogenesis

Activation of inflammatory pathways as a consequence of accumulation of intracellular metabolites and formation of hypoxic regions leads to altered cytokine and adipocytokine secretion. This conduces to inhibition of continued lipids and glucose uptake and stimulates angiogenesis. However, most of the products released by the adipose tissue under such conditions may also affect tumour physiology [30,47–49]. In this section, we review the alterations of these factors as a result of adipose tissue dysfunction and how they influence tumour formation and development.

Adiponectin

Adiponectin circulates in three distinct molecular forms, trimeric, hexameric and high-molecular-weight forms (several hexameric forms), and it is mainly produced by the adipose tissue [50–52]. Adiponectin levels, especially high-molecular-weight forms, were found to be lower in obesity, insulin resistance and type 2 diabetes and inversely correlated with abdominal perimeter and fat mass, more than BMI [1,14,15,53]. Adiponectin expression is decreased by TNF- α and IL-6 (NF- κ B activators) and increased by PPAR γ agonists (Figure 2). However, adiponectin also inhibits TNF- α and IL-6 expression in macrophages, inactivating the inflammatory cascade [14,20]. Adiponectin increases fatty acid oxidation and insulin sensitivity and decreases gluconeogenesis, as it activates AMPK and PPAR α [1,20].

Adiponectin decreases proliferation of several cell types including adipocytes, endothelial cells and cancer cells [20]. Beyond inhibition of tumour cell growth and survival, adiponectin inhibits angiogenesis through decreased VEGF and Bcl-2 expression (anti-apoptotic) and increased p53, Bax and caspase activation (pro-apoptotic), leading to endothelial cell apoptosis [1,20]. Likewise, adiponectin was shown to reduce TNF- α -induced effects on cell proliferation and migration [53]. In fact, several studies showed hypo adiponectinaemia as a determinant for larger tumours with increased angiogenesis [1,14,20]. Thus, adiponectin may act in decreasing not only inflammation but also angiogenesis, and these effects may be crucial for regulation of neovascularization in pathological conditions such as obesity, type 2 diabetes and also cancer. However, it is not known yet whether hypo adiponectinaemia is a risk factor for cancer or hypo adiponectinemia and cancer development are parallel events of metabolic dysregulation.

Leptin

Leptin is currently at the centre of the obesity–cancer link, as it is produced in proportion to fat mass (almost exclusively in adipocytes) and potently induces cell (normal and tumoral) mitogenesis, growth and motility [14,20,48,54–57]. Leptin activates JAK/STAT and MAPK pathways and also the SOCS-1 and SOCS-3,

inhibiting insulin and its own signalling cascade [1,15,56,57]. This mechanism is chronically activated in obesity and is one of the major causes of leptin and insulin resistance [1,14,58].

Clinical studies in Norwegian and Japanese populations showed leptin correlation with increased risk of breast and colon cancers, independently of BMI [45,59]. Furthermore, leptin was also positively correlated with increased metastasis [1,20,51,60]. In most of the patients, tumour cells express the Ob-Rb. Insulin, IGF-1 and oestradiol were observed to activate its expression [61]. In addition, leptin also promotes aromatase expression in adipose tissue and ER on tumour cells [14,62]. However, leptin is mitogenic for both ER-positive and ER-negative tumour cell lines, inhibiting apoptosis and increasing cell proliferation [14,20,48,53,62].

From this, we may conclude that leptin promotes tumour cell proliferation both directly and indirectly (through an increase of oestrogen levels and insulin resistance). However, leptin effects on cancer development are still controversial. Several studies reported elevated leptin levels in cancer patients, but these studies compared cancer patients with healthy controls without separation of the obesity component in the experimental design. Furthermore, existing studies did not reveal a pattern of leptin variation between premenopause and postmenopause. Zucker rats, which have a mutation in Ob-Rb and exhibit hyperleptinaemia, have increased formation of benign neoplasies but minimal formation of malign tumours [53]. In fact, it is possible that leptin does not act as a tumour inducer but more as a supporter of already formed tumours.

Resistin

Resistin has been pointed as a major link between innate immunity and glucose metabolism as it was shown to inhibit hepatic, muscle and adipose insulin signalling, consequently leading to inflammation and insulin resistance. Its circulating levels are usually increased in models of obesity. Antibody neutralization, overexpression of nonfunctional resistin or gene knockout in mice results in improved glucose metabolism and insulin sensitivity [15,18,63–65]. However, extrapolating possible effects of human resistin from murine experiments has several caveats. Murine resistin is produced in adipocytes, whereas human resistin is mainly produced in macrophages (Figure 2) [15,18,65]. Furthermore, human and murine resistin genes are located in different chromosomes, and studies searching for a human gene more similar to murine resistin have been unsuccessful [15].

Recently, Qatanani and colleagues developed mice with suppressed expression of endogenous resistin and transgenic expression of human resistin in macrophages [18]. After consuming a high-calorie diet, these rats had increased lipolysis in adipose tissue. They also developed increased triglyceride and diacylglycerol levels and insulin resistance in muscle, due to PKC-induced serine phosphorylation of insulin receptor and IRS-1 (Figure 2) [18]. Resistin promotes foam cell formation from macrophages

through increased lipid uptake, resulting from activation of scavenger receptors (SR-1 and CD36) involved in recruiting lipoproteins and FAP. Conversely, resistin increases proteasomal degradation of ABCA1 involved in cholesterol efflux [15,66].

Human resistin stimulates proliferation of vessel muscle cells and VECs [15,65,67]. In endothelial and choriocarcinoma cells, resistin also increases the expression of VEGF and its receptors while also elevating MMP, possibly through NF- κ B activation [65,68]. Thus, resistin overexpression is a physiological response to adipose tissue hypertrophy and hypoxia, but it may also contribute to tumour angiogenesis. Despite the data about this issue still being scarce, several studies have implicated resistin in the aetiology of obesity-related cancers, namely breast and colon [50,51,64,69–72]. Resistin may provide unique insights to the link between obesity, inflammation, insulin resistance and cancer risk in humans.

Visfatin

Visfatin is highly expressed in visceral fat of rodents and humans, whose plasma circulating levels are positively correlated with the size of visceral fat deposits [73]. Several studies reported increased visfatin levels in various pathological conditions, such as obesity, inflammation, diabetes mellitus, metabolic syndrome and cardiovascular diseases [74–78].

Visfatin was originally isolated from lymphocytes as nicotinamide phosphoribosyltransferase, an enzyme that synthesizes nicotinamide mononucleotide from nicotinamide [79]. The following studies reported an insulin-mimetic effect through binding to a different region of insulin receptor, activating IRS-1 tyrosine phosphorylation [73,80–86]. However, the original study reporting these effects was later retracted [73], and the mechanisms of visfatin action, beyond NAD⁺ synthesis, remain obscure. Nevertheless, visfatin expression was found to be stimulated by HIF-1 and the JNK/NF- κ B pathway, promoting angiogenesis through MMP expression in endothelial cells and VEGF expression in tumour cells [79,87–89]. Visfatin is also expressed in tumoral cells, and its circulating levels were found to be increased in breast and colon cancer patients [82,87]. Furthermore, visfatin was observed to increase cell proliferation and invasion during breast cancer progression [87]. Visfatin inhibitors have been clinically evaluated as anticancer agents, but new epidemiological and cellular studies are needed to understand the mechanisms of visfatin action on cancer [90].

Tumour necrosis factor alpha

TNF- α was the first recognized adipose-derived cytokine to be involved in metabolism regulation. In this field, Hotamisligril's laboratory has been particularly active, producing data and reviews about TNF- α 's role on adipocyte metabolism (see reviews in [31,47]). TNF- α is produced mainly in macrophages, but also in adipocytes and preadipocytes, after excessive lipid accumulation, activating several serine kinases (JNK and IKK). This results in NF- κ B activation and inhibition of insulin

signalling (Figure 2) [1,14,15,20]. NF- κ B stimulates the production of other cytokines and inhibits PPAR γ activity and subsequent expression of adiponectin, CD36 and LPL. Thus, TNF- α inhibits preadipocyte differentiation, lipid and glucose uptake and fatty acid esterification and increases lipolysis [1,53]. These mechanisms aim to inhibit continued adipocyte growth but may also be important for cancer progression because they lead to metabolism dysregulation (as focused on in Section 2.1) and hyperinsulinaemia, when chronically active. In fact, a link between TNF- α levels and breast and colon cancer has been suggested [14]. Beyond its metabolic effects, TNF- α seems to contribute to tissue alterations necessary for tumour growth and metastasis, namely cell proliferation. TNF- α stimulates aromatase expression in adipose tissue, increasing oestrogen-mediated tumour cell proliferation [20,91–93]. Furthermore, TNF- α stimulates VEC proliferation and migration in rat cornea [53]. Clinical trials with several TNF- α antagonists and antibodies have shown promising effects in reducing tumour development and metastasis [94].

Monocyte chemoattractant protein 1

MCP-1 is a protein that recruits and activates circulating monocytes to the adipose tissue [95–98]. Initially, MCP-1 was considered an activator of antitumour cytotoxic functions of macrophages. However, identification of two types of TAMs and the revelation of MCP-1 angiogenic potential altered the perspective of MCP-1 physiology on cancer (Figure 2) [95,99,100]. In the last years, several studies have found elevated MCP-1 levels, especially in advanced stages [96,99]. However, it is not established whether MCP-1 circulating levels are a systemic marker of poor prognosis or not. Some discrepancies may be caused by different studied populations, experimental designs, degree of metastasis, tumour phase and time of sample collection [99].

In adipose tissue, MCP-1 expression correlates with increased macrophage infiltration and angiogenesis [19,28,36]. In epithelial cells, MCP-1 expression is low, but it is acquired along with the transformation [98,99]. MCP-1 is also secreted by tumour stroma cells, such as macrophages and fibroblasts, acting through paracrine and autocrine pathways [95,99]. Recently, Niu and colleagues showed that MCP-1 promoted *in vitro* tube formation in HUVEC. In addition, Hong and colleagues showed that these effects were dependent on VEGF activation [101,102]. Furthermore, MCP-1 was observed to increase angiopoietin (Ang)-2 levels and MMP expression on monocytes, T cells and tumour cells, which also contributes to angiogenesis [96,99,103]. On tumour cells, MCP-1 does not induce proliferation but increases their migration ability. MCP-1 was also suggested to be involved in epithelial-to-mesenchymal transformation, an event essential for cell transformation [99]. Higher levels of MCP-1 are correlated with increased macrophage infiltration, MSC migration, vascularization, tumour progression and lymphatic invasion [95,96,99,104]. An anti-MCP-1 antibody decreased MSC migration to tumour,

angiogenesis and lung metastasis, increasing the survival ratio [99,104]. Thus, as in adipose tissue, MCP-1 and recruited macrophages change the tumour microenvironment to support cell proliferation and migration and angiogenesis.

Angiogenic mechanisms – growth factors and matrix remodelling

Adipose tissue profoundly depends on angiogenesis as it is subjected to constant expansion/regression. Capillary growth is a complex process involving matrix degradation, endothelial cell migration and tube formation and depends on the anastomosis of two growing tubes. This process is regulated by several factors, namely fibroblast growth factors, hepatic growth factor (HGF), VEGF, Angs (Ang-1 and Ang-2), epithelial growth factors, adipocytokines, insulin/IGF-1, oestrogens and MMP, some of them already discussed in this review [53].

Several adipose-derived factors have paracrine effects, acting directly on neighbouring endothelial cells to promote angiogenesis. Most of these factors are produced in response to adipocyte hypertrophy and consequent metabolic dysregulation and regional hypoxia. As we already discussed, the unifying point is inflammation, which results from both of these conditions. Inflammatory mediators directly induce angiogenesis and increase the expression of several angiogenic factors. Furthermore, most of these factors also recruit macrophages, which also produce their own set of angiogenic factors (VEGF, TNF- α , fibroblast growth factors, MMPs and resistin), further contributing to angiogenesis (Figure 2) [53,105,106].

Tumour angiogenesis is important for its growth, but vessels are usually dysfunctional with abnormal ramifications, probably due to the imbalanced expression of growth factors in hypertrophic adipose tissue and in the tumour. Furthermore, these vessels are usually very permeable and allow the passage of fluids, proteins and tumour cells, favouring metastization.

Growth factors

VEGF is the only angiogenic factor specific for VECs. Obese individuals commonly have increased VEGF levels [see review at 53]. VEGF is produced by adipose tissue cells, namely adipocytes, preadipocytes and macrophages, stimulating VEC proliferation and migration, expression of proteolytic enzymes and tube formation and inhibiting apoptosis (Figure 2) [53]. VEGF expression is stimulated by regional hypoxia (through HIF-1 stabilization), factors from the EGF family and insulin (via PI3K and Akt), to compensate for insulin-induced nutrient deposition and tissue expansion [53,107,108]. Recently, our group described the VEGF/Ang-2 ratio to determine vascular integrity. The imbalance of this ratio caused endothelial cell apoptosis and loss of vessel integrity [109]. However, increased tumour VEGF levels are also associated with poor prognosis [53]. Consequently,

several antitumour drugs based on its neutralization were developed. Antibody neutralization of VEGF receptor 2 also decreased both angiogenesis and preadipocyte differentiation [53].

Other growth factors such as bFGF and HGF have also been studied as possible players in tumour growth. bFGF and HGF are produced in adipocytes and stimulate VEC proliferation and MMP expression [20,53,110]. For bFGF, no correlations with BMI were found in previous studies, probably because it acts mainly through paracrine mechanisms [53]. HGF is known to act on its receptor Met (product of *c-met* gene) and to promote proliferation, motility and invasion of hepatic tumour parenchymal cells [53,110,111]. However, HGF is produced proportionally to BMI and correlates with bigger tumours and increased lymphatic invasion [20,53]. Furthermore, HGF promotes cell invasion independently of VEGF and also increases its expression [20,53,110,111]. HGF is highly produced in tumour or peritumoral adipose tissue, decreasing after tumour excision [20].

Proteolytic matrix degradation – plasminogen system

Besides MMPs, the plasminogen system also regulates extracellular matrix degradation, to allow vessel growth. This system is formed by the plasminogen, cleavage enzymes (tissue plasminogen activator and uPA and serine proteases), PAI-1 and membrane uPA receptor (uPAR), regulating fibrinolysis and matrix proteolysis [see review at 11]. Plasminogen is cleaved to plasmin by its tissue plasminogen activator in arterial walls and by uPA in the extracellular matrix, increasing matrix degradation [11]. Furthermore, uPA binding to uPAR leads to integrin binding and cytoskeleton arrangement favouring cell migration [11]. Thus, uPA and uPAR are markers of invasiveness and poor prognosis. uPA inhibition results in decreased tumour invasiveness. On the other hand, PAI-1 binds to and inhibits uPA. This complex binds to uPAR and is internalized [11]. Despite uPA inhibition effects, circulating PAI-1 levels are a marker of poor prognosis in breast cancer, and it was suggested as a potential target in cancer therapy [11]. Although the reasons for elevated PAI-1 levels are not known, it is known to be highly produced in dysfunctional adipose tissue, and it has been suggested to promote cell growth and migration, angiogenesis and migration of smooth muscle cells [11,112].

Insulin – mitogenesis and IGF-1 regulation

Insulin resistance is common in patients with elevated BMI and is a hallmark of type 2 diabetes, being usually associated with visceral obesity and inflammation in adipose tissue [7,14,15]. Also, the effects of insulin analogues in type 1 diabetic patients have been discussed in the last years. Long-action insulin analogues, such as glargine, are widely used because they do not cause hypoglycaemia. However, attempting to efficiently control postprandial glycaemia with these medications, authors have found that

patients are episodically hyperinsulinaemic [113]. Hyperinsulinaemia has been pointed as a major risk factor for breast, colon and prostate cancer [7,14,15,114–116]. Also, elevated C-peptide levels were found to be associated with a threefold increase in colon cancer risk [1,12].

Insulin has been shown to increase cell survival and growth (through the IRS-1-PI3K-Akt pathway), supporting tumour development [113]. Furthermore, Chen and colleagues recently reported that insulin-mediated activation of this pathway causes chemotherapy resistance, originating new questions about the role of insulin on cancer [117,118]. However, the impact of insulin mitogenic effects on obesity-related cancer through this pathway is controversial, as IRS-1 is partially inhibited on insulin resistance. Thus, the discovery of a noncanonical pathway for insulin-stimulated mitogenesis is a major hallmark on metabolism-related cancer. Most growth factors activate the canonical Ras-Raf-MAPK pathway. Besides increasing IGF-1 and VEGF circulating levels and aromatase expression in adipose tissue, insulin was also observed to increase isoprenylated (active) Ras levels in the cell membrane, thereby increasing cell response to other growth factors (see review in [113]).

The IGF family components include IGF-1 and 2, membrane receptors (IGFR1 and 2), IGFBPs (IGFBP1 to IGFBP6) and IGFBP proteases [14]. Like insulin, IGF-1 regulates cell survival, growth and differentiation and inhibits apoptosis [9]. On the other hand, IGFBPs decrease IGF bioavailability, inhibiting its effects and promoting apoptosis [14]. However, IGFBP1 and IGFBP1 expressions are downregulated by insulin, increasing free/active IGF-1 levels [3,8]. Insulin-resistant individuals commonly have higher IGF-1 circulating levels [3,8,9].

On epithelial cells, IGF-1-IGFR binding leads to receptor dimerization and to PI3K and Ras-Raf-MAPK activation [14]. Furthermore, oestrogens increase IGF-1 and IGFR1 expressions, acting synergistically in favour of tumour growth [14]. As insulin, IGF-1 also activates VEGF expression and thus indirectly acts on endothelial cells [1]. Elevated IGF-1 and decreased IGFBP-1 levels were previously correlated with increased cancer risk, tumour invasion and poor prognosis [1,14]. Also, breast tumours with elevated IGFR-1 levels, both premenopause and postmenopause, usually are more invasive [14]. In mice, IGF-1 overexpression resulted in increased tumour growth, whereas disruption of IGFR1 prevented it [8,9,14].

The role of macrophages on tissue remodelling – implications in adipose tissue and tumour

In obese individuals, adipose tissue commonly has extensive macrophage infiltration, recruited by TNF- α and particularly by MCP-1 (Figure 2). On the other hand, macrophage-secreted products such as TNF- α or resistin induce insulin resistance and promote angiogenesis (Figure 2). Macrophage coculture results in impaired

adipocytokine expression and increased production of pro-inflammatory cytokines in adipocytes [8].

Tumour environment is formed by several cell types, namely fibroblasts, cells from hematopoietic lineage, endothelial cells and adipocytes. These cells strongly interact, creating a microenvironment that is conducive to tumour growth [119,120]. Adipose-related tumours have large amounts of macrophages (up to 50% of the tumour cells) [100,105,119,120]. More than 80% of cancers have elevated macrophage infiltration, correlating with increased vessel density, tumour progression and poor prognosis [53,99,100,104,105,119]. Genetic macrophage elimination reduces tumour progression and metastasis in mice [8].

A tumour commonly has two distinct populations of macrophages: M1 macrophages are pro-inflammatory and secrete antitumour cytokines and M2 macrophages are immunosuppressive and produce a broad range of angiogenic and growth factors, including MCP-1, MMPs, TGF- β , VEGF and PDGF, promoting proliferation and migration of tumour cells and VEC, matrix degradation and ultimately angiogenesis [8,49,104–106,119–121]. The reason why this macrophage population acquires this behaviour is still unknown. However, this is also observed in macrophages recruited to the hypertrophic adipose tissue, which are involved in tissue remodelling and angiogenesis [36].

Macrophages intensely populate the fibrotic ring surrounding the tumour and regions of basal membrane rupture. They seem to regulate collagen synthesis in fibroblasts and also support blood vessel formation close to collagen fibres [119]. This leads to increased collagen deposition, anchored to blood vessels, allowing faster tumour cell migration to the circulation (Figure 1) [100,119]. These features are not found in macrophage-deficient mice [8,119]. Importantly, adipose tissue from obese animal models was recently described to develop extensive fibrotic regions, which may support growth of adipose-associated tumours [121]. Fibroblasts from tumour periphery also have high aromatase expression, which is increased by macrophage-released cytokines such as TNF- α [14,20].

The close relation between tumour cells and macrophages in the invasive front involves CSF-1 and EGF signalling [100,120]. Tumour cells express CSF-1 to increase macrophage activity, and macrophages express EGF to stimulate migration of tumour cells. Accordingly, macrophages express CSF-1R whereas tumour cells express EGFR1 [119]. Mice with CSF-1 depletion have decreased VEGF and MMP expression and metastasis and more benign phenotypes. CSF-1 expression restores phenotype, and CSF-1 overexpression in normal mice leads to more TAM and increased tumour growth and metastasis [100,119]. Moreover, macrophage depletion results in 40% decreased angiogenesis even in advanced tumours, suggesting that macrophages are involved in the formation of new blood vessels and in the remodelling of those already existing, as observed during adipose tissue expansion [36,100,119]. Macrophage-derived VEGF, beyond VEC

stimulation, also recruits other macrophages to support tumour growth [100,119]. Present data show that TAM and adipose tissue macrophages share important similarities, promoting tissue remodelling and angiogenesis. In addition, adipose tissue macrophages may possibly support tumour development.

Conclusions

Adipose tissue dysfunction in obesity occurs as a result of tissue hypertrophy. Hypertrophic adipose tissue commonly presents a dysregulated lipid metabolism and formation of hypoxic regions. These two events lead to the activation of inflammatory pathways and, consequently, to impaired adipocytokine expression, increased pro-inflammatory cytokine expression, angiogenesis, insulin resistance and macrophage recruitment. All these features are also observed in tumours, which usually are very close to dysfunctional adipose tissue, namely in breast and colon cancers. Despite the mechanisms discussed here being shown to be tumour supportive,

the trigger for tumour development is still unknown and should be a matter of interest for future studies.

Search strategy and selection criteria

Bibliographic search was performed using electronic databases, namely PubMed, with the reference words 'obesity', 'adipose tissue', 'adipocytokines', 'angiogenesis', 'insulin', 'estrogens', 'macrophages', which were also paired with 'cancer'. Original (both biochemical and epidemiological) and review papers with higher relevance and novelty from the last 3 years were considered. Furthermore, older articles with recognized importance in the field were also included.

Conflict of interest

The authors have declared that there is no conflict of interest.

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AMPK as a metabolic tumor suppressor: control of metabolism and cell growth

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AMPK is an evolutionarily conserved fuel-sensing enzyme that is activated in shortage of energy and suppressed in its surfeit. AMPK activation stimulates fatty acid oxidation, enhances insulin sensitivity, alleviates hyperglycemia and hyperlipidemia, and inhibits proinflammatory changes. Thus, AMPK is a well-received therapeutic target for metabolic syndrome and Type 2 diabetes. Recent studies indicate that AMPK plays a role in linking metabolic syndrome and cancer. AMPK is an essential mediator of the tumor suppressor LKB1 and could be suppressed in cancer cells containing loss-of-function mutations of *LKB1* or containing active mutations of B-Raf, or in cancers associated with metabolic syndrome. The activation of AMPK reprograms cellular metabolism and enforces metabolic checkpoints by acting on mTORC1, p53, fatty acid synthase and other molecules for regulating cell growth and metabolism. In keeping with *in vitro* studies, recent epidemiological studies indicate that the incidence of cancer is reduced in Type 2 diabetes treated with metformin, an AMPK activator. Thus, AMPK is emerging as an interesting metabolic tumor suppressor and a promising target for cancer prevention and therapy.

AMP-activated protein kinase acts as a fuel gauge that is activated under stresses such as hypoxia, ischemia, glucose deprivation and exercise [1]. Activation of AMPK stimulates fatty acid oxidation to generate more ATP to cope with acute energy demand and inhibits anabolic processes that consume ATP [1]. As a result, energy is preserved for acute cellular programs. In addition, AMPK activation enhances insulin sensitivity, inhibits hepatic glucose production, stimulates glucose uptake in muscle, inhibits fatty acid synthesis and esterification, and diminishes proinflammatory changes [2]. Thus, AMPK is a well-accepted target for the treatment of metabolic syndrome and Type 2 diabetes (for extensive reviews, refer to [1–3]). During the last 5 years, since our first review [3], great attention has been drawn to link AMPK and cancer, and substantial progress has been made. AMPK, by regulating a variety of downstream targets, such as mTORC1, p53 and fatty acid synthase (FASN), and associated metabolic processes, controls intracellular energy levels in order to maintain the cell growth rate at an appropriate level. Likewise, AMPK activation under metabolic stress or by pharmacological activators can regulate various processes, including cell cycle checkpoint, cell polarity, senescence, autophagy and apoptosis [4–7]. In this article, we aim to summarize recent evidence in support of the notion that AMPK

serves as a metabolic tumor suppressor and discuss the implications of AMPK in cancer prevention and treatment.

Activation of AMPK

AMP-activated protein kinase belongs to a family of serine/threonine protein kinases and is highly conserved from yeast to human. It consists of three subunits: a catalytic subunit (α) and two regulatory subunits (β and γ) [8,9]. In mammals, each subunit of AMPK contains two to three isoforms ($\alpha 1, \alpha 2; \beta 1, \beta 2; \gamma 1, \gamma 2$ and $\gamma 3$). When cells confront metabolic stress, the intracellular AMP level or the ratio of AMP to ATP is increased. AMP then binds to the γ -subunit, yielding two outcomes; first, it serves as allosteric activator and second, it protects AMPK against phosphatases to dephosphorylate threonine 172 in the activation loop of the catalytic α -subunit [9–11]. Another critical step for AMPK activation is the phosphorylation of threonine 172. Previous studies suggest that AMP binding enables the phosphorylation of threonine 172 by an upstream kinase [12,13]. However, this notion is challenged by recent *in vitro* studies showing that phosphorylation of AMPK by purified LKB1 complex is independent of AMP [11,14]. A possible explanation of this discrepancy is that the AMPK preparations used in previous studies may have been contaminated with phosphatases, of which the activity toward AMPK is inhibited by AMP.

Keywords

- acetyl CoA carboxylase
- AMPK ■ fatty acid synthase
- LKB1 ■ metabolic syndrome
- metabolism ■ mTOR ■ p53
- tumor suppressor
- tumorigenesis

AMP-activated protein kinase can also be activated by hormones and cytokines, including leptin (e.g., in skeletal muscle) and adiponectin secreted from adipocytes, IL-6 and ciliary neurotrophic factor (CNTF) [1,15]. Interestingly, leptin could exert opposite effects on AMPK depending on cell types. While it inhibits the enzyme in the arcuate and paraventricular regions of the hypothalamus of fasted mice, leptin activates α 2 isotype of AMPK in skeletal muscle [16,17]. In addition, AMPK can be activated by a variety of pharmacological agents. The prototypical activator is 5-aminoimidazole-4-carboxamide 1-D-ribonucleoside (AICAR), a cell permeable agent that is phosphorylated and converted to ZMP, an AMP analog, after entering the cell. Importantly, two clinically used antidiabetic drugs, metformin and thiazolidinediones (TZDs), have been known to activate AMPK [15]. Upon activation, AMPK phosphorylates a plethora of substrates and regulates their functions (FIGURE 1), which are far beyond the canonical ones that are known to promote fatty acid oxidation and simultaneously inhibit lipid synthesis [8,9].

Thus far, several kinases have been identified to phosphorylate threonine 172 on the catalytic α -subunit of AMPK leading to its activation. The first kinase is LKB1, which is originally found in liver and also known as serine/threonine kinase 11 (STK11) [13,18,19]. LKB1 is ubiquitously expressed and responsible for AMPK activation in most scenarios. The second kinase is calmodulin-dependent protein kinase- β (CaMKK- β), which phosphorylates AMPK in response to increases in intracellular Ca^{2+} levels instead of AMP [20,21]. Other kinases include TGF- β -activating kinase 1 (TAK1) and ataxia-telangiectasia mutated (ATM) [22–24]. Whether these kinases are bona fide kinases for AMPK remains to be further determined by genetic approach. Interestingly, a recent study has reported that TAK1 mediates TNF-related apoptosis-inducing ligand to activate AMPK, inducing autophagy independent of LKB1 and CaMKK [25].

AMPK mediates the tumor suppressive function of LKB1

LKB1 is a tumor suppressor [26], and its loss-of-function mutations, most of which cause a loss of kinase activity, are an etiological factor of Peutz–Jeghers syndrome, an autosomal dominant genetic disorder. This genetic syndrome is characterized by multiple hamartomatous polyps (benign overgrowth of differentiated

tissues) in the gastrointestinal tract and a markedly increased risk of gastrointestinal adenocarcinomas [26]. In addition, somatic mutations of the *LKB1* gene have been found in several other cancers, for example, in approximately 34% of lung adenocarcinomas, 19% of squamous cell carcinomas and 20% of cervical carcinomas and other cancers [27–30].

Although a complete ablation of *LKB1* causes embryonic lethality in mouse models, its heterozygous deletion increases the incidence of tumor in the intestine and stomach [26] and predisposes animals to carcinogenesis induced by 7,12 dimethylbenz(α)anthracene, thereby developing squamous cell carcinoma of the skin and lung [31]. In addition, tissue-specific deletion of *LKB1* in the endometrial epithelium of female mice or the prostate epithelium of male mice causes endometrial adenocarcinomas and prostate neoplasia, respectively [32,33]. AMPK plays an important role in mediating tumorigenic effects of LKB1. Thus, a hypomorphic mutation that decreases LKB1 markedly accelerates tumor development in *PTEN*^{+/-} mice, whereas pharmacological AMPK activators significantly delay tumor onset [34]. In LKB1-deficient lung cancer cells, AMPK activity is suppressed and refractory to its pharmacological activators, leading to increased mTORC1 signaling, whereas the ability of AMPK to inhibit cell growth is restored when wild-type LKB1 is expressed [35,36].

Downstream targets of AMPK

The number of AMPK targets has grown rapidly in recent years and their biological functions have been discussed in great detail elsewhere [1,5,8,9]. In this article, we highlight only some of the targets that are well characterized and known to have important roles in tumorigenesis.

mTORC1 signaling

Many studies have established that mTOR is a major downstream target of AMPK (FIGURE 2) [5]. Early studies have shown that AMPK activated by stress or pharmacological activators inhibits protein synthesis via regulation of mTOR/S6K [37] and translation elongation factor 2 [38]. mTOR is a key component of mTORC1 and mTORC2, both of which have essential roles in PI3K signaling [39]. While mTORC2 phosphorylates Akt at Ser 473, contributing to its activation, mTORC1 integrates nutrients and growth signals to function downstream of PI3K/Akt. Upon stimulation of cells with growth factors, activated Akt phosphorylates tuberous sclerosis complex 2 (TSC2), which forms a complex with TSC1 and thus

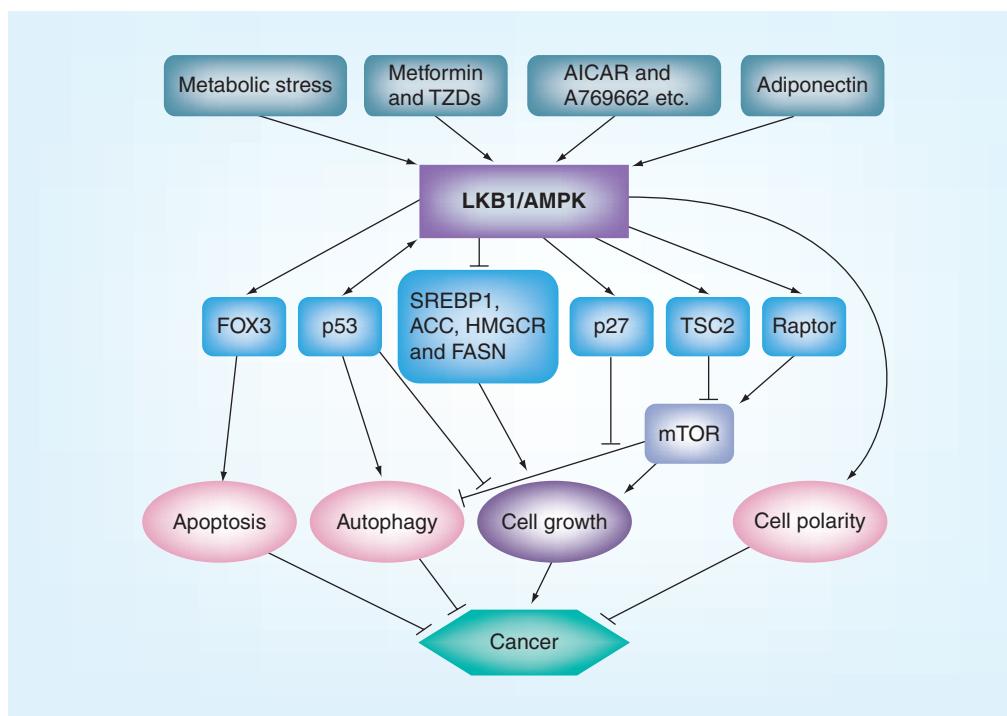


Figure 1. Essential Roles of LKB1/AMPK in controlling cell growth and tumorigenesis.

LKB1/AMPK by regulating mTORC1, p53 and other important molecules control cellular processes, such as cell cycle checkpoint, apoptosis, autophagy and cell polarity. Dysregulation of LKB1/AMPK causes loss of these control points and thus leads to unrestrained growth. Only a few representative molecules that are directly relevant to growth control are listed here.

TZD: Thiazolidinedione.

constitutes a GTPase-activating protein (GAP) for a small GTPase, RHEB, and an immediate activator of mTOR. This phosphorylation leads to inactivation of the GAP activity, causing activation of mTOR. Germinal mutations of TSC1 or TSC2 account for another autosomal genetic disease, in which patients develop hamartomas in multiple organs, such as the brain, lung, skin, heart and kidney [40].

AMP-activated protein kinase has been shown to phosphorylate TSC2 at a different site to the Akt site, which primes phosphorylation at an adjacent site by GSK3, leading to activation of the TSC GAP activity [41,42]. In addition, AMPK has recently been shown to phosphorylate Raptor, a scaffold in the mTORC1 complex. The phosphorylation results in 14-3-3 binding and inactivation of mTORC1 [43]. Thus, AMPK exerts a dual control of mTORC1, so as to enforce a metabolic checkpoint in response to nutrient deprivation. The identification of mTORC1 as a downstream target of LKB1/AMPK is of great interest in cancer biology and therapy, inasmuch as the pathway is activated in a large number of tumors owing to oncogenic activation of receptor-tyrosine kinase, PI3K, and loss-of-function

mutations of PTEN [44]. Indeed, deficiency of LKB1 in humans and mouse models of both Peutz–Jeghers syndrome and lung cancer is accompanied with deregulated mTORC1 activity and associated changes in gene expression, such as increased expression of SREBP1 and HIF-1 α [45,46]. When tested, these tumors are sensitive to the inhibition of mTORC1 [35,46,47].

p53 & other tumor suppressors

Another important partner of AMPK is the tumor suppressor p53, with which AMPK is mutually regulated. On one hand, AMPK activation by AICAR or glucose deprivation leads to upregulation of p53 as well as its phosphorylation at Ser15 [48,49]. Mouse embryonic fibroblasts (MEFs) bearing wild-type p53 are arrested at G1/S phase by glucose deprivation, AICAR and expression of a constitutively activated mutant of AMPK, whereas the metabolic checkpoint is ineffective in the p53-deficient MEF cells [48]. We have shown that expression of a dominant negative mutant of the AMPK $\alpha 1$ -subunit in prostate cancer cells accelerates their growth, concomitant with decreases in mRNA and protein levels of p53 [50]. On the other hand, AMPK is regulated by p53. The first example

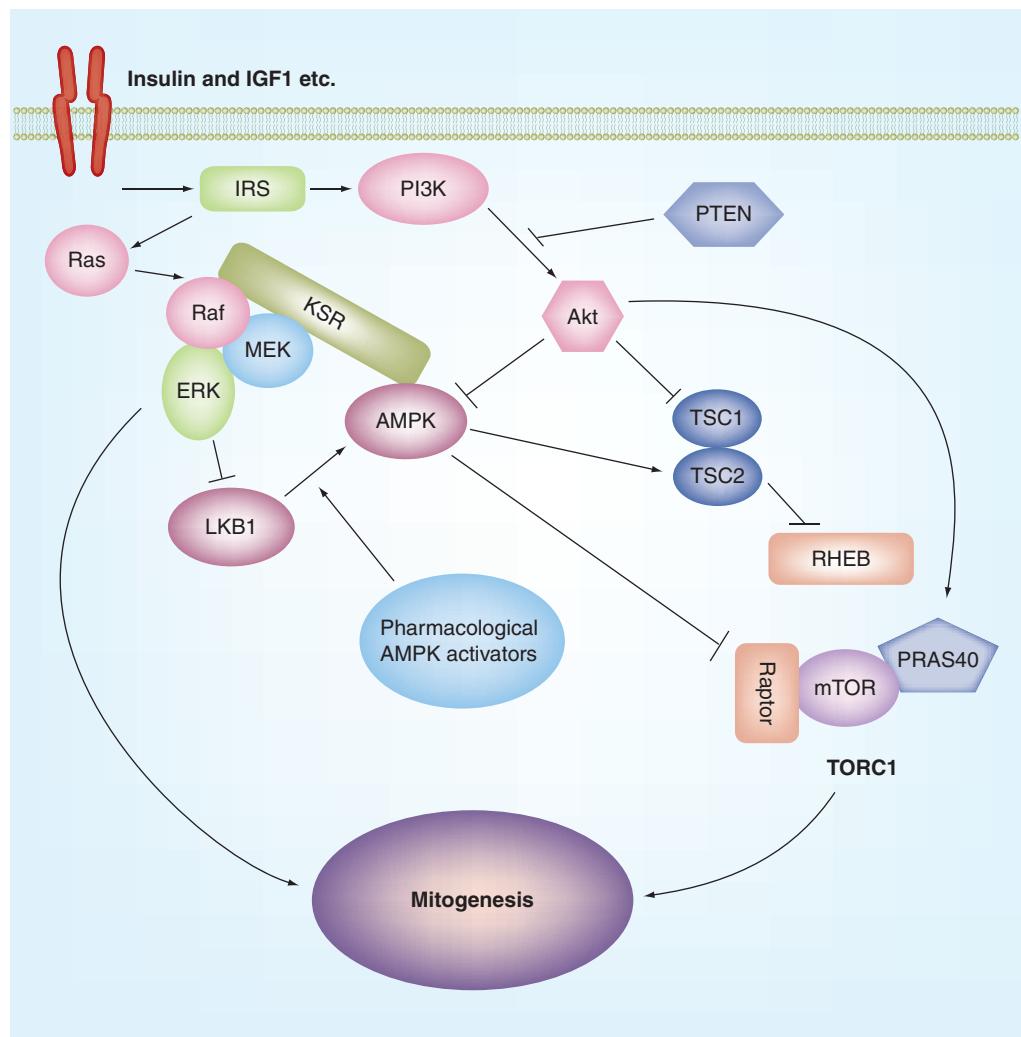


Figure 2. Interaction of AMPK with the ERK and PI3K pathways. Recent studies have demonstrated that the LKB1/AMPK axis functionally interacts with the mitogenic pathways: Akt could phosphorylate and inhibit AMPK; LKB1 is inhibited by oncogenic B-Raf in an ERK/RSK-dependent fashion; KSR associates with Raf/MEK, which is required for Raf/MEK activation. Recently, KSR2 has been shown to associate with AMPK and participate in its activation. It will be interesting to test if the KSR scaffolds, by tethering Raf/MEK and AMPK, play a role in determining cell growth or arrest; AMPK inhibits TORC1 by phosphorylating TSC2 and Raptor. This negative regulation is important, as TORC1 is a critical regulator for the growth of cancer cells bearing the loss-of-function mutations of PTEN or activating mutations of PI3K and Akt.

is the study showing that the $\beta 1$ -isoform of the regulatory subunit of AMPK is upregulated by p53 [51]. A second study has revealed that two downstream targets of p53, sestrin 1 and sestrin 2, are implicated in the activation of AMPK and concordant inhibition of mTOR [52]. These findings suggest that their mutual regulation enhances their tumor suppressive functions.

In addition, AMPK has been shown to phosphorylate and/or regulate several other molecules that regulate cell metabolism, growth, survival and autophagy, such as p300 histone acetyltransferase [53], FOXO3 [54] and the cell cycle inhibitor p27 [55,56]. It is not clear whether AMPK phosphorylation of p300

affects its histone acetyl transferase activity in addition to inhibition of p300 interaction with several nuclear receptors. AMPK phosphorylates FOXO3 and p27 at the same sites as Akt. However, phosphorylation by Akt elicits a negative effect on their function, in contrast to the positive net effect imposed by AMPK. Hence, it cannot be excluded that mechanisms other than direct phosphorylation account for AMPK's effects on these molecules.

Enzymes for fatty acid & cholesterol synthesis

AMP-activated protein kinase was first identified as a kinase that phosphorylates and inhibits

acetyl CoA carboxylase (ACC) and HMG-CoA reductase, rate-limiting enzymes for *de novo* synthesis of fatty acid and cholesterol, respectively. Both of these enzymes have important roles in tumorigenesis. In keeping with this, statins, HMG-CoA reductase inhibitors that lower cholesterol levels, have been suggested to prevent cancer in experimental models and reduce cancer risk in humans [57,58]. FASN, ACC and other enzymes required for *de novo* synthesis of free fatty acid and cholesterol are highly expressed in several types of cancers, including those arising from the breast, prostate, colon and ovary [59,60]. The increase of these enzymes is attributed to increased expression and maturation of SREBP-1 and SREBP-2, which are transcription factors. AMPK has been shown to inhibit SREBP1 [61]. However, the mechanism is not fully understood. One possible mechanism is through the inhibition of mTOR [45]. It has been reported that the inhibition of FASN activity by pharmacological agents or by siRNA attenuates proliferation of cancer cells and causes their apoptosis [62,63]. Thus, in light of these findings and the clinical observations that it is upregulated in cancer, *FASN* is regarded as a metabolic oncogene [60]. Interestingly, pharmacological inhibition of FASN in human ovarian cancer cells leads to a rapid activation of AMPK, followed by the induction of cytotoxicity [64]. The cytotoxic effect is suppressed by compound C, a chemical inhibitor of AMPK. Although further studies using molecular approaches are needed, these findings suggest that AMPK mediates the effect of pharmacological inhibition of FASN.

AMPK: journey from metabolic syndrome to cancer

Metabolic syndrome is a combination of metabolic disorders such as insulin resistance, hyperinsulinemia and proinflammatory and procoagulant changes that increase the risk of cardiovascular disease and diabetes. Studies from both human and rodents have shown that metabolic disorders, such as insulin resistance, obesity and Type 2 diabetes, are accompanied by decreases in AMPK activity, and that activation of AMPK by ACIAR, TZDs, metformin, polyphenols and exercise can correct or prevent these disorders [2,65–68]. Of note, AMPK activation stimulates fatty acid oxidation, which sounds paradoxical to its use in the treatment of diabetes and metabolic syndrome, as increased fatty acid oxidation may lead to ketosis. It should be pointed out that ketosis is

not as severe for Type 2 diabetes as for Type 1 diabetes. In Type 1 diabetes, peripheral tissues burn more fat as an alternative fuel source to compensate for the shortage of glucose in the absence of insulin, which results in intolerable increases in ketone bodies. By contrast, in Type 2 diabetes and metabolic syndrome, the major problems are insulin resistance in liver, muscle and adipose tissue (e.g., increased hepatic glucose production and decreased glucose uptake in skeletal muscle), and increased proinflammatory response in the cardiovascular system [2]. AMPK activation has been shown to mitigate all of these abnormalities. In fact, no severe ketosis has been found as a side effect of metformin and TZDs in the treatment of Type 2 diabetes.

In recent years, metabolic syndrome has been found to be associated with cancer, and new evidence suggests that AMPK could play a bridging role, as illustrated in FIGURE 3.

AMPK: a key regulator to link metabolic syndrome & cancer

A large body of epidemiological studies has indicated that metabolic syndrome is a risk factor for many types of cancer and is associated with increased mortality and poor prognosis [69–72]. Likewise, the association between metabolic syndrome and tumorigenesis has also been demonstrated by experimental studies [73–75]. Using a tumor xenograft mouse model in which obesity and insulin resistance is induced with a high-fat diet, Yakar *et al.* have shown that tumor growth is significantly increased in obese mice [73]. A second line of evidence came from the study with a fatless mouse model, where the expression of the transgene *A-ZIP/F-1*, encoding a dominant negative protein that prevents the DNA-binding of B-ZIP transcription factors of both C/EBP and Jun families, is under the control of the adipose-specific aP2 enhancer/promoter [74]. The transgenic mice without white fat but with increased ectopic adiposity developed typical symptoms of metabolic syndrome and diabetes. The mice are susceptible to skin carcinogenesis induced by 7,12 dimethylbenz(α) anthracene, and the development of breast cancer is accelerated when they are crossed with transgenic mice expressing the SV40 large tumor antigen transforming sequences in their mammary glands. The increased carcinogenesis and tumorigenesis is probably attributed to the metabolic disorders engendered by hyperinsulemia. This possibility is corroborated by a recent elegant study by Fierz *et al.* [75]. In this study, a dominant

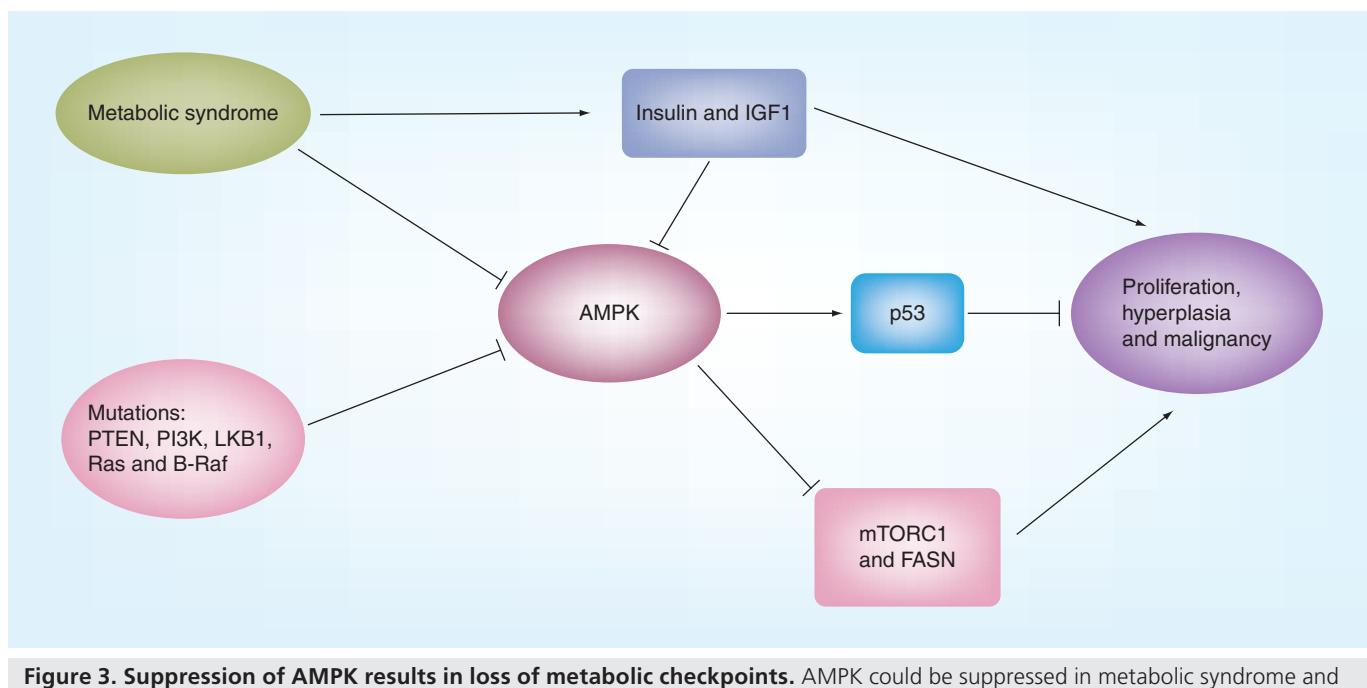


Figure 3. Suppression of AMPK results in loss of metabolic checkpoints. AMPK could be suppressed in metabolic syndrome and by activated Akt owing to mutation of PTEN or PI3K, or activated ERK/RSK due to Ras or B-Raf, leading to a loss of metabolic checkpoints. Likewise, increased insulin and IGF1 associated with metabolic syndrome can inhibit AMPK and stimulate cell proliferation, hyperplasia and malignant growth of cancer cells.

negative mutant of the IGF1 receptor is specifically expressed in skeletal muscle of transgenic mice ($\text{MKR}^{+/+}$), which results in insulin resistance and diabetes [76]. When the $\text{MKR}^{+/+}$ mice are crossed with mice expressing the Polyoma Virus middle T oncogene in the mammary gland or are orthotopically inoculated with mouse tumor cells, tumor growth is markedly enhanced. Intriguingly, treatment of animals with CL-316243, a potent insulin sensitizing drug that specifically activates the $\beta 3$ -adrenergic receptor, significantly reduced the elevated insulin levels in $\text{MKR}^{+/+}$ mice and concomitantly attenuated mammary tumor progression [75].

Thus far, both the animal and epidemiological studies support the contention that metabolic syndrome increases the risk of cancer and that correction of the metabolic disorders could be beneficial to cancer prevention and treatment. AMPK is a likely benevolent candidate to fulfill the latter mission. Indeed, this idea is supported by recent studies. First, retrospective investigations have reported that patients with Type 2 diabetes taking metformin display a reduced risk of cancer as compared with the normal population or with the patients who have never taken metformin [77,78], and a reduced trend of mortality compared with patients who take sulfonylureas or insulin [79]. In addition, reduced levels of adiponectin have been found in the plasma of patients with some cancers (e.g., breast and

prostate cancers), and treatment of cancer cells with adiponectin attenuated their growth, an event that is blocked by the dominant negative mutant of AMPK [80–83]. Finally, a large number of studies have shown that maneuvers that activate AMPK, such as treatment with pharmacological agents (e.g., AICAR, metformin and TZDs), exercise and dietary restriction, can attenuate cancer cell growth *in vitro* and inhibit tumor development *in vivo* [34,36,84–87].

Dysregulation of AMPK in cancer

In addition to loss-of-function mutations of LKB1 in Peutz–Jeghers syndrome and cancers, AMPK could be suppressed by oncogenic mutations (FIGURE 2). Previous studies have shown that AMPK is phosphorylated and inhibited by insulin-activated Akt [88]. However, this has not been reported in cancer cells containing constitutively activated Akt. Additionally, the mRNA levels of AMPK $\alpha 2$ inversely correlate with clinical prognosis in breast and ovarian tumors, and are diminished in cancer cells by activated PI3K pathways [89]. Since Akt is activated in many cancer cells where PTEN is inactivated or PI3K is constitutively activated, it will be interesting to expand the study by examining more human tumor specimens. In fact, recent investigations, despite their limited number, shed light on this direction [90,91]. One report showed that AMPK activation is reduced in lung cancer specimens

containing loss-of-function mutations of LKB1 [91]. A second study examined the activation status of AMPK in breast cancer specimens and showed that both phospho-AMPK and phospho-ACC signals were reduced, which inversely correlates with histological grade and axillary node metastasis [90]. It has not been investigated if the reduction of AMPK activity correlates with metabolic status of the breast cancer patients.

Recently, two independent studies have indicated that LKB1 is inhibited by an active mutant of B-Raf, an event mediated by ERK1/2 [92,93]. Intriguingly, opposite changes between phosphorylation of ERK and AMPK were observed in melanoma specimens [92]. This finding may have an important clinical implication, inasmuch as activating mutation of B-Raf accounts for approximately 6% of human cancer, with the highest incidence in malignant melanoma (50–70%) [94]. Furthermore, ERK1/2 is constitutively activated in cancer cells containing *Ras* mutations, which is found in 20–30% of human cancers [95].

A more complex inter-relationship between the mitogenic and metabolic pathways is highlighted by a most recent discovery of the interaction between kinase suppressor of ras 2 (KSR2) and AMPK [96]. KSR1 and KSR2 are best known for their scaffold function for the Raf/MEK/ERK signaling cascade, facilitating the activation of Raf and MEK [97,98]. Costanzo-Garvey *et al.* have found that AMPK associates with KSR1 and KSR2 with more preference toward the latter [96]. Knockout of the mouse *KSR2* gene causes obesity and insulin resistance. More interestingly, AMPK activation by AICAR is diminished in MEF cells isolated from the *KSR2* knockout mouse. KSR2 binds to AMPK and Raf/MEK through different domains: while AMPK binds to the domain near or superimposed with the membrane targeting domain, the binding sites for Raf and MEK are located to a more carboxy terminal region of KSR2 [96,99]. Hence, it is tempting to speculate that KSR2 functions as a switching point of cell fate for mitogenesis and metabolic checkpoint if all these proteins coexist in the same complex. In proliferating cells, KSR2 brings an active MEK/ERK complex to AMPK, preventing its activation by LKB1 (as illustrated in FIGURE 2). Conversely, under metabolic stress, binding of AMPK to KSR2 prevents Raf/MEK to be targeted to the plasma membrane for their activation. Alternatively, KSR2 could form independent complexes with AMPK and Raf/MEK.

AMPK & glucose metabolism in cancer cells

One of the prominent traits of cancer cells is aerobic glycolysis, which was first described by Otto Warburg in the 1920s [100]. Regardless of adequate oxygen supply, cancer cells rely on the glycolysis that takes place in the cytosol over oxidative phosphorylation in the mitochondria, although the former is much less efficient at generating ATP. It is still not clear whether the switching of ATP-producing systems is causal or consequent to malignancy. Nonetheless, increased glycolysis offers many growth advantages to cancer cells in addition to adaptation of hypoxia tumor microenvironment [101]. For example, the high rate of glycolysis is not just required for the production of ATP to compensate for impaired oxidative phosphorylation in mitochondria, but it is also important to provide intermediates for anabolic processes, including biosynthesis of glycogen, amino acids, nucleic acids, and lipids [101,102]. Second, increased glycolysis in tumor cells is accompanied by increased stability of the mitochondrial membrane. This is partly ascribed to mitochondria membrane associated-hexokinase (HK) I or II, which is upregulated in tumors. As a result, leakage of cytochrome C into the cytosol and subsequent activation of caspases are prevented. As such, cancer cells are resistant to apoptosis invoked by the intrinsic pathway. Third, increased secretion of lactic acid creates an extracellular acidic milieu, which is advantageous for cancer cell invasion but toxic to normal cells.

Complex mechanisms are involved in the adaptation of cancer cells to glycolysis. Two important modulators relevant to AMPK are HIF-1 and p53 (FIGURE 4). HIF-1 is a transcription factor that is activated by hypoxic, oncogenic and other stresses [103]. HIF-1 is a heterodimer consisting of two subunits, α and β . The α -subunits are degraded under normoxic conditions owing to the sequential action of oxygen-dependent prolyl tryosylases and the von Hippel-Lindau E3 ubiquitin ligase. Under hypoxic conditions, von Hippel-Lindau is inactivated, leading to stabilization of HIF-1 α , which stimulates expression of modulators required for glycolysis, including glucose transporter 1, HK1 and HK2, lactate dehydrogenase and pyruvate dehydrogenase kinase. The latter phosphorylates and inhibits pyruvate dehydrogenase, leading to a decrease in pyruvate entering mitochondria for oxidation. Therefore, the characteristic increases of glucose uptake and glycolysis in tumors distinguishes

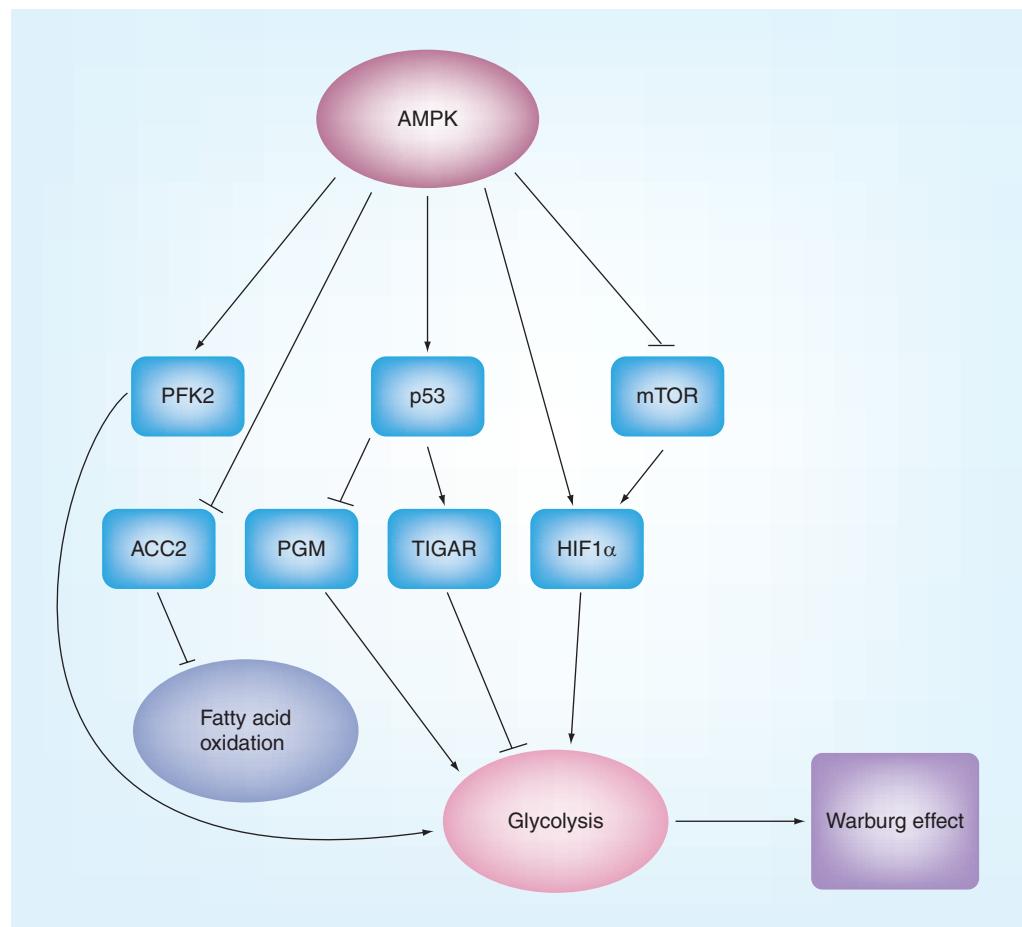


Figure 4. AMPK plays a role in reprogramming energy metabolism. AMPK activation could have two effects on energy metabolism: acute effect, AMPK that is activated under metabolic stress, such as hypoxia, ischemia and glucose deprivation, stimulates fatty acid oxidation and, in some circumstances, such as in the heart, enhances glycolysis to generate more ATP; chronic effect, AMPK may inhibit glycolysis via its action on mTOR and p53. Thus, suppression of AMPK in some cancer might result in increased glycolysis, which may contribute to the Warburg effect.

them from normal tissues, thereby offering a tracing advantage by ^{18}F 2-fluoro-2-deoxy-D-glucose PET scanning, and these characteristic increases are also targeting points for cancer therapy [104].

AMP-activated protein kinase has been implicated in the regulation of HIF1 α . This may be mediated by its action on mTORC1 [105]. Thus, in LKB1- or AMPK-deficient fibroblasts, the levels of HIF1 α and its downstream targets are elevated, which is diminished by rapamycin [46]. A similar increase of HIF1 α is also found in the epithelia of gastrointestinal hamartomas from *LKB1 $^{+/-}$* mice [46]. These studies suggest that AMPK suppresses glycolysis in tumor cells by inhibiting mTOR. However, AMPK has also been shown to stimulate glycolysis in ischemic hearts by activating a cardiac specific enzyme, phosphofructokinase 2 (PFK2) [106]. In addition, studies have asserted that AMPK activation upregulates HIF-1 α and VEGF expression

in hypoxia [107,108]. Thus, it needs to clarify whether these findings are reflective of acute stress response and examine whether the net effect of these opposite regulations by AMPK depends on cellular context and metabolic state of the cells.

Another paradigm of AMPK regulation of glycolysis is its possible action on p53. In addition to regulating cell growth and apoptosis, p53 has been recently implicated in the regulation of glycolysis and mitochondrial oxidative phosphorylation through at least three mechanisms [109]. First, p53 positively regulates the expression of the protein synthesis of cytochrome C oxidase 2 (SCO2), a key regulator of cytochrome C oxidase complex that is essential for mitochondrial respiration [110]. Second, p53 stimulates transcription of TP53-induced glycolysis and apoptosis regulator (TIGAR), a molecule that shares functional similarities with the bisphosphatase domain of the bifunctional

enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase [111]. Thus, the upregulation of TIGAR leads to a decrease in the levels of fructose-2,6-phosphates, thereby inhibiting glycolysis. Third, p53 downregulates the expression of phosphoglycerate mutase, another key enzyme in the glycolytic pathway [112]. Collectively, the net effect of p53 mutation is to enhance glycolysis. Therefore, AMPK, via regulating p53, could inhibit aerobic glycolysis and thus diminish the utilization of glycolytic intermediates for the biosynthesis of building blocks of cancer cells (e.g., protein, lipid and nucleic acids). However, data directly linking AMPK, p53 and glycolysis are currently still in scarcity.

Implication of AMPK in cancer prevention & treatment

Many studies have shown that exercise, AICAR and metformin, all of which cause AMPK activation, reduce insulin secretion and IGF1 production and alleviate hyperlipidemia and hyperglycemia [50,65,113–118]. We have provided direct evidence that AICAR suppresses the expression of IGF1 and its receptor in prostate cancer cells, while expression of the dominant negative mutant of AMPK causes their upregulation [50]. In addition, AMPK reduces reactive oxidation species, which can cause DNA damage and thus induce mutagenesis [3]. Hence, these studies suggest an important role of AMPK in tumor prevention. As noted earlier, this notion is supported by several recent epidemiological investigations in patients with Type 2 diabetes receiving metformin treatment and experimental studies using animal models.

AMP-activated protein kinase might also be a promising target for cancer therapy, as many recent studies have shown that pharmacological activators of AMPK, such as metformin, phenformin, AICAR and A769662, inhibit or delay the onset of tumors in animal models. Interestingly, one clinical study assessed the response of breast cancer patients with diabetes to nonadjuvant chemotherapy in combination with metformin or with nonmetformin antidiabetic drugs and demonstrated that the patients receiving metformin showed complete pathological responses [119]. This finding is intriguing and warrants further investigation. Indeed, an expanded use of metformin as an adjuvant in the treatment of breast cancer patients with or without diabetes is progressing at the Phase III clinical trial [120]. As an adjuvant of cancer therapy, several factors may

determine the efficacy of pharmacological activators of AMPK and response to the treatment. For example, the presence of LKB1 is critical for the response of cancer cells to AMPK-targeted treatment. AMPK is unable to be activated by many pharmacological agents in the absence of LKB1 unless they directly bind to and activate AMPK. In some cancer cells, failure of AMPK activation may be beneficial to therapy, as it sensitizes the cells to apoptosis induced by chemotherapeutic agents [121]. Second, the cells containing hyperactive downstream targets of AMPK might be especially sensitive to AMPK activators when LKB1 is present. These include cancer cells containing hyperactivated mTORC1 and upregulated FASN [34,36,64]. In addition, the status of p53 might also be a determining factor. In keeping with this, a recent study has shown that metformin induces apoptosis of p53-null HCT116 colon cancer cells and selectively suppresses the growth of xenografts derived from these cells [87]. By contrast, metformin or AICAR fails to inhibit the tumor growth of HCT116 cells containing wild-type p53 but, instead, elicits autophagy [87]. However, another study has reported that p53 plays a critical role in mediating AMPK-induced apoptosis of thymocytes in response to glucose deprivation [122]. These results may reflect differences in cell types, but points to different treatment regimens needed to be considered when the genetic context varies.

Conclusion

In summation, AMPK appears to serve as a metabolic tumor suppressor to keep cell metabolism and growth at appropriate levels. Thus, in response to energy stress in the microenvironment of tumors, AMPK activation may lead to the reprogramming of cellular metabolism and elicits a metabolic checkpoint on the cell cycle through its action on mTORC1, p53 and other modulators for cell growth and survival. The checkpoint presumably allows a cell to fix the problems. Thus, when it is lost, the cell will undergo two directions of fate: apoptosis or unrestrained growth. In this regard, it is of no doubt that AMPK is a promising target for cancer prevention. Moreover, many lines of evidence suggest that AMPK could also serve as a target for cancer therapy, which will require more support from both preclinical and clinical investigations. The clinical study is just at its beginning, and hopefully, more exciting outcomes are expected to come along in the near future.

Future perspective

A great deal of experimental studies support the notion that AMPK is an important mediator of LKB1 in inhibiting cancer cell growth and tumorigenesis. It is noteworthy, however, that unlike LKB1, AMPK may not have emerged as a tumor suppressor by genetic studies, which is probably attributed to redundancy between the multiple isoforms [123,124]. Thus, to further delineate the tumor suppressive function of AMPK, it will be interesting to cross the AMPK $\alpha 1$ or $\alpha 2$ null mice or transgenic mice expressing the dominant negative mutant of AMPK α -subunit with p53 or PTEN-null mice and test whether ablation of AMPK accelerates tumor development or whether deletion of the *AMPK* α -genes increases the susceptibility to carcinogenesis. Another important direction will be to gain more evidence on AMPK dysregulation in tumor microenvironment caused by metabolic or genetic changes. Inspired by the epidemiological studies showing the reduced risk of cancer and improved prognosis in breast cancer patients with diabetes who have taken metformin and *in vitro* studies using animal models with AMPK activators, extended studies of metformin use as an adjuvant to cancer therapy will hopefully be launched soon. An attractive reason to consider AMPK as a therapeutic target is the fact that two pharmacological activators, metformin and TZDs, have already been used in the clinical treatment of Type 2 diabetes, and tolerance of their side effects has

been documented. Since metformin inhibits Complex I of the respiratory chain, one of its side effects is the production of lactic acidosis. Owing to the fatal acidosis side effect, phenformin, a potent analog of metformin, has been removed from the market in the USA. Although metformin also produces this side effect, it is to a much lower degree that is within the tolerable range. Furthermore, it is reasonable to expect more AMPK activators with higher specificity and potency to appear in the market in the near future. We should be aware that, as tumors have high degrees of heterogeneity, different genetic backgrounds may determine the sensitivity and efficacy, and the combination of AMPK activators with other treatment regimens will always be a better choice. Lastly and not surprisingly, as tremendous interest is invested, more AMPK targets and new functions will be identified.

Acknowledgements

The authors thank Ms Rong Tao and Miss Laura Stevens for checking typographical and grammatical errors.

Financial & competing interests disclosure

This work is supported by an NIH grant (R01CA118918 to Zhijun Luo). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

- AMPK serves as a critical downstream substrate of LKB1, a tumor suppressor whose mutations are found in Peutz–Jeghers syndrome and cancer.
- AMPK has emerged as a therapeutic target for metabolic syndrome and Type 2 diabetes.
- AMPK can be suppressed in metabolic syndrome, which is a risk factor for cancer, and by oncogenic activation of the Raf/ERK/RSK pathway.
- Activation of AMPK by energy shortage (e.g., hypoxia and low levels of nutrients) reprograms cellular metabolism and enforces a metabolic checkpoint on the cell cycle. Loss of such a checkpoint could lead to unrestrained cell growth.
- AMPK may function as a metabolic tumor suppressor regulating glucose, lipid and protein metabolism. It does so by acting on mTORC1, p53 and FASN.
- AMPK is a promising target for cancer prevention and therapy.

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Polymorphisms in the Reduced Folate Carrier, Thymidylate Synthase, or Methionine Synthase and Risk of Colon Cancer

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Abstract

Folate metabolism supports the synthesis of nucleotides as well as the transfer of methyl groups. Polymorphisms in folate-metabolizing enzymes have been shown to affect risk of colorectal neoplasia and other malignancies. Using data from a population-based incident case-control study (1,600 cases and 1,962 controls), we investigated associations between genetic variants in the reduced folate carrier (*RFC*), thymidylate synthase (*TS*), methionine synthase (*MTR*), and 5,10-methylenetetrahydrofolate reductase (*MTHFR*) and colon cancer risk. The *TS* enhancer region (*TSER*) variant was associated with a reduced risk among men [2rpt/2rpt versus 3rpt/3rpt wild-type; odds ratio (OR), 0.7; 95% confidence interval, 0.6-0.98] but not women. When combined genotypes for both *TS* polymorphisms (*TSER* and 3'-untranslated region 1494delTTAAAG) were evaluated, ORs for variant genotypes were generally below 1.0, with statistically significantly reduced risks among women.

Neither *MTR* D919G nor *RFC* 80G>A polymorphisms were associated with altered colon cancer risk. Because folate metabolism is characterized by interrelated reactions, we evaluated gene-gene interactions. Genotypes resulting in reduced *MTHFR* activity in conjunction with low *TS* expression were associated with a reduced risk of colon cancer. When dietary intakes were taken into account, individuals with at least one variant *TSER* allele (3rpt/2rpt or 2rpt/2rpt) were at reduced risk in the presence of a low folate intake. This study supports findings from adenoma studies indicating that purine synthesis may be a relevant biological mechanism linking folate metabolism to colon cancer risk. A pathway-based approach to data analysis is needed to help discern the independent and combined effects of dietary intakes and genetic variability in folate metabolism. (Cancer Epidemiol Biomarkers Prev 2005; 14(11):2509-16)

Introduction

Folate is an essential micronutrient in humans, the primary function of which is as a carrier of single-carbon units. Folate-dependent reactions include the biosynthesis of thymidylate, purines, methionine, and glycine thus linking it to nucleotide synthesis as well as the provision of methyl groups (1). High dietary folate intakes, or biomarkers thereof, have been associated with a reduced risk of colon cancer or its precursors in most, although not all, studies (2-5). Several studies showing associations with genetic polymorphisms in folate-metabolizing enzymes lend support to a causal relationship between folate and colorectal carcinogenesis (6-10). Biological mechanisms linking folate to colorectal carcinogenesis include an altered provision of S-adenosylmethionine for methylation reactions, including DNA methylation, and changes in the availability of nucleotides, such as thymidylate, for DNA synthesis and repair (11, 12).

We have previously reported on associations between polymorphisms in 5,10-methylenetetrahydrofolate reductase (*MTHFR*) and risk of colon cancer (13). Here, we extend this work to common genetic variants in thymidylate synthase (*TS*), the reduced folate carrier (*RFC*), and methionine synthase (*MTR*) in relation to colon cancer risk. *TS* is a key enzyme in folate metabolism that catalyzes the conversion of dUMP to dTMP for the provision of thymidine, a rate-limiting nucleo-

tide essential for DNA synthesis and repair (see Fig. 1). *TS* is also a primary target for major chemotherapeutic agents, including 5-fluorouracil. We investigated the role of a polymorphism in the 5'-untranslated region (5'-UTR) enhancer region (three or two repeats of a 28-bp sequence), resulting in reduced *TS* expression among those with fewer repeats (14) and a 6-bp insertion or deletion (1,494 bp in the 3'-UTR) that affects mRNA stability (15, 16).

RFC is responsible for the active transport of 5-methyltetrahydrofolate from plasma to cytosol. A polymorphism in the *RFC* gene (80G>A, Arg²⁷His) seems associated with a higher affinity for folate (17). Among 169 healthy individuals who were stratified by *MTHFR* 677C>T genotype, the variant A allele was consistently and linearly associated with higher plasma folate concentrations (17). Furthermore, concentrations of methotrexate 24 to 48 hours after administration were higher among children with acute leukemia homozygous for the variant A allele, providing additional support for differential carrier activity among those with variant genotypes (18).

MTR catalyzes the methylation of homocysteine to methionine with simultaneous conversion of 5-methyl-tetrahydrofolate to tetrahydrofolate (Fig. 1). A variant in the *MTR* gene (2756A>G, Asp⁹¹⁹Gly; ref. 19) may affect plasma homocysteine concentrations. Some studies (20, 21) but not others (22-24) have found that homocysteine concentrations tend to decrease linearly across genotypes, with the AA genotype associated with the highest homocysteine concentrations. Studies on colorectal neoplasia have been inconsistent: the GG genotype has been associated with a somewhat reduced risk of colorectal cancer (22, 25) yet a possible increased risk of colorectal adenoma (26).

In this large population-based case-control study of colon cancer, we sought to evaluate the role of these polymorphisms in defining colon cancer risk, either alone or in interaction with specific nutrient intakes and other genotypes. Furthermore, as

Received 4/14/05; revised 8/10/05; accepted 9/8/05.

Grant support: NIH grants R01 CA48998 and R01 CA59045.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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doi:10.1158/1055-9965.EPI-05-0261

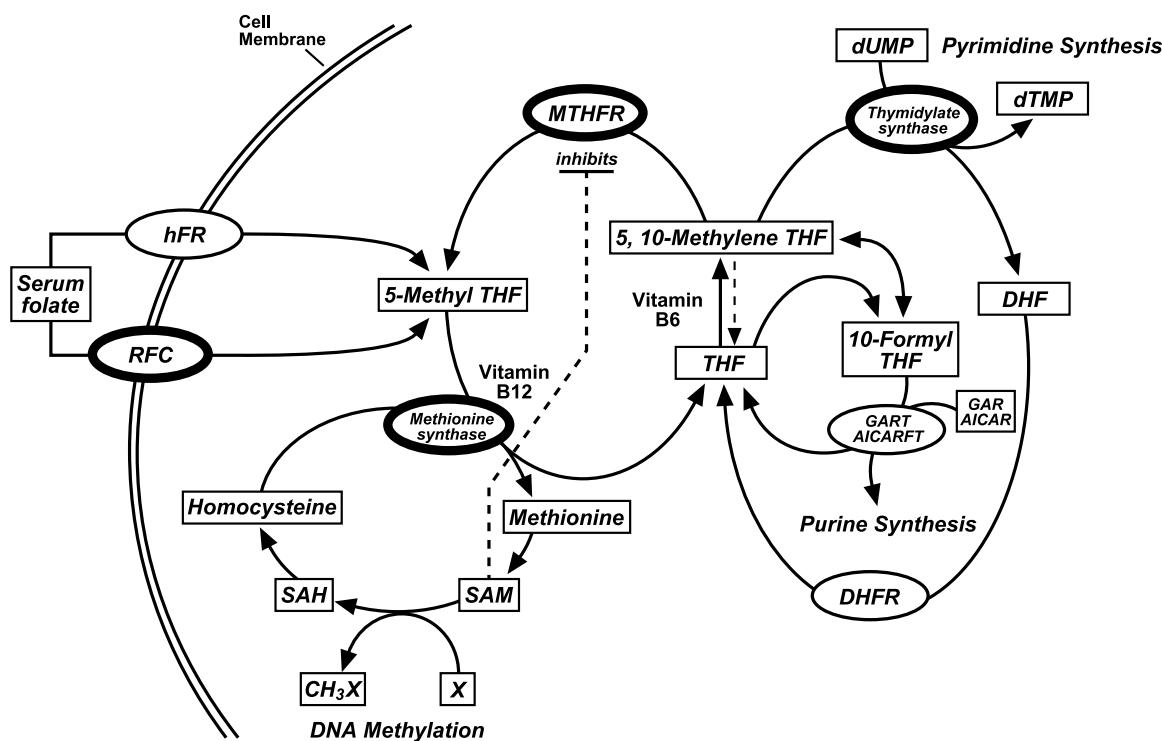


Figure 1. Simplified version of folate-mediated one-carbon metabolism, highlighting proteins with polymorphisms investigated in this study (figure modified from ref. 36). Key enzymes are denoted as ovals, substrates as rectangles. THF, tetrahydrofolate; DHF, dihydrofolate; DHFR, dihydrofolate reductase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; X, a variety of substrates for methylation; RFC, reduced folate carrier; hFR, human folate receptor; MTHFR, 5,10-methylenetetrahydrofolate reductase; GART, phosphoribosylglycinamide formyltransferase; AICARFT, 5-aminomidazole-4-carboxamide ribonucleotide; 5-FU, 5-fluorouracil.

some of the associations of folate metabolism may differ by estrogen exposure (13), possibly because of mechanisms attributable to hypermethylation of the estrogen receptor (27), we evaluated interactions with postmenopausal hormone (PMH) use.

Materials and Methods

Participants were African American, Caucasian, or Hispanic subjects from the Kaiser Permanente Medical Care Program of Northern California, an eight-county area in Utah, and the metropolitan Twin Cities area of Minnesota. Eligibility criteria for cases included diagnosis with first-primary incident colon cancer (*International Classification of Diseases for Oncology*, 2nd edition codes 18.0, 18.2-18.9) between October 1, 1991 and September 30, 1994; between 30 and 79 years of age at time of diagnosis; and mentally competent to complete the interview. Proximal tumors were defined as cecum through transverse colon; tumors in the splenic flexure and descending and sigmoid colon were categorized as distal. Cases with adenocarcinoma or carcinoma of the rectosigmoid junction or rectum (defined as the first 15 cm from the anal opening) or with known familial adenomatous polyposis, ulcerative colitis, or Crohn's disease were not eligible. Of all cases identified, 65% of those contacted consented to participate in the study. Controls who had never had a previous colorectal tumor were randomly selected in proportion to the cases within the geographically defined areas from Kaiser Permanente Medical Care Program membership lists in California; driver's license lists, random digit dialing, or Centers for Medicare and Medicaid Services lists, formerly known as the Health Care Finance Administration, for Utah; and driver's

license or state identification lists in Minnesota. Controls were frequency matched to cases by sex and 5-year age group. These methods have been described in detail (28). Of all controls selected, 64% participated.

Data Collection. Trained interviewers collected diet and lifestyle data in person using laptop computers. Study quality control methods have been described (29, 30). The reference period for the study was the calendar year ~2 years before date of diagnosis (cases) or date of selection (controls). Dietary intake data were ascertained using an adaptation of the validated Coronary Artery Risk Development in Young Adults diet history questionnaire (31). Participants were asked to determine which foods were eaten and the frequency with which foods were eaten. Nutrients were calculated using the Minnesota Nutrition Coordinating Center's nutrient database, version 19.

TS, MTR, and RFC genotyping. Of 4,403 cases and controls with valid study data, 3,680 (84%) had blood collected primarily during the in-person interview, or during a clinical visit (83% of cases and 85% of controls). Genomic DNA was extracted using methods described in (refs. 14, 32). All samples were genotyped for two polymorphisms in the *TS* gene (*TSER*, 3'-UTR 1494delTTAAAG), *MTR* D919G, and *RFC* 80G>A. A total of 3,562 (97% of cases and 97% of controls with blood collected) had genotype information for both *TSER* and 3'-UTR 1494delTTAAAG. 5'-Nuclease assays that had been previously used to genotype other polymorphisms in the folate pathway (*MTHFR* 677C>T, *MTHFR* 1298A>C, and *MTR* D919G) have been described (13, 26).

Both *TS* polymorphisms were analyzed using fluorescent size discrimination. For the analysis of the *TSER* 28-bp repeat polymorphism, a fragment containing the repeats was

amplified using the following primers: forward primer, 5'-6FAM-GTGGCTCCTGCGTTCCCCC-3'; reverse primer, 5'-GGCTCCGAGCCGGCACAGGCATGGCGCGG-3'(14). The PCR reactions contained 1× GeneAmp buffer (Applied Biosystems, Foster City, CA), 1.5 mmol/L MgCl₂, 200 μmol/L deoxyribonucleotide triphosphates, 100 nmol/L each primer, 10% DMSO, 1 unit AmpliTaq DNA polymerase (Applied Biosystems), and 100 ng of genomic DNA. Cycling conditions were one cycle of 94°C for 2 minutes; 35 cycles of 94°C for 30 seconds, 63°C for 30 seconds, and 72°C for 30 seconds; and a final extension at 72°C for 5 minutes. The amplified fragments were analyzed on an ABI 3100 genetic analyzer. A fragment containing the 3'-UTR deletion was amplified using the following primers: forward primer, 5'-6FAM-CAAATCTGAGGGAGCTGAGT-3'; reverse primer, 5'-CAGATAAGTGGCACTACAGA-3'. The PCR reactions contained 1× GeneAmp buffer, 2 mmol/L MgCl₂, 150 μmol/L deoxyribonucleotide triphosphates, 300 nmol/L each primer, 1 unit AmpliTaq DNA polymerase, and 50 ng genomic DNA. Cycling conditions were one cycle of 94°C for 5 minutes; 30 cycles of 94°C for 30 seconds, 60°C for 45 seconds, and 72°C for 60 seconds; and a final extension at 72°C for 10 minutes. The amplified fragments were analyzed on an ABI 3100 genetic analyzer. For both *TS* polymorphisms, the correlation between fragment size and repeat number was confirmed by sequencing.

The 80G>A polymorphism in *RFC* was detected by allelic discrimination using the 5' nuclease assay on a 7900HT sequence detection system (ABI). The 5'-nuclease genotyping assay was validated by genotyping 100 individuals by both 5'-nuclease assay and RFLP. There were no discrepancies between the two assays. Genotyping of the 80G>A polymorphism was done in 20-μL reactions containing 1× Taqman PCR core reagents (ABI), 3 mmol/L MgCl₂, 200 nmol/L each PCR primer (forward primer, 5'-AGCCCAGCGGTGGA-GAAG-3' and reverse primer, 5'-AGCCGTAGAACAAAGG-TAGCA-3'), 150 nmol/L MGB probe 5'-VIC-TCTTGGC-GGCC-3' (Applied Biosystems; G allele), 100 nmol/L MGB probe 5'-6-FAM-TGGCGGCACCTCG-3' (A allele), 0.5 unit AmpliTaq Gold, 0.2 unit AmpErase UNG, and 5 ng genomic DNA. The amplification cycles were 50°C for 5 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Positive controls for all the genotypes as well as four negative controls were included in each plate. For quality control of all the polymorphisms, genotyping for 94 randomly selected samples was repeated. There were no discrepancies.

Statistical Methods. Logistic regression models were used to estimate associations in various ways. We stratified the data by sex and estimated the risk of colon cancer given a certain *TS*, *MTR*, or *RFC* genotype and examined risk estimates further stratified by other population characteristics (e.g., tumor site and age). The combined effects of *TSER* and 3'-UTR 1494delTTAAAG were calculated using individuals who were homozygous for the common allele at both loci as the reference group. We assessed the joint interaction between genotype and level of nutrient intake by using those with low nutrient intake and homozygous for the most common (wild type) allele for *TSER*, 3'-UTR, *MTR*, or *RFC* as a common reference point. We also assessed gene-gene interactions in the folate pathway using the homozygous genotype for the most common allele as the reference. Similarly, the interaction between genotype and recent estrogen status in postmenopausal women was assessed using as the reference group no PMH use and wild-type *TS*, *MTR*, or *RFC* genotype.

Maximum likelihood estimates of population *TS* haplotype frequencies from unphased genotype data were obtained from an expectation maximization algorithm, assuming Hardy-Weinberg equilibrium, according to Excoffier and Slatkin (33) using SAS/Genetics software, 2002 (SAS/Genetics, Cary, NC).

Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated from unconditional logistic regression models. In these models, age at diagnosis or selection, body mass index reported for the reference period (kg/m²), long-term vigorous leisure time physical activity, total energy intake, dietary fiber, dietary calcium, and number of cigarettes smoked per day on a regular basis were included as covariates to adjust for potential confounding. Haplotype-specific relative risks were assessed according to methods described in Stram et al. (34) using logistic regression software (SAS, release 8.2).

Separate analyses were done for men and women to determine whether differences existed by sex, as most of the literature has focused on either men or women. Assessment of interactions among genotypes, diet, and the risk of colon cancer were based on departure from additive risks using the relative risk due to interaction formulation of Hosmer and Lemeshow extended to more than two allelic combinations and/or environmental exposures (35). Interaction using a multiplicative scale was also examined. Interactions between

Table 1. Characteristics of the study population (*n* = 3,562)

	Cases (<i>n</i> = 1,600)	Controls (<i>n</i> = 1,962)	P*
Tumor site, <i>n</i> (%)			
Proximal	791 (49)		
Distal	771 (48)		
Unknown	38 (3)		
Age at diagnosis or selection (range, 30-79), y [†]	64.9 ± 9.8	65.0 ± 10.2	0.86
Sex, <i>n</i> (%)			
Men	897 (56)	1,036 (53)	
Women	703 (44)	926 (47)	0.05
Race/ethnicity, <i>n</i> (%)			
Non-Hispanic White	1,462 (91)	1,825 (93)	
Other	138 (9)	137 (7)	0.05
Recent PMH use, postmenopausal women [‡] , <i>n</i> (%)			
No	467 (77)	549 (69)	
Yes	143 (23)	242 (31)	<0.01
Kilocalories [†]			
Men	2,773 ± 1,217	2,638 ± 1,162	0.01
Women	2,046 ± 874	1,974 ± 832	0.09
<i>TSER</i> genotype, <i>n</i> (%)			
3/3 repeats	488 (30)	542 (28)	
3/2 repeats	764 (48)	983 (50)	
2/2 repeats	348 (22)	437 (22)	0.16
Frequency, 2 repeat allele [§]	0.46	0.48	0.15
<i>TS</i> 3'-UTR 1494delTTAAAG genotype, <i>n</i> (%)			
ins/ins	720 (45)	881 (45)	
ins/del	690 (43)	866 (44)	
del/del	190 (12)	215 (11)	0.65
Frequency, del allele [§]	0.32	0.32	0.77
<i>MTR</i> D919G genotype, <i>n</i> (%)			
DD	1,015 (63)	1,264 (64)	
DG	529 (33)	608 (31)	
GG	56 (4)	90 (5)	0.15
Frequency, G allele [§]	0.20	0.20	0.97
<i>RFC</i> 80G>A genotype, <i>n</i> (%)			
GG	513 (32)	585 (30)	
GA	788 (49)	976 (50)	
AA	299 (19)	401 (20)	0.24
Frequency, A allele	0.43	0.45	0.10
<i>TS</i> haplotype, estimated (expected) ^{,¶}			
3 repeat/ins	0.30 (0.36)	0.28 (0.35)	
3 repeat/del	0.24 (0.17)	0.24 (0.17)	
2 repeat/ins	0.38 (0.31)	0.40 (0.33)	
2 repeat/del	0.08 (0.15)	0.08 (0.15)	0.56

*Based on χ² or *t* test.

†Mean ± SD.

‡Postmenopausal hormone use within 2 years of diagnosis/selection.

§Allele frequencies are reported for non-Hispanic Whites.

||Maximum likelihood estimate (expected assuming linkage equilibrium).

¶*P* < 0.01 (χ² test of linkage disequilibrium for cases or controls, separately).

genotypes and PMH use in postmenopausal women were assessed using a Wald χ^2 test of the difference between slopes from the (assumed linear) change in ORs, keeping the wild-type *TS* genotype constant across the varying genotypes for the respective other *TS* polymorphism.

Results

Selected characteristics of the study population and *MTHFR* genotype frequencies by case or control status are presented in Table 1. The study participants were predominantly self-identified as non-Hispanic Caucasian (92%), with the remainder Hispanic (4%) and African American (4%). All genotypes were in Hardy-Weinberg equilibrium (assessed separately for cases and controls), and allele frequencies were consistent with previous reports (36). The *TS* polymorphisms were in linkage disequilibrium ($D' = 0.46$ among non-Hispanic Caucasians).

Table 2 describes the main associations seen with the polymorphisms, stratified by gender. Among men, *TSER* variant genotypes were associated with a significantly decreased risk (3rpt/2rpt: OR, 0.8; 95% CI, 0.6-0.98; 2rpt/2rpt: OR, 0.7; 95% CI, 0.6-0.98). No such risk reduction was observed among women. When combined genotypes (or diplotypes) for both *TS* polymorphisms were considered, almost all of the ORs for variant genotypes among both men and women were below 1.0, with statistically significantly reduced risks for women. Risk estimates for *TS* haplotypes, including variant alleles, compared with wild-type haplotype were not significantly different from 1.0 (data not shown).

Neither the *MTR* D919G nor *RFC* 80G>A polymorphism was associated with altered colon cancer risk among men or women (Table 2).

Folate metabolism involves circulation of folate metabolites through multiple cycles, as well as feedback mechanisms between these cycles (Fig. 1). Therefore, we evaluated gene-gene interactions between the polymorphisms investigated here, as well as those we have reported on previously (8, 13). ORs different from 1.0 were seen largely for stratifications of *TS*, *RFC*, or *MTR* by *MTHFR* 677C>T or 1298A>C genotypes,

and these are presented in Table 3. Among men, reduced risks associated with variant *TS* genotypes (e.g., the presence of *TSER* 2rpt/2rpt or *TS* 3'-UTR deletion) were most pronounced for those with *MTHFR* TT genotypes. The *MTHFR* 1298CC genotype was associated with a decreased risk among women; however, this risk reduction seemed independent of *TS* genotypes. There was no evidence for interactions between *MTR* D919G or *RFC* 80G>A and *MTHFR* genotypes.

We also investigated whether risk estimates associated with polymorphisms in these folate-metabolizing enzymes differed by dietary intakes of folate, methionine, alcohol, or vitamins B6, B2, or B12. Consistent with our previous report on colorectal adenoma (37) among men, the *TSER* variant conferred a reduced risk in the presence of low folate intake (lowest tertile < 318 µg/d; *TS* 2rpt/2rpt or 2rpt/3rpt: OR, 0.7; 95% CI, 0.5-0.9 compared with wild-type 3rpt/3rpt). A similar risk reduction with the *TSER* variant genotypes was observed among men with low methionine intakes (<2.0 g/d): *TS* 2rpt/2rpt or 2rpt/3rpt (OR, 0.6; 95% CI, 0.4-0.9) compared with wild-type 3rpt/3rpt. However, none of these patterns was observed among women for the *TS* genotypes nor for *TS* haplotypes in either sex. There were no clear patterns or associations following stratification by vitamin B6 or B12 intake. No meaningful differences in risk were observed for *MTR* genotypes when stratified by nutrient intakes.

Among women in the lowest tertile of folate intake (≤ 273 µg/d), the *RFC* variant genotypes were associated with a decreased risk (wild-type GG: OR, 1.0; GA or AA: OR, 0.7; 95% CI, 0.5-1.0). Among women, we observed a significant gene-nutrient interaction in that only those with the GG genotype benefited from a diet higher in folate, whereas no difference in risk with variable folate intake was seen among those with the combined GA or AA genotypes ($P_{\text{interaction}} = 0.04$, multiplicative scale; $P = 0.01$, additive scale). This pattern was not seen among men.

Because of the observed differences in risk patterns among men and women and our past findings regarding an interaction between postmenopausal hormone use (PMH use) and

Table 2. Association between *TS*, *MTR*, and *RFC* genotypes and colon cancer

Genotype	Men			Women		
	Cases (n)	Controls (n)	OR (95% CI)	Cases (n)	Controls (n)	OR (95%CI)
<i>TSER</i>						
3rpt/3rpt	295	283	1.0 (reference)	190	254	1.0 (reference)
3rpt/2rpt	421	519	0.8 (0.6 to <1.0)	337	462	0.9 (0.7-1.2)
2rpt/2rpt	173	231	0.7 (0.6 to <1.0)	171	205	1.1 (0.8-1.5)
<i>TS</i> 3'-UTR 1494delTTAAAG						
ins/ins	387	468	1.0 (reference)	328	409	1.0 (reference)
ins/del	392	465	1.0 (0.8-1.2)	293	398	0.9 (0.7-1.1)
del/del	110	100	1.2 (0.9-1.7)	77	114	0.8 (0.6-1.2)
Combined <i>TSER</i> and <i>TS</i> 3'-UTR						
3rpt/3rpt	77	82	1.0 (reference)	71	61	1.0 (reference)
ins/del or del/del	218	201	1.2 (0.8-1.7)	119	193	0.5 (0.3-0.8)
3rpt/2rpt or 2rpt/2rpt						
ins/ins	310	386	0.9 (0.6-1.2)	257	348	0.6 (0.4-0.9)
ins/del or del/del	284	364	0.8 (0.6-1.2)	251	319	0.7 (0.5 to <1.0)
<i>MTR</i> D919G						
DD	555	668	1.0 (reference)	458	600	1.0 (reference)
DG	308	319	1.1 (0.9-1.4)	220	288	1.0 (0.8-1.3)
GG	30	54	0.7 (0.4-1.1)	25	37	0.9 (0.5-1.5)
<i>RFC</i> 80G>A*						
GG	301	317	1.0 (reference)	210	267	1.0 (reference)
GA	425	511	0.8 (0.7-1.0)	361	468	1.0 (0.8-1.2)
AA	167	211	0.8 (0.6-1.1)	132	190	0.9 (0.6-1.1)

NOTE: Adjusted for age, body mass index, lifetime vigorous leisure activity, energy intake, dietary fiber, dietary calcium, usual number of cigarettes smoked, and other *TS* polymorphism where appropriate (*TSER*, *TS* 3'-UTR).

*Men and women combined: GG, OR = 1.0 (reference); GA, OR = 0.9 (95% CI, 0.8-1.0); AA, OR = 0.8 (95% CI, 0.7-1.0).

Table 3. Association between polymorphisms in the folate pathway and colon cancer

Genotype		Men			Women		
		Cases (n)	Controls (n)	OR (95% CI)	Cases (n)	Controls (n)	OR (95% CI)
<i>TSER</i>	<i>MTHFR</i> 677C>T						
3 rpt/3 rpt	CC or CT	266	242	1.0 (reference)	172	226	1.0 (reference)
	TT	28	41	0.6 (0.4-1.0)	18	28	0.9 (0.5-1.7)
3/2 rpt or 2/2 rpt	CC or CT	544	668	0.7 (0.6-0.9)	456	592	1.0 (0.8-1.3)
	TT	50	81	0.6 (0.4-0.9)	52	75	0.9 (0.6-1.4)
<i>TS</i> 3'-UTR	<i>MTHFR</i> 1298A>C						
3 rpt/3 rpt	AA or AC	261	254	1.0 (reference)	175	219	1.0 (reference)
	CC	34	29	1.2 (0.7-2.0)	15	35	0.5 (0.3 to <1.0)
3/2 or 2/2 rpt	AA or AC	535	676	0.8 (0.6 to <1.0)	463	589	1.0 (0.8-1.2)
	CC	59	74	0.8 (0.5-1.1)	45	78	0.7 (0.5-1.1)
<i>TS</i> 3'-UTR	<i>MTHFR</i> 677C>T						
ins/ins	CC or CT	347	418	1.0 (reference)	297	359	1.0 (reference)
	TT	40	49	1.0 (0.6-1.6)	31	50	0.8 (0.5-1.2)
Any deletion	CC or CT	463	492	1.1 (0.9-1.3)	331	459	0.9 (0.7-1.1)
	TT	38	73	0.6 (0.4-0.9)	39	53	0.9 (0.6-1.4)
<i>TS</i> 3'-UTR	<i>MTHFR</i> 1298A>C						
ins/ins	AA or AC	346	420	1.0 (reference)	298	354	1.0 (reference)
	CC	41	48	1.0 (0.6-1.5)	30	55	0.6 (0.4-1.0)
Any deletion	AA or AC	450	510	1.0 (0.8-1.2)	340	454	0.9 (0.7-1.1)
	CC	52	55	1.1 (0.7-1.7)	30	58	0.6 (0.4 to <1.0)
<i>MTR</i> D919G	<i>MTHFR</i> 677C>T						
DD	CC or CT	507	583	1.0 (reference)	402	533	1.0 (reference)
	TT	47	83	0.7 (0.5 to <1.0)	56	67	1.1 (0.8-1.6)
DG or GG	CC or CT	307	333	1.0 (0.9-1.3)	230	289	1.1 (0.9-1.3)
	TT	31	40	0.9 (0.6-1.5)	15	36	0.6 (0.3-1.1)
<i>TS</i>	<i>MTHFR</i> 1298A>C						
DD	AA or AC	495	597	1.0 (reference)	417	523	1.0 (reference)
	CC	60	70	1.0 (0.7-1.5)	41	77	0.7 (0.4 to <1.0)
DG or GG	AA or AC	304	340	1.1 (0.9-1.3)	226	289	1.0 (0.8-1.2)
	CC	34	33	1.2 (0.7-2.0)	19	36	0.7 (0.4-1.2)
<i>RFC</i> 80G>A	<i>MTHFR</i> 677C>T						
GG	CC or CT	274	279	1.0 (reference)	194	236	1.0 (reference)
	TT	27	38	0.7 (0.4-1.1)	16	31	0.7 (0.3-1.2)
GA or AA	CC or CT	540	636	0.8 (0.7-1.0)	438	586	0.9 (0.7-1.1)
	TT	51	85	0.6 (0.4-0.9)	55	72	0.9 (0.6-1.4)
<i>TS</i>	<i>MTHFR</i> 1298A>C						
GG	AA or AC	267	283	1.0 (reference)	194	236	1.0 (reference)
	CC	34	34	1.1 (0.7-1.9)	16	31	0.6 (0.3-1.1)
GA or AA	AA or AC	532	653	0.8 (0.7 to >1.0)	449	576	0.9 (0.7-1.2)
	CC	60	69	0.9 (0.6-1.3)	44	82	0.6 (0.4 to <1.0)

NOTE: Adjusted for age, BMI, lifetime vigorous leisure activity, energy intake, dietary fiber, dietary calcium, usual number of cigarettes smoked, and other *TS* polymorphisms where appropriate (*TSER*, *TS* 3'-UTR). *P*_{interaction} values of gene-gene associations were not statistically significant on an additive or multiplicative scale (data not shown).

MTHFR genotype, we investigated whether risk estimates of *TS*, *MTR*, or *RFC* genotypes differed by PMH use. Among PMH users, the variant *TS* genotypes were associated with substantially reduced risk of colon cancer, whereas much weaker associations were observed among non-PMH users (Table 4). No such interactions were observed for *MTR* or *RFC* (data not shown).

Discussion

Within this large population-based study of colon cancer, we investigated polymorphisms in three folate-metabolizing enzymes (*TS*, *MTR*, and *MTHFR*), as well as the relevant carrier protein (*RFC*), thus addressing genetic variability in multiple key proteins in this biological pathway. There is strong evidence for the functional effect of *MTHFR* 677C>T and *TSER* variant genotypes (14, 38-41), with some, yet less well defined, evidence for the *in vivo* functional relevance of *MTR*, *RFC*, and the *TS* 3'-UTR variant (16-18, 20-23, 42). Our evaluations of colon cancer risk confirm this assessment, in that there were no significant alterations in risk for *MTR*, *RFC*, and *TS* polymorphisms, with the exception of a risk reduction associated with the *TSER* variants among men. The ORs for *TSER* are comparable with those from a previous

report by Chen et al. (43) among male physicians (OR, 0.9; 95% CI, 0.6-1.3 for the 2 rpt/3 rpt genotype and OR, 0.6; 95% CI, 0.4-0.98 for the 2 rpt/2 rpt genotype). A statistically significant trend towards reduced risk with the variant *TSER* alleles was observed both here and in that population (43). These results indicate that the variant *TSER* 2 rpt/2 rpt genotype reduces risk of colon or colorectal cancer among men to a degree comparable with that of the *MTHFR* 677TT genotype (44). As risk reductions for *TSER* variants were only seen among women who are on postmenopausal hormones but not among women overall, estrogen status may play a role.

Further complexity is added as a result of the presence of a second functionally relevant polymorphism in *TS* (15, 16). We evaluated the combined effects of these genotypes as well as haplotypes to discern possible risk patterns. The combined *TS* wild type/wild type genotype constitutes only 8.3% of our population. When compared with this wild type/wild type reference group of putatively highest *TS* expression and *TS* mRNA stability, the variant *TSER* genotypes were associated with statistically significantly reduced risk among women (OR, 0.6; 95% CI, 0.4-0.9) yet not among men. Our sample sizes for these sex-specific associations were limited, and results should be followed up in large study populations that have the ability to investigate combined genotypes as well as sex-specific ORs.

Table 4. Association among combined *TS* genotype, postmenopausal hormone (PMH) use, and colon cancer in postmenopausal women

TSER genotype	TS 3'-UTR 1494delTTAAAG genotype					
	ins/ins			ins/del and del/del		
	Cases (n)	Controls (n)	OR (995% CI)	Cases (n)	Controls (n)	OR (95% CI)
No, PMH						
3rpt/3rpt	41	42	1.0 (reference)	80	113	0.7 (0.4-1.2)
3/2 or 2/2rpt	178	208	0.9 (0.5-1.4)	164	182	0.9 (0.5-1.4)
Yes, PMH						
3rpt/3rpt	21	9	2.2 (0.9-5.4)	21	56	0.4 (0.2-0.7)
3/2 or 2/2rpt	48	89	0.5 (0.3 to <1.0)	53	87	0.6 (0.3 to > 1.0)

NOTE: Adjusted for age, body mass index, lifetime vigorous activity, energy intake, dietary fiber, dietary calcium, and usual number of cigarettes smoked. $P < 0.01$ (Wald χ^2 test of slopes) for TSER 3rpt/3rpt genotype across 3'-UTR genotypes. $P = 0.03$ (Wald χ^2 test of slopes) for 3'-UTR insertion/insertion genotype across TSER genotypes.

The presence of two common functional variants within *TS* suggests that it is essential to take both of these into account simultaneously.

The *MTR* polymorphism is less common (allele frequency = 0.20) and has been investigated in three epidemiologic studies, including a large Norwegian cohort (7, 25, 45). Similar to our findings, Le Marchand et al. (45) and Chen et al. (7) did not report any associations between this variant and colon cancer risk, whereas Ulvik et al. (25) observed a significantly reduced risk among those with a GG genotype compared with wild-type AA (OR, 0.65; 95% CI, 0.47-0.90). We observed reduced risks among men (OR, 0.7), but the 95% CI included 1.0. As only about 5% of the population have the homozygous variant genotype, very large studies are needed to quantify the strength of this association.

For gene-gene interactions, only combinations with the *MTHFR* polymorphisms showed interesting patterns. This is not surprising, because *MTHFR* is a key regulatory enzyme in folate-mediated one-carbon metabolism, the activity of which determines the distribution of folate metabolites toward nucleotide synthesis or methylation reactions. There is strong evidence that the *MTHFR* 677C>T variant alters the balance of metabolites within the pathway (39, 46). In combined analyses of *TS* and *MTHFR* polymorphisms, we observed that men carrying at least one variant *TS* allele (either *TSER* 2rpt or *TS* 1494del) in addition to the *MTHFR* 677TT genotype were at relatively lowest risk compared with all other groups (both OR, 0.6; 95% CI, 0.4-0.9). This confirms our previous observation in colorectal adenoma, where individuals with low *TS* expression and low *MTHFR* activity genotypes also experienced the lowest adenoma risk (OR, 0.56; ref. 10). If this statistical interaction reflects biological mechanisms, then we may hypothesize that the observed pattern suggests that a greater diversion of folate metabolites (specifically 5,10-methylene-tetrahydrofolate) toward purine synthesis is protective for the development of colorectal neoplasia. Recent findings by Quinlivan et al. suggest that folate depletion adversely affects purine synthesis in humans and a greater relative rate of adenine synthesis among individuals with the *MTHFR* TT genotype (46). Depurination is the most common type of DNA damage with ~10,000 depurinations/cell/d (47, 48). Although efficiently repaired, apurinic sites are present in DNA. We recognize that one other study did not observe this risk pattern, but their sample size was limited to 270 cases, with consequent restricted power for studying gene-gene interactions (43).

Our investigations of gene-diet interactions confirmed, to some extent, associations we have previously observed with respect to *TSER*, and folate intake that reduced *TS* expression (*TSER* 2rpt/2rpt) is associated with a reduced risk in the presence of a low folate intake (10). However, this pattern was seen only among men and also has not been observed in the

Health Professionals study (43). Again, if that pattern reflects a biological mechanism, it would point toward purine synthesis as a key link between one-carbon metabolism and colorectal neoplasia. We were unable to confirm previously observed gene-diet interactions for *MTR* in colorectal adenoma (26) and did not see a clear pattern for *RFC*-diet interactions. However, the *RFC* is the transporter for naturally occurring folates (in the form of 5-methyl-tetrahydrofolate) but plays a smaller role in the transport of folic acid (49). Thus, in populations, such as the one described here, where folate intake from supplements in the form of folic acid comprises a substantial proportion of the overall folate intake, genetic variability in the *RFC* may not be as relevant for the overall supply of folate metabolites. Unfortunately, no quantitative information on supplement use was available for this population.

Lastly, we observed differences in risk patterns dependent on the past use of postmenopausal hormones. Interactions between folate metabolism and PMH use are not implausible, as there are links between homocysteine concentrations and PMH use (50-53), and methylation of the estrogen receptor is an early event in colorectal carcinogenesis, which may less frequently occur in the presence of PMH (27, 54). We have previously reported on a significant difference in risk patterns of PMH-associated risks by *MTHFR* genotypes (13). However, these interactions need to be confirmed by others, because sample sizes were in parts insufficient to yield stable estimates.

Although this study is quite comprehensive with respect to investigations of genetic variability in one-carbon metabolism and risk of colon cancer, there are three important limitations. First, our investigations did not include other genetic polymorphisms in folate-metabolizing enzymes that may be of possible relevance, such as methionine-synthase reductase (*MTRR*) or serine-hydroxymethyltransferase (*SHMT*). Thus far, *MTRR* does not seem related to colon cancer risk (45), and the functional relevance of the *cSHMT* polymorphisms is unclear. The study presented here focused on candidate polymorphisms in key enzymes with substantial evidence for functional effect; we hope to expand our investigations to other relevant candidate polymorphisms as they are reported.

Second, there is now strong evidence that a subset of colorectal cancer cases arises as part of a CpG island methylator phenotype (55, 56). Information on CpG island methylator phenotype status should be taken into account in future studies investigating links between genetic variability in folate metabolism and risk of colorectal cancer.

A final limitation is our current inability to integrate knowledge of biochemical relationships within the pathway into the statistical analysis. Although an approach that uses stratification for gene-nutrient or gene-gene interactions is valuable, in that it allows for an empirical investigation of the associations, it is also limited in that statistical power for higher-order interactions is lacking, even within this large

study population. Because folate metabolism consists of several interconnected cycles (see Fig. 1), such interactions are to be expected. Our approach toward solving this problem is to use, in the future, results from a mathematical model of one-carbon biochemistry for investigations of multiple genetic variants on selected biomarkers. Although this model is still under development, preliminary results show that it replicates the biochemical relationships in the folate cycle and methionine cycle with reasonable accuracy (57, 58). Furthermore, our group and others are developing methods to address this key problem for molecular epidemiologic studies (59). Thus, we hope that in the future, we will be able to achieve closer integration of the biochemistry and statistical analysis. Because there is strong evidence that disturbances in this biochemical pathway can modify risk of several types of malignancies (60–63), birth outcomes (64–66), and possibly cardiovascular disease (67) and autism (68), a more thorough understanding of the interplay of multiple genetic polymorphisms under specific dietary conditions and their combined effect on biomarkers and disease end points will be highly relevant.

Acknowledgments

We thank Sandra Edwards and Leslie Palmer at the University of Utah for data collection efforts of this study, Clayton Hibbert and Linda Massey for word processing assistance, and Juanita Leija for genotyping assays.

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Polymorphisms in the Reduced Folate Carrier, Thymidylate Synthase, or Methionine Synthase and Risk of Colon Cancer

Cornelia M. Ulrich, Karen Curtin, John D. Potter, et al.

Cancer Epidemiol Biomarkers Prev 2005;14:2509-2516.

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Molecular and Cellular Endocrinology 177 (2001) 145–159

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Functionally relevant polymorphisms in the human nuclear vitamin D receptor gene

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Abstract

The functional significance of two unlinked human vitamin D receptor (hVDR) gene polymorphisms was evaluated in twenty human fibroblast cell lines. Genotypes at both a *Fok I* restriction site (*F/f*) in exon II and a singlet (A) repeat in exon IX (*L/S*) were determined, and relative transcription activities of endogenous hVDR proteins were measured using a transfected, 1,25-dihydroxyvitamin D₃-responsive reporter gene. Observed activities ranged from 2–100-fold induction by hormone, with higher activity being displayed by the *F* and the *L* biallelic forms. Only when genotypes at both sites were considered simultaneously did statistically significant differences emerge. Moreover, the correlation between hVDR activity and genotype segregated further into clearly defined high and low activity groups with similar genotypic distributions. These results not only demonstrate functional relevance for both the *F/f* and *L/S* common polymorphisms in hVDR, but also provide novel evidence for a third genetic variable impacting receptor potency. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Vitamin D receptor; Gene polymorphisms; Pharmacogenomics

1. Introduction

The biological actions of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] are mediated largely, if not entirely, by the vitamin D receptor (VDR), a member of the superfamily of nuclear hormone receptors (Whitfield et al., 1999). This protein is found in tissues known to play a role in calcium homeostasis, and also in numerous other tissues, where it appears to regulate a variety of processes, including cell proliferation and differentiation (Haussler et al., 1998). The significance of the nuclear VDR in calcium homeostasis, as well as in certain differentiation and proliferation processes in skin and uterus, has been confirmed by gene knockout studies in mice (Li et al., 1998; Kato et al., 1999).

A simplified diagram that illustrates how nuclear hVDR mediates transcriptional activation by the 1,25(OH)₂D₃ hormone is shown in Fig. 1A. The key features of this model are: (i) liganding of nuclear VDR

by 1,25(OH)₂D₃; (ii) recruitment by 1,25(OH)₂D₃-VDR of its retinoid X receptor (RXR) heteropartner that, in turn, facilitates high-affinity interaction of the dimeric complex with vitamin D responsive elements (VDREs) upstream of target genes; (iii) attraction by VDR of basal transcription factor IIB (TFIIB), the rate-limiting component of the transcription preinitiation complex; and (iv) recruitment by the heterodimer of a number of transcription coactivators, some with histone acetyl transferase (HAT) activity to modify nucleosome/chromatin organization, such as SRC-1 (Gill et al., 1998), and others like the DRIPs (Rachez et al., 1999) that target the VDR supercomplex to the TATA-box/TBP and RNA polymerase II transcription initiation machinery. The net result of this 1,25(OH)₂D₃-triggered response is the regulation of genes coding for proteins that carry out intestinal calcium absorption, bone remodeling, cell differentiation, etc. (Jurutka et al., 2001).

A modular diagram of the functional domains within the hVDR protein is presented in Fig. 1B. The details of the hVDR subdomain arrangement (see figure legend) basically follow the general pattern for the sub-

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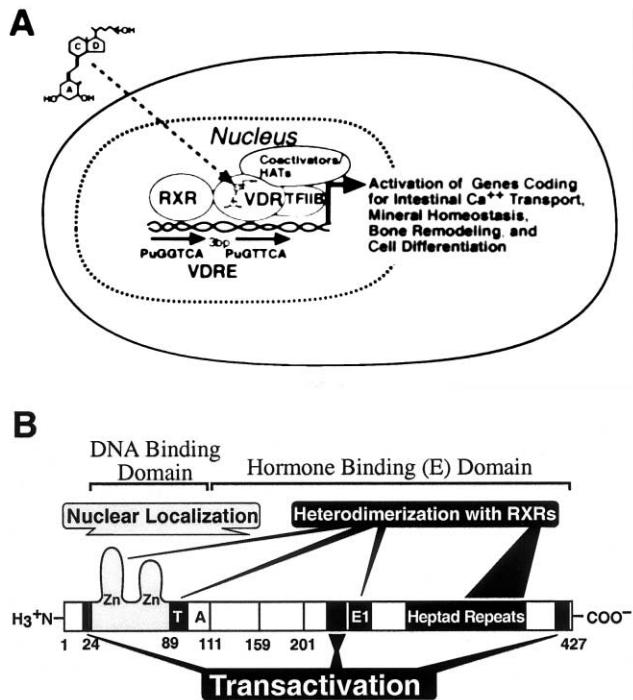


Fig. 1. (A) Model for transcriptional activation by $1,25(\text{OH})_2\text{D}_3$ as mediated by a heterodimer of VDR and RXR bound to VDREs upstream of target genes in vitamin-D responsive cells. As the primary receptor, VDR is activated by $1,25(\text{OH})_2\text{D}_3$ binding, but the RXR coreceptor apparently can remain unliganded. The receptor heterodimer associates with direct repeat-type responsive elements upstream of the target genes and the liganded heterocomplex attracts various coactivators, some with histone acetylase (HAT) activity. VDR itself recruits basal transcription factor IIB (TFIIB). Finally, the ensemble of protein-protein-DNA interactions promotes transcriptional initiation of a battery of target genes, leading to the pleiotrophic effects of the $1,25(\text{OH})_2\text{D}_3$ hormone (Haussler et al., 1998). (B) Schematic illustration of functional domains in the VDR protein. The DNA binding domain, with two zinc finger motifs, is located near the N-terminus and also contains residues that promote nuclear localization of the receptor. The central and C-terminal region of the receptor contain subdomains that mediate ligand binding (Rochel et al., 2000). The heterodimerization (with RXR) and transactivation functions of hVDR appear to be mediated by widely separated regions of the receptor. Heterodimerization is supported by heptad repeats in the helix 7–10 region (Nakajima et al., 1994), an E1 domain (Whitfield et al., 1995), as well as residues in the T-box and first zinc finger (Hsieh et al., 1995). Transactivation regions include: (i) the extreme N-terminus, which possesses a TFIIB docking site (Jurutka et al., 2000); (ii) a centrally located domain corresponding to helix 3 in the rat TR α , hRAR γ and hVDR ligand binding domain crystals (Renaud et al., 1995; Wagner et al., 1995; Kraichely et al., 1999; Rochel et al., 2000); and (iii) the extreme C-terminus, corresponding to helix 12 in the rat TR α , hRAR γ and hVDR crystals (Renaud et al., 1995; Wagner et al., 1995; Jurutka et al., 1997; Rochel et al., 2000). ‘A’ designates the A-box, which contains important DNA-binding amino acids (Hsieh et al., 1999). The residue 159–201 segment, which is unconserved in VDRs, is encoded by a novel exon (V), not seen in other nuclear receptors for which the gene structure is known (Haussler et al., 1998).

family of nuclear receptors that heterodimerize with RXR, such as the all-trans retinoic acid receptors (RARs) and the thyroid hormone receptors (TRs) (Whitfield et al., 1999). For the purposes of the present communication, the most relevant regions of hVDR are the hormone-binding domain, encoded by exons VI–IX of the human gene (see also Fig. 2), the DNA binding domain/zinc fingers, encoded by exons II–IV, and a set of discontinuous transactivation domains, including regions at the N-terminus (for TFIIB docking) (Jurutka et al., 2000), and in helices 3 and 12 (for coactivator recruitment). Since transactivation is the ultimate biochemical action of the liganded VDR and depends on all of the other capabilities of the receptor (ligand binding, nuclear localization, heterodimerization and VDRE/DNA binding), the present study focuses on this parameter of receptor activity in order to probe for functional significance of hVDR gene polymorphisms.

The chromosomal gene for VDR has been cloned (Miyamoto et al., 1997), and several common genetic variants have been described in humans, most of which are identified by a biallelic variation in a restriction endonuclease site (Fig. 2). Genetic variation in the 3' region of the hVDR gene is observed in specific intronic sites for *Bsm I* (Morrison et al., 1992) and *Apa I* (Faraco et al., 1989), a silent *Taq I* site in exon IX (Morrison et al., 1992), as well as in a singlet(A) repeat in the portion of exon IX encoding the 3' UTR (Ingles et al., 1997a) (see Fig. 2, right). All of these variations near the 3' end of the gene are in linkage disequilibrium (Morrison et al., 1992; Verbeek et al., 1997), although this linkage is weaker in some ethnic groups such as African-Americans (Ingles et al., 1997a). Interestingly, none of these polymorphisms affect the structure of the VDR protein itself, although the singlet(A) repeat in the 3' UTR is expressed in the mature mRNA for hVDR. Singlet(A) variants are classified according to length by the number of consecutive A's in the repeat, with ≥ 17 A's scored as ‘long’ (L), and ≤ 15 A's considered ‘short’ (S).

Another polymorphic site has been found in exon II near the center of the hVDR gene (Saijo et al., 1991). This site, which is genetically unlinked to the above *Bsm/Apa/Taq*/singlet(A) cluster, is unique among common hVDR variants described so far, in that it results in an alteration of the hVDR protein structure (Fig. 2, bottom center). Presence of the *Fok I* site (designated f) predicts that a 427-residue VDR protein will be produced beginning at Met-1 (M1 according to the numbering scheme of Baker et al. (1988), whereas absence of this site (denoted F) dictates translation from Met-4 (M4), producing a protein of 424 amino acids (Arai et al., 1997).

In an initial report (Morrison et al., 1994), allelic variation in the chromosomal gene for the vitamin D receptor was proposed to represent a major part of the genetic predisposition for low bone mineral density (BMD), and perhaps for osteoporosis and/or skeletal

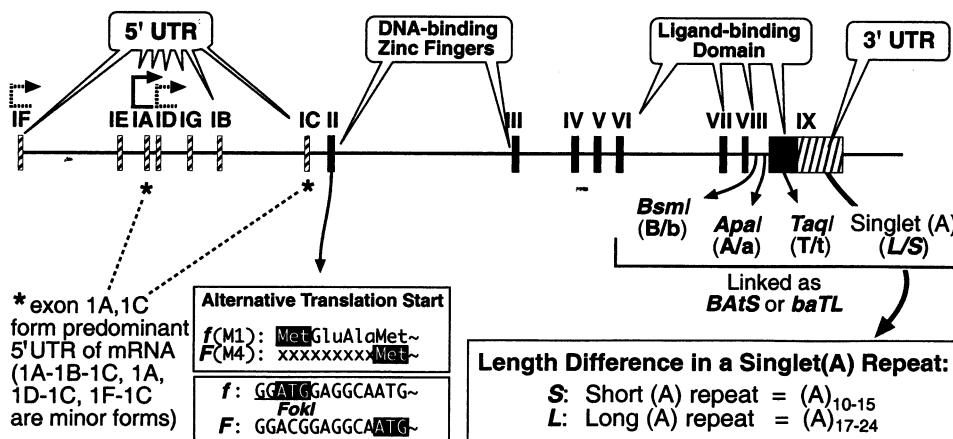


Fig. 2. The human VDR gene, with key features relevant to polymorphic variation in VDR expression and activity. The fifteen known exons are depicted at the top (Miyamoto et al., 1997; Crofts et al., 1998). The 5' untranslated region of hVDR mRNAs is observed to be alternatively spliced in all tested human tissues (Crofts et al., 1998). The predominant hVDR mRNA in tissues tested to date contains a 5' UTR consisting of exon IA spliced to IC; several other less abundant spliced forms have been described, implying the existence of at least three promoters for the hVDR gene, depicted by arrows above exons IF, IA and ID, respectively, (Crofts et al., 1998). Exons II and III encode the translation start site, a short N-terminal domain, and the two zinc finger motifs of the DNA binding domain (one in each exon). The overlapping ligand-binding and strong heterodimerization domains are encoded by exons VI–IX, with exon IX also containing the entire 3' UTR. At right are shown four linked polymorphic sites in or somewhat 5' to exon IX. The present study focuses on the singlet(A) repeat, which lies about 1 kb upstream of the polyadenylation site and exists in either a long (*L* = 17–24 A's) or a short (*S* = 10–15 A's) form (Ingles et al., 1997a). An additional site of interest to the current study is the dimorphic translational start site (Saijo et al., 1991), the two forms of which (*F* or *f*, illustrated at bottom center) are unlinked to the *L/S* variants (Gross et al., 1996).

fractures, although these associations have been disputed by other studies [reviewed in (Wood and Fleet, 1998)]. More recently, correlations have been reported between VDR allelic variants and risk of prostate cancer (Ingles et al., 1997b; Watanabe et al., 1999), breast cancer (Ingles et al., 1997c; Ruggiero et al., 1998; Curran et al., 1999), sporadic primary hyperparathyroidism (Correa et al., 1999), and sarcoidosis (Niimi et al., 1999). However, conflicting reports have appeared that minimize or even contradict these associations (Cheng and Tsai, 1999; Correa et al., 1999). Likewise, direct testing of hVDR alleles for activity has yielded somewhat variable results, although, when a difference is found, the *b* and *F* hVDR alleles appear to be more active than the *B* or *f* alleles (see Section 4).

One caveat in most of the above-cited studies is that correlations were sought between a single, specific polymorphism, or between the *Bsm-Apa-Taq* linkage group, and the physiological parameter of interest. Very few studies have attempted to control for hVDR genotype at both the *Bsm/Apa/Taq*/singlet(A) cluster and the *Fok I* site. In one example (Ferrari et al., 1998), a correlation between *Fok I* alleles and BMD could not be demonstrated, but 'cross-genotyping' with *Bsm I* alleles revealed a potentially important positive association in prepubertal girls between the *ffBB* hVDR genotype and low BMD (Ferrari et al., 1998).

Another caveat in the above cited studies is that a direct influence of allelic variation on VDR expression or activity was not demonstrated, leaving open the possibility that the observed correlation might be due

to linkage to another nearby site or even to a different gene. In the only two extant studies in which the potential relationship between genotype and activity of the hVDR protein was evaluated (Verbeek et al., 1997; Gross et al., 1998), no functional influence of specific alleles was observed, but again, only a single polymorphic site was examined in isolation.

In the present communication, we report an evaluation of a panel of twenty human fibroblast lines. The current protocol includes simultaneous consideration of the hVDR genotypes at both the singlet(A) and the *Fok I* loci, which are then correlated with activity of the endogenous VDR in the corresponding cell line. From these data, we conclude that (a) biallelic variants at the *Fok I* and the singlet(A) sites, in combination, affect transcriptional activation by the endogenous hVDR in the tested human fibroblasts; (b) the singlet(A) *L* allele is more active than the *S* allele; and (c) a third, unknown genetic variable appears to influence VDR activity.

2. Materials and methods

2.1. Plasmid DNAs used for transfection and *in vitro* transcription

The 1,25(OH)₂D₃-responsive reporter plasmid, (CT4)₄TKGH, contains four copies of the rat osteocalcin VDRE (Terpening et al., 1991) linked upstream of the thymidine kinase promoter-GH reporter gene

(Nichols Institute, San Juan Capistrano, CA). The hVDR expression vector, pSG5-hVDR, expressing the *F/M4* isoform of hVDR, has been described earlier (Hsieh et al., 1991). This construct was adapted for expression of the *f/M1* hVDR isoform by inserting the appropriate DNA codons via in vitro site-directed mutagenesis (Jurutka et al., 2000). For monitoring the efficiency of transfection, a commercial plasmid expressing β -galactosidase (CMV- β gal) was obtained from Promega Corp. (Madison, WI).

2.2. Cell lines

Cell lines DNF-BJ, DWF-CV and DWF-TW were provided courtesy of C. Bloch at the Children's Hospital, Denver, CO. Patients DWF-CV and DWF-TW are reported to have features of William's Syndrome. Other cell lines were obtained from the American Type Culture Collection, Manassas, VA, with patients Ber Lin, Be Sal and Ran Nor reported to have late-onset osteoporosis.

2.3. Transfection of cultured cells and transcriptional activation assay

Human fibroblast cell lines were cultured in DMEM:Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were transfected by electroporation (see Fig. 3, top left). Briefly, cells were collected by trypsinization, pelleted at low speed and

resuspended at 5×10^6 cells per ml in 1X HeBS buffer (20 mM HEPES, pH 7.1, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 , 6 mM dextrose). Suspended cells (0.8 ml) were then combined with 40 μ g (CT4)₄ TKGH reporter plasmid, 10 μ g of CMV- β gal plasmid, and 450 μ g carrier DNA (pTZ18U plasmid), and adjusted to a total volume of 1 ml in 1X HeBS buffer in a 0.4 cm electrode gap electroporation cuvette. Each cuvette was then subjected to electroporation in a Bio-Rad Gene Pulser II apparatus (with capacitance extender attachment) at settings of 200 V and 950 μ F. After 10 min of incubation at room temperature, the electroporated cells were suspended in culture medium and then divided into six 60 mm culture dishes and incubated at 37°C for 72 h in the presence of 10^{-8} M 1,25(OH)₂D₃ (three plates) or ethanol vehicle (also in triplicate plates). The levels of growth hormone secreted into the culture medium were then assessed by radioimmunoassay using a commercial kit (Nichols Institute) according to the manufacturer's protocol. To normalize results for the efficiency of transfection in each plate, β -galactosidase levels were assayed in cell lysates (freeze-thaw method) using reagents and instructions from a commercial kit (Promega Corp.). These steps are represented as a flow chart in Fig. 3 (left).

For the experiment depicted in Fig. 7, ROS 2/3 cells (8×10^5 cells per 60 mm dish) were transfected by calcium phosphate coprecipitation as described earlier (Jurutka et al., 2000) using 10 μ g of a reporter plasmid containing 1100 bp of the natural rat osteocalcin promoter linked to the human growth hormone gene [de-

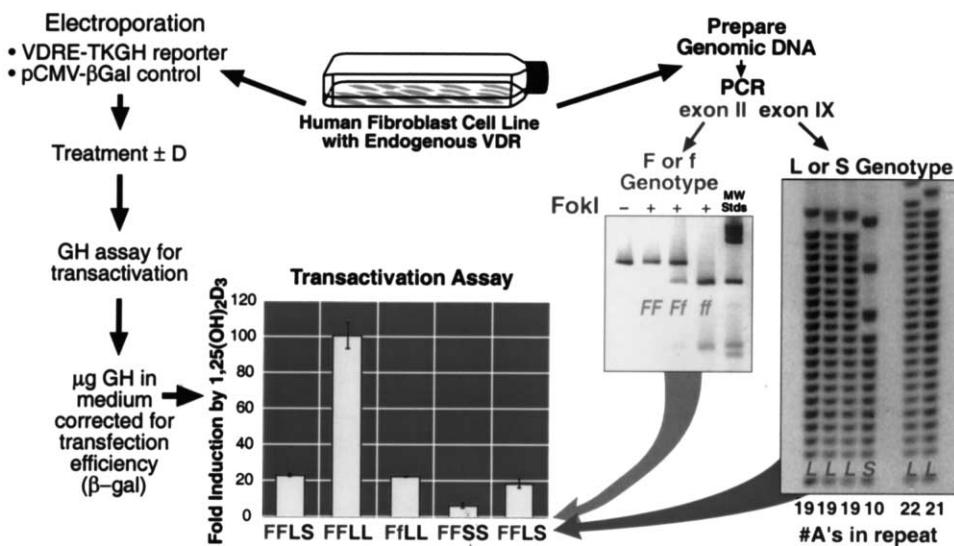


Fig. 3. Protocol for VDR genotype/phenotype analysis of human fibroblast cell lines. Cultured cells (top center) were transfected, incubated and assayed for relative transcriptional activity as depicted schematically at the left and detailed in Section 2. Results were expressed as fold-induction by $1,25(\text{OH})_2\text{D}_3$. Cells of each line were also harvested for genomic DNA isolation and genotyping by PCR as shown at the upper right. For determination of singlet(A) repeat length, multiple PCR products from exon IX of each line were sequenced individually (see sample gel at far right). PCR products of exon II were digested with the *Fok I* restriction enzyme and resolved by electrophoresis (see sample gel at center-right). Final results for each cell line thus included the genotype at both the 3' UTR singlet(A) site (*L/S*), and the translation initiation site (*F/f*), along with relative transactivation activity of the endogenous VDR (see sample summary graph at bottom center).

noted BGP-TK GH (Terpening et al., 1991)], along with 1.0 µg of pSG5-hVDR expressing either *F/M4* or *f/M1* hVDR. Sixteen hours post-transfection, the cells were washed, and refed (DMEM:Ham's F-12 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin) and treated with 10⁻⁸ M 1,25(OH)₂D₃ or ethanol vehicle. After 24 h, the level of secreted growth hormone was assayed in the culture medium from each plate as described above.

2.4. Genotyping of human fibroblasts

DNA was prepared from cultured human fibroblasts (10⁷ cells) using the QIAamp tissue kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. For *F/f* genotyping, isolated genomic DNA (500 ng) was combined with 100 ng each of primers 2a and 2b (Gross et al., 1996), along with 5 ml of 10X buffer (Perkin Elmer, Norwalk, CT) plus 1.5 mM MgCl₂, 2.5 mM each of dATP, dCTP, dTTP and dGTP, and 0.25 ml Taq DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN). PCR conditions were: 20 cycles at 94°C for 30 s, 74°C for 30 s (with –0.1°C per cycle) and 72°C for 60 s. This was followed by 25 cycles at 94°C for 30 s, 63°C for 30 s and 72°C for 60 s. Approximately, 200 ng of unpurified PCR product was then incubated with 1 µl *Fok I* enzyme (New England Biolabs, Beverly, MA) and 1 µl 10X buffer in a total volume of 10 µl for 1.5 h at 37°C. The digestion mixture was electrophoresed on a 4% NuSieve (3:1) Agarose gel in TBE buffer (90 mM Tris–borate, pH8, 2 mM EDTA) to determine whether the PCR product was completely digested (indicating the *ff* genotype), partially digested (*Ff*) or completely undigested (*FF*).

For *L/S* genotyping, isolated genomic DNA (500 ng) was combined with 5' and 3' primers (100 ng each) (Ingles et al., 1997b) using the same PCR profile described above. PCR products at approximately 400–425 bp were resolved on 0.8% Agarose gels, excised from the gel and isolated into 20 µl of Tris–HCl, pH 8.5, using a QIAEX II extraction kit (Qiagen Inc.) according to the protocol of the manufacturer. The isolated PCR products (7.5 µl) were then cloned into the *T*-vector and transformed into the XL-1 Blue strain of *E. coli* using a *T*-vector kit (Promega Corp.). Plasmid DNA was isolated from transformed bacteria by standard methods and sequenced ('A' reaction only) using a T7 Sequenase kit (Amersham Pharmacia Biotech, Piscataway, NJ). Typical results are depicted in Fig. 3 (right).

2.5. GST coprecipitation assays

The ability of either *F/M4* or *f/M1* hVDRs to interact with human TFIIB was assessed as described earlier (Jurutka et al., 2000). Briefly, TFIIB-glutathione-S-

transferase (GST) fusion protein was expressed from pGEX-2T-hTFIIB (Baniahmad et al., 1993) and GST alone was expressed from pGEX-4T, both in *E. coli* strain DH5α. Each protein was then coupled to glutathione Sepharose. For the GST 'pull-down' assays, pSG5-hVDR vectors expressing either *F/M4* or *f/M1* hVDRs were used to generate [³⁵S] methionine-labeled proteins utilizing the TNT Coupled Reticulocyte Lysate kit (Promega Corp.). The desired ³⁵S-labeled protein was then incubated with the beads in the absence or presence of 1,25(OH)₂D₃ (10⁻⁶ M). Next, the unbound proteins were washed from the beads with 4 × 1 ml wash buffer [0.15 M KCl, 10 mM Tris–HCl, pH 7.6, 1 mM EDTA, 0.3 mM ZnCl₂, 1 mM dithiothreitol, 0.1% Tween-20, 1 mg/ml BSA, and the following protease inhibitors, obtained from Roche Molecular Biochemicals (Indianapolis, IN): 0.5 µg/ml Pefabloc SC, 15 µg/ml aprotinin, 1 µg/ml pepstatin A, 1 µg/ml leupeptin]. The bound proteins were extracted from the beads into 40 µl loading buffer (2% SDS, 5% β-mercaptoethanol, 125 mM Tris–HCl, pH 6.8, and 20% glycerol), boiled for 3 min, separated by SDS-PAGE and visualized via autoradiography.

3. Results

3.1. Genotyping of human fibroblast lines

As described in Section 2 and depicted schematically in Fig. 3, genomic DNA samples were extracted from twenty human fibroblast cell lines, subjected to PCR using two independent sets of primers, and analyzed for their hVDR genotype at the polymorphic sites in exon II (*F/f*) and exon IX (*L/S*). *F/f* genotypes were determined by digestion of the PCR products from each line with the restriction enzyme *Fok I* (Gross et al., 1996). Two independent PCR reactions were performed for each line, and digestion experiments included a *ff* and *Ff* line as positive controls to monitor activity of the *Fok I* restriction endonuclease. A typical digestion pattern is shown at the center-right of Fig. 3, displaying the undigested PCR product (265 bp), partially digested DNA from a heterozygote, and completely digested DNA (69 and 196 bp fragments) from an *ff* subject. The frequencies of the *F* and *f* alleles in the present sample group were 62.5 and 37.5%, respectively. This distribution of hVDR alleles is similar to that characterized for Caucasian populations in other studies (Gross et al., 1996; Eccleshall et al., 1998; Gennari et al., 1999).

L/S genotypes were determined by sequencing of PCR products to ascertain the exact length of the singlet(A) repeat (Ingles et al., 1997b). As reported by others (Ingles et al., 1997a), we observed (Fig. 4) multiple alleles at this locus which segregate into a 'long' (*L*)

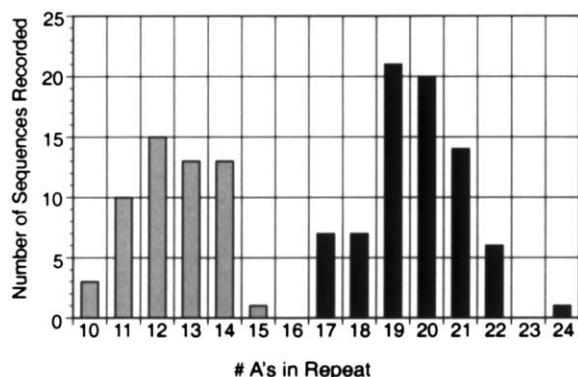


Fig. 4. Compilation of sequencing results from *L/S* genotyping (approximately 4–5 sequences per cell line). Variations of 1–2 A's were observed in separate sequencing reactions from the same cell line, presumably because of errors in PCR (Ingles et al., 1997a) or in the bacterial replication of these repeated sequences. Allele frequencies in the present sample of 20 cell lines were 40% *S* and 60% *L*, nearly identical to published results of 41% *S* for Caucasians living in the USA (Ingles et al., 1997a).

group with 17–24 A's in the repeat, and a 'short' group (*S*) with 10–15 A's in the repeat. The frequencies of the *L* and *S* hVDR alleles in the present panel of cell lines were 60 and 40%, respectively, similar to that earlier published for Caucasian populations (Ingles et al., 1997a).

3.2. Relative VDR transcriptional activities of human fibroblast cell lines

Fig. 5 shows the hVDR-mediated transcription results from 20 human fibroblast cell lines after transfection.

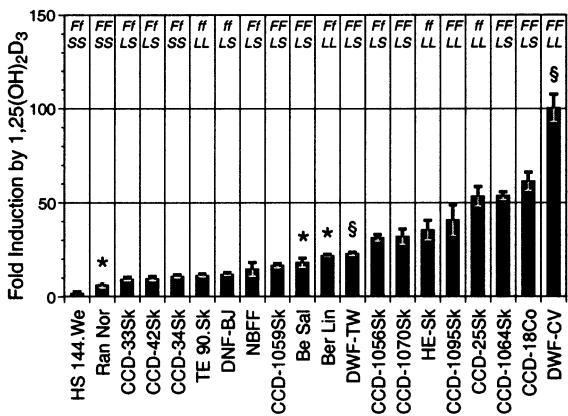


Fig. 5. Relative transcriptional activity of endogenous VDR in 20 human fibroblast lines. Results from all cell lines (average of ≥ 6 experiments, each in triplicate (\pm S.E.M.)) are arranged in order of increasing fold-induction by $1,25(\text{OH})_2\text{D}_3$. Cell lines DWF-TW and DWF-CV (\$) were obtained from patients with features of William's Syndrome, and patients Ran Nor, Be Sal and Ber Lin (*) had late-onset osteoporosis. All other lines were taken from patients with no known disorders of bone or calcium metabolism. Genotypes of each line are given at the top.

tion with a $1,25(\text{OH})_2\text{D}_3$ -responsive reporter plasmid and incubation in the presence or absence of 10^{-8} M $1,25(\text{OH})_2\text{D}_3$, expressed as fold-induction by the hormone. The assay conditions, including quantitative monitoring of transfection efficiency using a β -galactosidase vector, were designed to permit comparison of endogenous VDR activity between cell lines. The data (Fig. 5) reveal a striking spectrum of activities, ranging from only a 1.75-fold induction of the growth hormone reporter within the 72-h treatment period to a 100-fold effect of hormone, with a mean of 28 ± 24 (S.D.) fold induction. The lowest activity (1.75-fold induction by $1,25(\text{OH})_2\text{D}_3$) was observed in a fibroblast line derived from a 5-week-old embryo. As denoted in Fig. 5, a subset of the tested fibroblast cell lines are from patients with either osteoporosis (*), in which subjects are usually normocalcemic, but can have low blood calcium, or with William's Syndrome (\$), a condition frequently presenting with hypercalcemia. Interestingly, one of the William's patients (DWF-CV) contains endogenous VDR with the highest activity (100-fold induction by $1,25(\text{OH})_2\text{D}_3$), while the other patient displays a near-average (23-fold) induction by $1,25(\text{OH})_2\text{D}_3$. Conversely, one of the osteoporosis patients (Ran Nor) yielded a very low VDR activity in the assay (6-fold induction), while the two other osteoporotic individuals (Be Sal, Ber Lin) displayed inductions by hormone that were only slightly below average (18- and 22-fold, respectively). No other associations were noted between patient status (e.g., age, sex or medical condition) and VDR activity (see Section 4).

3.3. Correlations between either *Fok I* or *L/S* genotype and activity of endogenous VDR

Given the current interest in the *Fok I* and *L/S* hVDR polymorphisms, correlations were next sought between the genotypes of each cell line at these two loci and the relative activities of the corresponding endogenous VDRs. Accordingly, all twenty cell lines examined were grouped into *ff* (4 lines), *Ff* (7 lines) or *FF* (9 lines). The average fold-inductions by $1,25(\text{OH})_2\text{D}_3$ for each group are displayed (\pm S.E.M.) in Fig. 6A. Although no clear trend is evident, it is notable that the *FF* group displays the highest average fold-induction by the hormonal ligand. These *F/f* groupings were then subdivided into sets with the *LS* genotype (Fig. 6C, left panel) and the *LL* genotype (Fig. 6C, right panel), thereby controlling for the *L/S* genotype when evaluating the activity of endogenous *F/f* hVDR. Again, in both groups of data, the *FF* cells display the highest activity. However, none of the differences between groupings in either Fig. 6A or 6C achieve statistical significance (at the 95% confidence interval), presumably because of the small number of cell lines in each grouping or the possible existence of a novel

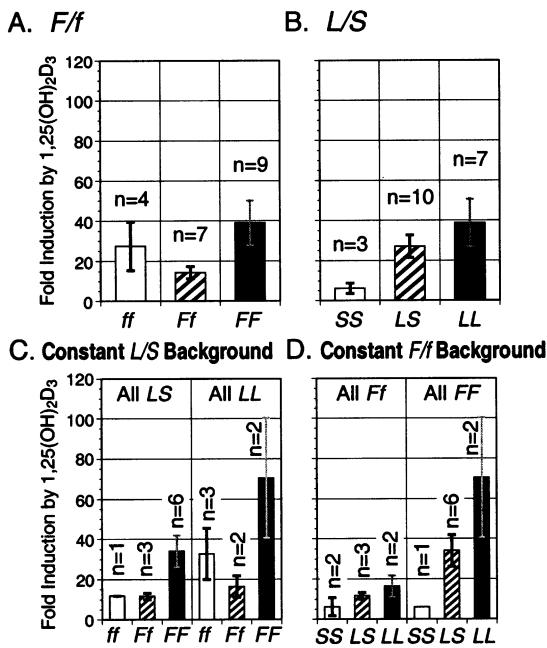


Fig. 6. Correlation of transcriptional activity with hVDR genotype. (A) All 20 cell lines are grouped by *F/f* genotype. Each grouping consists of the indicated number of lines, and the fold-induction values for these lines (taken from Fig. 5) are represented as the average \pm S.E.M. (B) As in (A), but all 20 cell lines are grouped by *L/S* genotype. (C; left panel) The ten cell lines with genotype *LS* are grouped by *F/f* genotype, and the average fold-induction by 1,25(OH)₂D₃ \pm S.E.M. is shown. (C; right panel) The seven lines with an *LL* genotype are similarly grouped by *F/f* genotype and plotted. The three *SS* hVDR cell lines are omitted from this analysis, since they do not form a complete set of *F/f* groupings (*ffSS* is missing). (D; left panel) The seven cell lines with genotype *Ff* are grouped by *L/S* genotype. (D; right panel) The nine lines with a *FF* genotype are similarly grouped by *L/S* genotype and plotted. The four *ff* cell lines are omitted, as they do not form a complete set of *L/S* groupings (again, *ffSS* is missing).

genetic variable in hVDR activity. Nevertheless, the *FF* groupings show higher fold-inductions than the corresponding *Ff* groups, with the exception of lines with the *SS* hVDR genotype (see Fig. 6D), appearing to confirm an effect of the *F/f* polymorphism and suggesting that the *F* allele is more active.

When 1,25(OH)₂D₃-stimulated transcription activities in the twenty lines are grouped by *L/S* genotype (Fig. 6B), a much clearer, but still not statistically significant, trend emerges, with *SS* having the lowest fold-induction, *LL* possessing the highest, and the *LS* genotype exhibiting intermediate activity. This trend persists when the groupings are subdivided into those with constant *Ff* or *FF* genotypic backgrounds (Fig. 6D), leading to the tentative conclusion that the *L* hVDR allele is more active than the *S* allele. The *SS* and *ff* subsets were not included in Fig. 6C and Fig. 6D, because of the low number of samples of both *SS* and *ff* homozygotes (3 and 4, respectively), as well as the complete lack of the *ffSS* hVDR genotype in the current series of fibroblasts lines.

3.4. The *F* hVDR isoform is more active in transfected cells

Although the above *F/f* groupings did not show a rigorous association between hVDR genotype and fold-induction by 1,25(OH)₂D₃ (Fig. 6A and C), recent results from other laboratories (Arai et al., 1997; Colin et al., 2000) have demonstrated an apparent higher activity for the *F* isoform of hVDR relative to the *f* isoform. The results presented in Fig. 6 are generally consistent with this conclusion. In addition, Fig. 7A depicts an in vitro experiment in which the *F* and *f* hVDR isoforms were expressed from a pSG5-hVDR construct and directly tested for transcriptional activity. The original pSG5-hVDR vector expresses the *F/M4* isoform; a cDNA insert expressing the *f/M1* was engineered into this same vector via site-directed mutagenesis [see Section 2 and (Jurutka et al., 2000)]. These two vectors were separately transfected into the VDR-deficient rat osteosarcoma line, ROS 2/3, along with the BGP-TKGH reporter plasmid. The results of this analysis (Fig. 7A) show a significant ($P < 0.001$) difference in fold-induction by 1,25(OH)₂D₃, with the *F* hVDR construct displaying a greater response to 1,25(OH)₂D₃ (4.2-fold) than the *f* allele construct (2.6-fold).

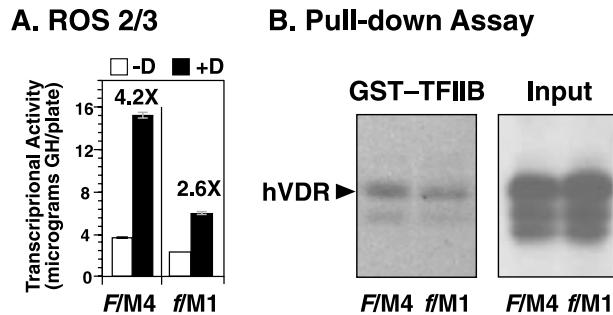


Fig. 7. Relative activities of *F/M4* hVDR and *f/M1* hVDR, expressed from an engineered construct. (A) An *f/M1* hVDR cDNA, differing at the translational start site, was created from an existing *F/M4* cDNA by site-directed mutagenesis. Both cDNAs, cloned in the vector pSG5, were used to express *F/M4* and *f/M1* hVDRs in VDR-deficient ROS 2/3 cells (Jurutka et al., 2000). Assays for relative transcriptional activity were performed as described above for endogenous VDR in human fibroblast cell lines, except that the BGP-TKGH reporter vector was used (see Section 2). The results shown (triplicate assays \pm S.E.M.) are representative of at least three independent experiments. (B) Using the same cDNAs as in (A), ³⁵S-labeled *F/M4* and *f/M1* hVDRs were expressed in a coupled in vitro transcription/translation system (see Section 2) and assayed for their ability to bind a human TFIIB-glutathione S-transferase fusion protein that had been immobilized on Sepharose beads (Jurutka et al., 2000). Washed beads were denatured and subjected to electrophoresis on 5–15% SDS-PAGE gels (see Section 2), and ³⁵S-labeled protein bands were visualized by autoradiography (left panel of B). The right panel of B displays autoradiograms of ³⁵S-labeled proteins used for the pull-down assays (5% of total input).

3.5. Relative ability of *F/f* hVDR isoforms to interact with TFIIB, *in vitro*

It has earlier been reported by our group (Jurutka et al., 2000) that *F* hVDR interacts more efficiently with TFIIB than does the *f* hVDR isoform, thus providing a plausible mechanism for the greater transactivation potency of the *F* hVDR. Fig. 7B illustrates a typical experiment, utilizing the GST pull-down technique to compare the abilities of *in vitro*-synthesized, ³⁵S-labeled *F/M4* and *f/M1* hVDR isoreceptors to interact with an immobilized TFIIB fusion protein. Even in the face of a higher input of ³⁵S-labeled *f/M1* protein, the *F/M4* protein shows a reproducibly greater (approximately 2-fold) ability to interact with TFIIB, when compared with the *f/M1* protein under the same conditions (Jurutka et al., 2000). Whether this difference in activity reflects that occurring under *in vivo* conditions is not known; however, these results provide a reasonable mechanism by which to explain the enhanced transactivation ability of the *F/M4* hVDR isoform, *in vitro*, and are consistent with a proposed bioactivity for *F* hVDR that is also greater than that of *f* hVDR, *in vivo* [(Gross et al., 1996; Arai et al., 1997; Harris et al., 1997; Tao et al., 1998; Videman et al., 1998; Correa et al., 1999; Ferrari et al., 1999; Gennari et al., 1999; Kurabayashi et al., 1999; Lucotte et al., 1999; Colin et al., 2000; Sosa et al., 2000), see Section 4].

3.6. Correlation between genotype at both polymorphic loci and transactivation by endogenous VDRs

Considering the lack of genetic linkage between the *Fok I* and *L/S* polymorphisms (Gross et al., 1996; Ferrari et al., 1998; Cheng and Tsai, 1999), plus the fact that both loci appear to affect function of the endogenous receptor, *in vivo* (Fig. 6), as well as evidence indicating that *F* hVDR is more active than *f*, *in vitro*, we attempted next to correlate the *combined* genotypes at both loci with hVDR transactivation ability. In order to condense genotypic information from both sites into a single variable, an ‘allele score’ was devised based on which allelic variants appear more active in the literature and in the present experiments. Since the *F* genotype is more active than *f* both *in vivo* and *in vitro*, each *F* hVDR allele was assigned a value of 1, while *f* alleles were scored as zero. Likewise, because the data in Fig. 6, panels B and D, indicate the *L* hVDR alleles to be more active than the *S* alleles, *L* and *S* alleles received scores of 1 and 0, respectively. Since the hVDR gene resides on an autosome (chromosome 12) (Szpirer et al., 1991), possible total allele scores range from 0 to 4 for both sexes. After grouping all twenty cell lines according to this formula, the average fold-induction by 1,25(OH)₂D₃ was plotted versus the allele score (Fig. 8A). A striking trend emerges from this analysis, with

each increasing increment in allele score yielding a higher average fold-induction by 1,25(OH)₂D₃. A qualitatively similar trend was seen if 1,25(OH)₂D₃-stimulated values for reporter gene production were plotted instead of fold-induction values (data not shown). Thus, the dramatic escalation of hVDR functional activity appears to correlate to the combined hVDR genotypic allele score at the *F/f* and *L/S* loci. Importantly, the difference between the two groups with allele scores of 2 and 4 achieves statistical significance by the two-tailed Student’s *t*-test (*P* = 0.035).

Fig. 8B depicts the identical data set analyzed in A, but with each cell line plotted as an individual point. When a linear regression line is calculated for all 20 lines, the allele score shows a moderately strong, and statistically significant, positive correlation with transactivation (correlation coefficient = 0.595; *P* = 0.012). If, however, the cell lines are divided into a high group (above the *n* = 20 regression line) and low group (below the *n* = 20 line), the respective correlation coefficients are markedly improved, to 0.958 for the high group (*n* = 8, *P* < 0.001) and 0.858 for the low group (*n* = 12, *P* < 0.001). This apparent segregation of values into high and low groups argues for the existence of a new, third variable, other than the *Fok I* or 3' cluster of polymorphisms containing *L/S*, in determining hVDR functional activity.

4. Discussion

A goal of the present study was to examine endogenous hVDR transcriptional activity in relation to hVDR genotype at unlinked polymorphic sites in both exons II and IX. The results reveal a strong correlation between genotype and VDR activity that emerges when both polymorphic sites are simultaneously considered (Fig. 8). A corollary of this conclusion is that variation at both polymorphic sites is important to hVDR functional activity, *in vivo*, but that considering each site separately may not reveal significant effects. Thus, the current data may explain why many attempts to correlate hVDR activity with genotype at a single locus have been unsuccessful.

The allelic distributions found in the present sample are similar to genotype frequencies in the published literature. The 62.5% frequency reported here for the *F* allele is comparable to the 63.5% published for Italian women (Gennari et al., 1999), the 62% for French women (Eccleshall et al., 1998) and the 61% for Mexican-American Caucasian women (Gross et al., 1996), but lower than the incidences of the *F* allele (68.5%) observed for Japanese women (Arai et al., 1997), or the 80.5% reported for African-American women (Harris et al., 1997). Concerning the singlet(A) *L/S* alleles, the frequency of the *L* allele in the present sample, 60%,

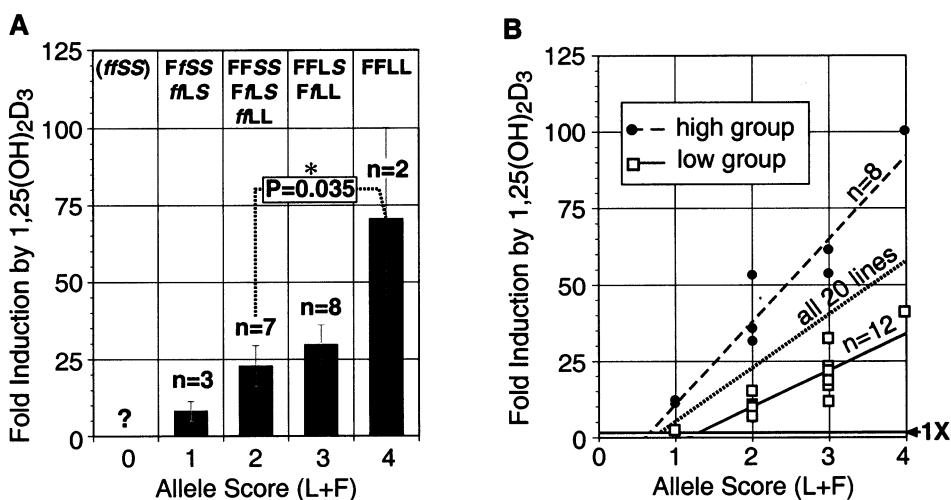


Fig. 8. Correlation of transcriptional activity with hVDR genotype at both *L/S* and *F/f* loci. (A) Results from Fig. 5 were grouped according to an 'allele score', computed as the sum of *F* and *L* alleles in each cell line. As indicated earlier, no cell lines with the *ffSS* hVDR genotype are included in the cell lines studied; hence, no cell line with a score of zero could be evaluated. The average of the group with a score of 4 ($P = 0.035$) as assessed by the two-tailed Student's *t*-test. (B) The data from (A) were plotted as individual points instead of the average of each group, with each point representing a single cell line. The linear regression fit for all 20 points is represented by a dotted line. The regression line for an apparent 'high' group (●, $n = 8$) is shown as a dashed line, while the remaining lines appear to form a 'low' group (□, $n = 12$), with its regression fit illustrated as a solid line.

again resembles published results for Caucasians (59%), but is less than that noted for Hispanics (69%), African-Americans (71%), Japanese-Americans (91%), and Chinese (91%) (Ingles et al., 1997a).

The assignment of *F* as the more active hVDR allele is based not only on the current analysis of fibroblast lines, but also on in vitro data collected with *F* and *f* proteins expressed in transfected cells [Fig. 7 and (Arai et al., 1997; Jurutka et al., 2000)]. Another group (Colin et al., 2000) has also studied *F* versus *f* hVDR proteins, and reported a lower ED₅₀ for $1,25(\text{OH})_2\text{D}_3$ with the *F* allele. These data indicating a more active *F* hVDR allele are consistent with a number of epidemiological studies which suggest that the *F* allele, when compared with the *f* allele, is associated with increased BMD (Gross et al., 1996; Arai et al., 1997; Harris et al., 1997; Tao et al., 1998; Ferrari et al., 1999; Lucotte et al., 1999), higher rates of bone turnover (Kurabayashi et al., 1999), lower risk for primary hyperparathyroidism (Correa et al., 1999; Sosa et al., 2000), lower risk for intervertebral disc degeneration (Videman et al., 1998) and lower incidence of vertebral fracture (Gennari et al., 1999). However, it should be acknowledged that not all studies have found these associations. For instance, one group (Eccleshall et al., 1998) did not observe a correlation between hVDR genotype and BMD in a large cohort of French women. Also, another group (Gross et al., 1998) was unable to correlate any hVDR-related functional parameter with *F/f* genotype in either cells transfected with vectors expressing *F* versus *f* hVDRs, or in a small panel of human fibroblast lines, although perhaps because of methodological limita-

tions, small differences in activity may have escaped detection. Additionally, as discussed above, the fact that *only* the *F/f* genotype was considered in the above investigations implies that the *L/S* genotype could have been a significant confounder in these studies.

The assignment of *L* hVDR as more active than *S* is based exclusively on observations with the present panel of fibroblast cell lines. *L* and *S* hVDR alleles do not produce different proteins, and, therefore, cannot be tested in the same fashion as *F/f* isoforms. As a provocative test of this assignment, we instead entertained the alternative hypothesis that *S* hVDR alleles are more active, and replotted the data in Fig. 8A. However, the plot of this modified allele score (*F+S*) versus transcriptional activity appeared to show an inverse correlation between hVDR allele score and bioactivity (data not shown). This exercise, plus the present data (Fig. 6 and Fig. 8), strongly support the premise that *L* is the more active hVDR allele. Since this polymorphism occurs in exon IX, but is expressed only in the 3' UTR of hVDR mRNA, the working hypothesis presented herein states that the *L* allele may produce receptor mRNA that is more stable and/or is translated more efficiently into hVDR protein than the *S* allele.

Pertinent to the hypothesis above, the mRNA stabilities of allelic variants in or adjacent to exon IX [i.e., considering one or more sites in the *Bsm/Taq/Apa*/singlet(A) cluster] have been evaluated in recent studies using various strategies (Morrison et al., 1994; Mocharla et al., 1997; Verbeek et al., 1997; Carling et al., 1998; Gross et al., 1998; Durrin et al., 1999).

Mirroring the epidemiological investigations, these studies have yielded conflicting results. In one set of experiments (Verbeek et al., 1997), lymphocytes heterozygous for the *Taq* polymorphism were examined. It was found that, whereas mRNA stabilities were similar for both alleles, the mRNA from the *t* allele (linked to *S*) was consistently 30% less abundant. This result was interpreted as suggesting a possible difference in transcriptional regulation between the two allelic forms studied, although no mechanism for this effect was proposed (Verbeek et al., 1997). In contrast, another study (Carling et al., 1998) examining pituitary adenomas from 42 patients showed that *B* and *t* alleles were associated with higher hVDR mRNA levels, reaching statistical significance when homozygous *BB* or *tt* lines were compared with *bb* or *TT* homozygotes. Results similar to those of Carling et al. were obtained by others (Morrison et al., 1994), using a heterologous system in which 3.2 kb of 3' UTR from two subjects homozygous for either *BAtS* or *baTL* were linked to a luciferase reporter gene. In transfected COS-7 cells, the *BAtS* construct displayed higher luciferase activity, suggesting to these authors that either transcriptional activity of the construct itself, or mRNA stability of its transcript, were more favorably affected by attachment of the *BAtS* as opposed to the *baTL* 3' UTR (Morrison et al., 1994). Finally, three further groups reported no significant effect of 3' UTR allelic variants on hVDR mRNA. These studies examined *B* versus *b* mRNA abundance in blood monocytes (Mocharla et al., 1997), *B* versus *b* hVDR protein and mRNA abundance in cultured skin fibroblasts (Gross et al., 1998), and stability of globin mRNAs attached to *L* or *S* 3' UTRs in transfected NIH3T3 cells (Durrin et al., 1999). In particular, the last study, in which methodology similar to that of Morrison et al. was used, strongly suggests that the *baTL* and *BAtS* 3' UTRs do not confer different mRNA stabilities, at least when attached to a heterologous (rabbit β globin) mRNA (Durrin et al., 1999).

Drawing conclusions from the above investigations with respect to the *L/S* polymorphism must be done cautiously, since only two of the six studies cited above (Morrison et al., 1994; Durrin et al., 1999) actually determined *L/S* genotype in their subjects. Nevertheless, given the reasonably tight linkage between *Bsm I* and singlet(A) polymorphisms (Ingles et al., 1997a), the above discussed results, when taken together, do suggest that mRNA stability may not be a major mechanism distinguishing the activity of *L* versus *S* alleles.

The possibility remains, however, that the *L* allele in some fashion produces more VDR protein from a given unit of mRNA. While there is a paucity of data to support or refute such a conclusion, it is notable that ligand binding assays (Gross et al., 1998) seem to indicate a trend toward higher VDR abundance (ex-

pressed as N_{\max}) in *bb* versus *BB* fibroblast lines, although these differences were not statistically significant. Should it be the case that *L* alleles (linked to *b*) produce more hVDR protein, what could be the mechanism for such an effect? Recent observations regarding mammalian and yeast poly(A) binding proteins (PABPs) indicate that binding of PABP to mRNA enhances translatability of mRNAs via an interaction with other proteins that interact with the 5' end of the message (Munroe and Jacobson, 1990; Le et al., 1997). Usually, multiple PABP monomers bind to poly(A)⁺ RNAs, with each monomer occupying approximately 27 adenylate residues (Baer and Kornberg, 1980). Intriguingly, further studies with human PABP suggest that as few as 11 consecutive A's can bind to PABP, with 25 A's giving maximum affinity (Deo et al., 1999). Thus, one could speculate that: (a) PABP may be capable of binding to the singlet(A) repeat in the hVDR gene; and (b) its ability to bind may be enhanced in long (*L*) alleles (17–24 A's) versus short (*S*) alleles (10–15 A's). Greater association of PABP with *L* alleles would then lead to more efficacious translation via a more potent interaction with translation factors such as EF-4B (Le et al., 1997).

Regardless of the potential mechanism, the endogenous VDR activities, as measured in the present experiments using a 1,25(OH)₂D₃-responsive reporter plasmid, showed a surprising range of activities, from 1.75- to 100-fold induction by 1,25(OH)₂D₃. It is asserted that the current results are valid when comparing cell lines within the studied group, particularly because the data were normalized for transfection efficiency as monitored by inclusion of an expression plasmid for β -galactosidase. It is possible, nonetheless, that the presence of four closely-spaced vitamin D responsive elements in the (CT4)₄TKGH reporter construct used could exaggerate small differences in activity that may be much more subtle under in vivo conditions, especially considering that natural promoters often possess a single, or at most, two vitamin D responsive elements (Haussler et al., 1998). Indeed, the results with the BGP-TKGH, which contains a single VDRE element, revealed more modest transactivation levels in transfected cells (see Fig. 7A) and a less dramatic difference between *F* versus *f* hVDR activity.

The absence in the current sample set of a cell line with an allele score of zero (i.e. *ffSS*) is notable because it did not allow an evaluation of the activity of this genotype. The obvious explanation for this absence is the fact that, since hVDR *ff* and *SS* homozygotes are relatively uncommon among Caucasians (19 and 15%, respectively), the combination of *ffSS* would, therefore, represent a rare genotype (estimated frequency about 3% in Caucasians). Given the observed correlation between allele score and fold-induction by 1,25(OH)₂D₃ (Fig. 8B), it is predicted that cells with this genotype

would have very low relative transcriptional activity. A recent epidemiological study (Hutchinson et al., 2000), in which a large cohort with malignant melanoma was genotyped at the *T/t* and *F/f* loci, supports this conclusion. It was found that *fftt* (recall that *t* is often linked to *S*) had significantly thicker tumors ($P = 0.001$). These results intimate that the *fftt* allele combination might be associated with less active hVDR, as VDR has been reported to have antiproliferative effects that might be expected to counter the malignant phenotype (Haussler et al., 1998). Clearly, more fibroblast lines must be examined to answer these questions, and it will be interesting to determine if *ffSS* hVDR cell lines actually possess very low hVDR activity. Should this be the case, the provocative possibility could be raised that this rare *ffSS* hVDR genotype might be so disadvantageous with respect to calcium and bone metabolism that it has been sharply reduced in the gene pool.

The two fibroblast lines in the present panel from patients with William's Syndrome exhibited very different hVDR activities, with the endogenous VDR in the DWF-CV line displaying extremely high (100-fold) induction by 1,25(OH)₂D₃, and the DWF-TW line displaying near-average activity (23-fold induction). The extremely high activity of the DWF-CV line (three standard deviations above the mean of 28 ± 24 fold-induction) suggests a possible association with the hypercalcemia often seen in patients with William's Syndrome. However, because the great majority of William's Syndrome cases involve a chromosomal deletion at 7q11.23 (Ewart et al., 1993), and the hVDR gene resides on chromosome 12, this syndrome would appear to be unrelated to VDR action (as seems to be the case with patient DWF-TW). However, it is conceivable that patient DWF-CV, whose fibroblasts exhibit extremely elevated VDR activity, may represent an atypical William's case that does involve increased sensitivity to 1,25(OH)₂D₃, a mechanism that has already been speculated for isolated cases resembling Williams's Syndrome (Ghirri et al., 1999).

Concerning the three osteoporotic patients in the current sample set, two fibroblast lines showed normal hVDR activity, but a third (Ran Nor, from a 69-year-old male) displayed very low induction of the reporter gene (6.2-fold versus the average of 28-fold). The low activity of hVDR in the Ran Nor cell sample could, in theory, be related to the low bone density of osteoporosis. However, osteoporosis is a multifactorial disease and, therefore, a very large study would be required to test any relationship between its etiology and hVDR alleles.

The lowest induction of the tested reporter construct by 1,25(OH)₂D₃ (1.75-fold) was observed in the transfected cell line HS 144.We, taken from a 5-week-old embryo. These cells represent one of two prenatal cell lines in the present panel; thus, one possible explana-

tion for the very low hVDR activity is that cells from this early gestational stage may not yet be differentiated sufficiently to express VDR at levels seen postnatally. However, the other fetal cell line, HE-SK (exact fetal age unknown), displayed a slightly above average induction of 35.4-fold. Further arguing against the above interpretation is the observation from this laboratory that, with the exception of tissues like intestine that are phenotypically responsive to vitamin D in the adult, VDR expression has actually been shown to diminish in rat and chick tissues such as muscle and liver when they mature beyond the embryonic stage (M.R. Haussler and K. Yamaoka, unpublished data). Other groups have also observed VDR expression in various embryonic and fetal tissues (Takeuchi et al., 1994; Johnson et al., 1995; Delvin et al., 1996; Johnson et al., 1996; Veenstra et al., 1998; Segura et al., 1999). Finally, given that the HS 144.We line has an allele score of 1 (genotype *FfSS*), its observed fold-induction of 1.75 resides between a predicted value of 5.3-fold stimulation by 1,25(OH)₂D₃ using the regression line for all 19 other lines, and a prediction of negligible stimulation (i.e. close to 1.0-fold) for the 'low group' regression line (both values calculated from plots similar to that of Fig. 8B, but omitting the HS 144.We data point). Thus, the observed HS 144.We hVDR transcriptional activity is not outside the predicted range for its allele score. Regardless, the data do not rule out early developmental-stage variations in human VDR expression, a topic that deserves further study.

The presence of two distinct groupings of hVDR activity versus allele score at the *F/f* plus *L/S* loci in Fig. 8B argues in favor of the existence of another variable that influences innate hVDR activity, at least in fibroblasts. There are a number of potential variables to consider, including such parameters as age and sex of the patients from whom the cells were taken. An analysis of these variables in the current sample set reveals that, although the gender distribution between the high and low groups of Fig. 8B is similar (60 and 50% male, respectively), the average age is somewhat higher in the low group, but this difference is without statistical significance (20.9 versus 4.8 years, $P = 0.11$). Thus, based on the current data, it is contended that age and sex of the cell donors would not be satisfactory explanations for the existence of a high and a low activity group.

Instead, the hypothesis put forth in the present communication (Fig. 9) is that there exists one or more additional polymorphic variations in the hVDR gene beyond those at the *F/f* locus and in the 3' cluster (*Apa/Bsm/Taq* and *L/S*) that affect(s) hVDR activity. The coding exons of the hVDR gene have been studied rather extensively, and have yielded numerous point mutations causing hereditary vitamin D resistant rickets (Hawa et al., 1996; Lin et al., 1996; Whitfield et al.,

1996; Haussler et al., 1998), but no evidence has been presented for the occurrence of common polymorphic sites other than the *Fok I* site in exon II and the linked cluster of sites in the exon VIII–IX region. Yet recent investigations into the portion of the hVDR gene encoding the 5' untranslated region (5' UTR) have revealed a surprising complex of at least seven exons (denoted IA-IG, see Fig. 9, top left), with evidence for alternative splicing (Miyamoto et al., 1997; Crofts et al., 1998). It is possible that undiscovered common polymorphic sites may exist in this newly described complex of multiple exons at the 5' end of the gene. Such polymorphisms could even alter VDR protein structure by introducing in-frame initiator methionine codons leading to the expression of VDRs with N-terminal extensions, as already proposed by one research group (Crofts et al., 1998).

Still another possibility is that polymorphisms in the 5' region of the VDR gene might affect the activity of one of the three proposed hVDR promoters (Crofts et al., 1998), leading to the expression of altered quantities of VDR proteins under physiologic conditions. Here again, a precedent exists from a recent report (Arai et al., 1999 J. Bone Miner. Res. 14, S191, Abstract T084), which describes a polymorphism in a binding site for

Cdx-2, a homeodomain protein related to *caudal* (position of Cdx binding site in the hVDR gene is shown in Fig. 9, top left). The importance of Cdx-2 for intestine-specific expression of hVDR was demonstrated earlier (Yamamoto et al., 1999). It is, of course, not possible to invoke this polymorphism to explain the present results in fibroblast lines, given the intestine-specific nature of Cdx-2 regulated expression (Suh et al., 1994). However, the existence of this polymorphism should certainly be considered in epidemiological studies relating VDR-mediated intestinal absorption of calcium and phosphate as they impact BMD and parathyroid gland function. Indeed, in a large cohort of Japanese women, the *A* allele at the Cdx-2 locus correlated with higher BMD in the lumbar spine, consistent with a slightly greater activity of a VDR promoter construct incorporating the Cdx-*A* type element (Arai et al., 1999, J. Bone Miner. Res. 14, S191, Abstract T084).

Rather than evoking Cdx-2 or any other known polymorphism to explain the present results, we prefer the interpretation that a novel polymorphism exists, likely located in the incompletely characterized 5' region of the hVDR gene. A full recognition of the genetic complexity of VDR action in humans may eventually allow for accurate prediction of VDR activ-

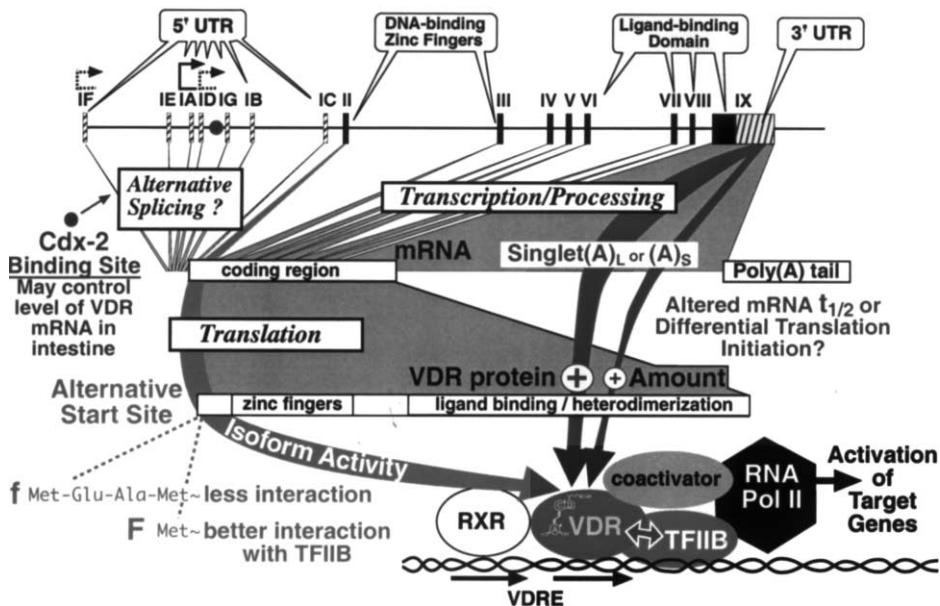


Fig. 9. Summary of common polymorphic variations in the hVDR gene that may influence VDR activity. The exon arrangement (top) is described in Fig. 2 and the associated text. The long (*L*) variant of the singlet(A) repeat in exon IX is proposed herein to be associated with increased VDR amount. A speculative mechanism for this effect is the enhanced ability of ≥ 17 consecutive As to recruit poly(A) binding protein, which would presumably stabilize VDR mRNA and/or promote its translation into hVDR protein (see text). The *F/f* polymorphism in exon II has likewise been shown to be associated with VDR activity, with the *F* isoform displaying both moderately higher transcriptional activity, as well as measurably greater interaction, *in vitro*, with TFIIB (Jurutka et al., 2000). Recently, a third hVDR polymorphism has been described upstream of exon IG in a binding site for the intestine-specific enhancer protein Cdx-2 (Arai et al., 1999, J. Bone Miner. Res. 14, S191, Abstract T084). This polymorphism may be significant for its effects on hVDR expression in intestinal cells (Yamamoto et al., 1999). All three of the illustrated polymorphisms are proposed to affect either the quantity of expressed VDR in human tissues (Cdx-2 and *L/S*), or the ability of VDR to interact with other proteins such as TFIIB (*F/f*, as illustrated at bottom right), in order to impact the transcription of target genes. Finally, it is suggested that other functionally relevant VDR gene polymorphisms may exist, especially in the recently-described complex of exons encoding the 5'-UTR.

ity in individual patients based on genotype, along with an enhanced ability to assess disease risk, as well as response to pharmacologic agents related to VDR action.

Acknowledgements

This work was supported by National Institutes of Health grants to Mark R. Haussler.

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Modeling of Environmental Effects in Genome-Wide Association Studies Identifies *SLC2A2* and *HP* as Novel Loci Influencing Serum Cholesterol Levels

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Abstract

Genome-wide association studies (GWAS) have identified 38 larger genetic regions affecting classical blood lipid levels without adjusting for important environmental influences. We modeled diet and physical activity in a GWAS in order to identify novel loci affecting total cholesterol, LDL cholesterol, HDL cholesterol, and triglyceride levels. The Swedish (SE) EUROSPAN cohort ($N_{SE} = 656$) was screened for candidate genes and the non-Swedish (NS) EUROSPAN cohorts ($N_{NS} = 3,282$) were used for replication. In total, 3 SNPs were associated in the Swedish sample and were replicated in the non-Swedish cohorts. While SNP rs1532624 was a replication of the previously published association between CETP and HDL cholesterol, the other two were novel findings. For the latter SNPs, the *p*-value for association was substantially improved by inclusion of environmental covariates: SNP rs5400 ($p_{SE,\text{unadjusted}} = 3.6 \times 10^{-5}$, $p_{SE,\text{adjusted}} = 2.2 \times 10^{-6}$, $p_{NS,\text{unadjusted}} = 0.047$) in the *SLC2A2* (Glucose transporter type 2) and rs2000999 ($p_{SE,\text{unadjusted}} = 1.1 \times 10^{-3}$, $p_{SE,\text{adjusted}} = 3.8 \times 10^{-4}$, $p_{NS,\text{unadjusted}} = 0.035$) in the *HP* gene (Haptoglobin-related protein precursor). Both showed evidence of association with total cholesterol. These results demonstrate that inclusion of important environmental factors in the analysis model can reveal new genetic susceptibility loci.

Citation: Igl W, Johansson Å, Wilson JF, Wild SH, Polašek O, et al. (2010) Modeling of Environmental Effects in Genome-Wide Association Studies Identifies *SLC2A2* and *HP* as Novel Loci Influencing Serum Cholesterol Levels. PLoS Genet 6(1): e1000798. doi:10.1371/journal.pgen.1000798

Editor: Paolo Gasparini, IRCCS Burlo Garofolo, University of Trieste, Italy

Received July 29, 2009; **Accepted** December 3, 2009; **Published** January 8, 2010

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Funding: The European Special Populations Research Network (EUROSPAN) was supported by European Commission FP6 STRP grant number 018947 (LSHG-CT-2006-01947). High-throughput genome-wide association analysis of the data was supported by joint grant from Netherlands Organisation for Scientific Research and the Russian Foundation for Basic Research (NWO-RFBR 047.017.043). Lipidomic analysis was supported by the European Commission FP7 grant LipidomicNet (2007-202272). The NSPHS study was supported by grants from the Swedish Natural Sciences Research Council, the European Commission through EUROSPAN, the Foundation for Strategic Research (SSF), and the Linneaus Centre for Bioinformatics (LCB). The ORCADES study was supported by the Scottish Executive Health Department and the Royal Society. DNA extractions were performed at the Wellcome Trust Clinical Research Facility in Edinburgh. The VIS study in the Croatian island of Vis was supported through the grants from the Medical Research Council UK to HC, AW, and IR and the Ministry of Science, Education, and Sport of the Republic of Croatia to IR (number 108-1080315-0302). The MICROS study was supported by the Ministry of Health and Department of Educational Assistance, University and Research of the Autonomous Province of Bolzano and the South Tyrolean Sparkasse Foundation. The ERF study was supported by grants from the NWO, Erasmus MC, and the Centre for Medical Systems Biology (CMSB). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Genome-wide association studies (GWAS) have identified more than 38 larger genetic regions which influence blood levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) [1–3]. These studies modeled basic anthropometric confounders, such as sex and age, while leaving out important

environmental influences, such as diet and activity. This strategy is statistically suboptimal since the unexplained variation in the phenotype can increase the measurement error and as a result require larger sample sizes to detect a significant effect. Manolio [4] argued strongly for modeling of environmental covariates in GWAS and recommended lipid levels as a paradigmatic phenotype for studying the genetic and environmental architecture of quantitative traits.



Author Summary

In this article we report a genome-wide association study on cholesterol levels in the human blood. We used a Swedish cohort to select genetic polymorphisms that showed the strongest association with cholesterol levels adjusted for diet and physical activity. We replicated several genetic loci in other European cohorts. This approach extends present genome-wide association studies on lipid levels, which did not take these lifestyle factors into account, to improve statistical results and discover novel genes. In our analysis, we could identify two genetic loci in the *SLC2A2* (Glucose transporter type 2) and the *HP* (Haptoglobin-related protein precursor) gene whose effects on total cholesterol have not been reported yet. The results show that inclusion of important environmental factors in the analysis model can reveal new insights into genetic determinants of clinical parameters relevant for metabolic and cardiovascular disease.

In order to explore the usefulness of including both environmental and genetic factors in the analysis model, we used lipid measurements from the EUROSPAN study, comprising 3,938 individuals for whom genome-wide SNP data ($N_{SNP} = 311,388$) were available [5]. We measured daily intake of food and physical activity at work and at leisure and modeled the influence of those environmental covariates on serum lipid levels in a GWAS. First, data from the Northern Sweden Population Health Study (NSPHS) were used as a discovery cohort to screen for SNPs that displayed the lowest *p*-values when the model was adjusted for environmental covariates. We then used the other, non-Swedish EUROSPAN cohorts for replication of our strongest associations in a candidate gene association study (CGAS).

We chose a population living in northern Sweden for the selection of candidate loci because it shows strong natural heterogeneity in certain lifestyle factors (e.g. diet, activity), but homogeneity in other environmental aspects such as climate [6]. Whereas one group is living a modern, sedentary lifestyle found also in the southern part of Sweden and other western European countries, a subgroup of Swedes follows a traditional, semi-nomadic way of life based on reindeer herding. Reindeer herders typically show higher intake of game meat (reindeer, moose), which has a high protein and low fat content, and lower intake of non-game meat, fish, and dairy products among other, lesser differences. They also exert more physical activity at work to tend their reindeer herds, but less activity at leisure [7].

Results

Exploratory GWAS in NSPHS

We performed a GWAS with a lifestyle-adjusted model which included not only sex and age, but also daily intake of game meat, non-game meat, fish, milk products, physical activity at work and at leisure as covariates. We focused on the 0.05% of all SNPs with the lowest *p*-values in the diet- and activity-adjusted model (corresponding to about 150 SNPs per lipid). For total cholesterol, 88 of these were located in a gene and 14 in genes that have been associated with energy metabolism (<http://www.ncbi.nlm.nih.gov/omim/>). For LDL-C, 65 SNPs were located in a gene, of which 8 were functionally relevant. Several of the SNPs for LDL-C were identical with those affecting total cholesterol, as expected from the high correlation ($r = 0.91$) between both phenotypes. For HDL-C, SNP rs2292883, located in the *MLPH* gene (Melanophosphorylase), showed a genome-wide significant *p*-value ($p = 1.06 \times 10^{-07}$).

69 SNPs for HDL-C were located in a gene and 14 of those genes were reported as having a metabolic effect. Finally, for triglycerides, 63 SNPs were located in a gene, but only 4 SNPs in genes with a functional annotation of interest (Table 1 and Table S1A, S1B, S1C, S1D).

P-value changes

In order to evaluate the effect of including diet and activity covariates in the association analysis, we overlaid the *p*-values in the Manhattan plots from the NSPHS for the unadjusted and adjusted GWAS models (Figure 1, Figure 2, Figure 3, Figure 4). More refined GWAS results separating the effect of adjusting for either diet or physical activity are presented in Figure S1A, S1B, S1C, S1D; and Figure S2A, S2B, S2C, S2D. As expected, the *p*-values for a number of SNPs were sensitive to the inclusion of both diet and activity covariates in the model. We matched the 0.05% SNPs with the lowest *p*-values (top SNP list) between the unadjusted and the adjusted model. For TC, 83 (53%) SNPs were found in both top SNP lists. Those lists contained 102 (64%) identical SNPs for LDL-C and 103 (65%) for HDL-C. The analyses resulted in the same 74 (47%) top SNPs for TG levels (Table S1A, S1B, S1C, S1D). Finally, we compared the *p*-value changes of the resulting 39 candidate SNPs that are located in genes with a metabolic effect between the diet and activity-adjusted (full) model and the unadjusted (restricted) model resulting in an up to 27-fold *p*-value decrease (Table 1).

Confirmatory CGAS in EUROSPAN

A food- and activity-adjusted candidate gene association study of the final 39 candidate SNPs in the Scottish (SC) sample ($N = 714$) was applied using similar lifestyle covariates (Table 2; Table S1E, S1F, S1G, S1H; Table S2). We replicated the effect of rs2000999 ($p_{SC,unadj} = 6.16 \times 10^{-03}$, $p_{SC,adj} = 4.33 \times 10^{-03}$) in the *HP* gene (Haptoglobin-related protein Precursor) on TC level and the effect of rs1532624 ($p_{SC,unadj} = 2.40 \times 10^{-09}$, $p_{SC,adj} = 1.96 \times 10^{-09}$) in *CETP* (Cholesteryl ester transfer protein) on HDL-C. In the Swedish cohort (SE), the unadjusted genetic effect of rs2000999 in the *HP* gene is equivalent to a moderately large difference in average TC level of 20.21 mg/dl between the homozygous genotypes ($Mean_{SE,unadj}[TC|A/A] - Mean_{SE,unadj}[TC|G/G] = 243.16 - 222.95$, Effect Size_{SE,unadj} = 0.41, Effect Size_{SE,adj} = 0.44)(Effect Size (ES) = $(M_{A/A} - M_{B/B})/SD_{pooled}$). Equivalent effects were observed in the Scottish replication sample ($M_{SC,unadj}[TC|A/A] - M_{SC,unadj}[TC|G/G] = 235.36$ mg/dl – 222.54 mg/dl = 12.82 mg/dl, ES_{SC,unadj} = 0.29, ES_{SC,adj} = 0.52). SNP rs1532624 in the *CETP* gene is associated with a large, unadjusted difference in HDL-C level of 9.99 mg/dl ($M_{SE,unadj}[HDL-C|A/A] - M_{SE,unadj}[HDL-C|C/C] = 68.14$ mg/dl – 58.15 mg/dl, ES_{SE,unadj} = 0.73, ES_{SE,adj} = 0.48) in the discovery cohort and similar effects regarding direction and size in the replication cohort ($M_{SC,unadj}[HDL-C|A/A] - M_{SC,unadj}[HDL-C|C/C] = 69.79$ mg/dl – 60.75 mg/dl = 9.04 mg/dl, ES_{SC,unadj} = 0.59, ES_{SC,adj} = 0.57).

We also performed an unadjusted candidate gene analysis of the 39 candidate SNPs in all non-Swedish (NS) EUROSPAN cohorts (Scotland, Croatia, The Netherlands, and Italy, $N_{NS} = 3,282$) and aggregated the results in a meta-analysis (Table 2; Table S1I, S1J, S1K, S1L). We confirmed the effects of rs5400 ($p_{NS} = 4.68 \times 10^{-02}$) in *SLC2A2* on TC. We again found that rs2000999 ($p_{NS,unadj} = 3.54 \times 10^{-2}$) in *HP* influences TC levels and rs1532624 ($p_{NS,unadj} = 2.87 \times 10^{-20}$) in *CETP* (Cholesteryl ester transfer protein) affects HDL-C levels. The unadjusted genetic effect of rs5400 is equivalent to a moderately large difference in mean TC level of 27.11 mg/dl between homozygous genotypes ($M_{SE,unadj}[TC|A/A] - M_{SE,unadj}[TC|G/G] = 249.30$ mg/dl – 222.95 mg/dl, ES_{SE,unadj} = 0.11, ES_{SE,adj} = 0.07).

Table 1. Candidate SNPs ($n=39$) selected from the Swedish discovery cohort.

SNP	p-value, unadjusted ^a	p-value, adjusted ^b	p-value ratio ^c	Gene Symbol	Product name (Product Symbol)
TC					
rs10513684	2.91E-05	1.08E-06	27	SLC2A2	Glucose transporter type 2 (GLUT-2)
rs1684885	3.47E-03	2.01E-04	17	PRKCI	Protein kinase C iota type (nPKC-iota)
rs5400	3.57E-05	2.18E-06	16	SLC2A2	Glucose transporter type 2 (GLUT-2)
rs47137	3.69E-03	2.63E-04	14	SLC2A12	Glucose transporter type 12 (GLUT-12)
rs669552	3.46E-03	2.87E-04	12	FNDC3B	Factor for Adipocyte Differentiation 104
rs2303324	1.49E-03	1.65E-04	9	GALNT14	Polypeptide GalNAc transferase 14
rs12617790	2.26E-03	2.53E-04	9	GALNT14	Polypeptide GalNAc transferase 14
rs10041333	3.04E-03	3.74E-04	8	FABP6	Gastrotropin (GT), alt. Fatty Acid-Binding Protein 6
rs222014	2.93E-03	3.71E-04	8	GC	Vitamin D-binding protein Precursor (DBP)
rs2000999	1.12E-03	3.84E-04	3	HP	Haptoglobin-related protein Precursor
rs2070657	1.70E-04	1.43E-04	1	APP	Alzheimer disease amyloid protein (ABPP)
rs2186830	2.66E-04	2.78E-04	1	COLEC12	Collectin-12
rs2478571	3.07E-04	4.42E-04	1	SLC39A12	Zinc transporter (ZIP12)
LDL-C					
rs1684885	1.53E-04	1.61E-05	10	PRKCI	Protein kinase C iota type (nPKC-iota)
rs1684881	2.80E-04	3.14E-05	9	PRKCI	Protein kinase C iota type (nPKC-iota)
rs10513684	1.98E-04	2.61E-05	8	SLC2A2	Glucose transporter type 2 (GLUT-2)
rs5400	4.22E-04	6.10E-05	7	SLC2A2	Glucose transporter type 2(GLUT-2)
rs12617790	1.65E-03	2.96E-04	6	GALNT14	Polypeptide GalNAc transferase 14
rs7583934	2.78E-04	1.84E-04	2	LRP1B	Low-density lipoprotein receptor-related protein (LRP-DIT)
rs1864616	8.64E-05	1.36E-04	2	TGFB2	Transforming growth factor-beta receptor type II (TGFR-2)
rs843319	6.74E-05	2.89E-04	4	MBOAT1	O-acyltransferase domain-containing protein 1
HDL-C					
rs2292883	1.48E-06	1.06E-07*	14	MLPH	Melanophilin
rs12712846	1.14E-03	3.02E-04	4	MTA3	Metastasis-associated protein
rs365578	8.39E-04	2.92E-04	3	NDUFS4	NADH dehydrogenase 8 iron-sulfur protein 4 (CI-AQDQ)
rs9866473	4.34E-04	2.55E-04	2	CETP	Cholesteryl ester transfer protein
rs10519336	6.34E-04	3.82E-04	2	MCC	Colorectal mutant cancer protein
rs2054247	3.73E-05	3.70E-05	1	APL2	Amyloid-like protein 2 Precursor
rs11708205	2.67E-04	3.57E-04	1	PLD1	Phospholipase D1
rs9863761	5.20E-06	7.74E-06	1	CETP	Cholesteryl ester transfer protein
rs2124147	1.49E-04	2.23E-04	2	CETP	Cholesteryl ester transfer protein
rs1567385	1.32E-04	1.99E-04	2	MAP4K4	Mitogen-activated protein kinase 4 (MEKK4)
rs3776817	6.82E-05	1.31E-04	2	ADAMTS2	Procollagen I N-proteinase
rs1999088	7.19E-05	1.51E-04	2	MBNL2	Muscleblind-like protein 2
rs1782644	1.39E-04	3.10E-04	2	ZMIZ1	Zinc finger MIZ domain-containing protein 1
rs1532624	1.06E-06	2.55E-06	2	CETP	Cholesteryl ester transfer protein
TG					
rs4304239	1.63E-03	2.40E-04	7	IGF2BP3	Insulin-like growth factor 2 mRNA-binding protein 3
rs11770192	1.82E-03	2.40E-04	8	IGF2BP3	Insulin-like growth factor 2 mRNA-binding protein 3
rs12540730	7.79E-04	2.43E-04	3	IGF2BP3	Insulin-like growth factor 2 mRNA-binding protein 3
rs3823763	9.58E-05	4.45E-05	2	BBS9	Parathyroid hormone-responsive B1 gene protein (PTHB1)

All candidate SNPs show strongest associations (p-value, top 0.05% SNPs per lipid trait) and are located in a gene which has been reported to be relevant for energy metabolism. SNPs are sorted by p-value ratio (unadjusted:unadjusted).

* $p \leq 1.6E-07$ = genome-wide significant; All SNPs in genes with at least one replicated SNP are displayed in bold, replicated SNPs are formatted in bold italics. a) unadjusted = covariates include sex and age; b) adjusted = covariates include sex, age, game meat, non-game meat, fish, milk products, physical activity at work and at leisure; c) p-ratio = $\max(p_{\text{unadjusted}}:p_{\text{adjusted}}, p_{\text{adjusted}}:p_{\text{unadjusted}})$; $p_{\text{unadjusted}}:p_{\text{adjusted}}$ ratios are aligned left, $p_{\text{adjusted}}:p_{\text{unadjusted}}$ ratios are aligned right.

doi:10.1371/journal.pgen.1000798.t001

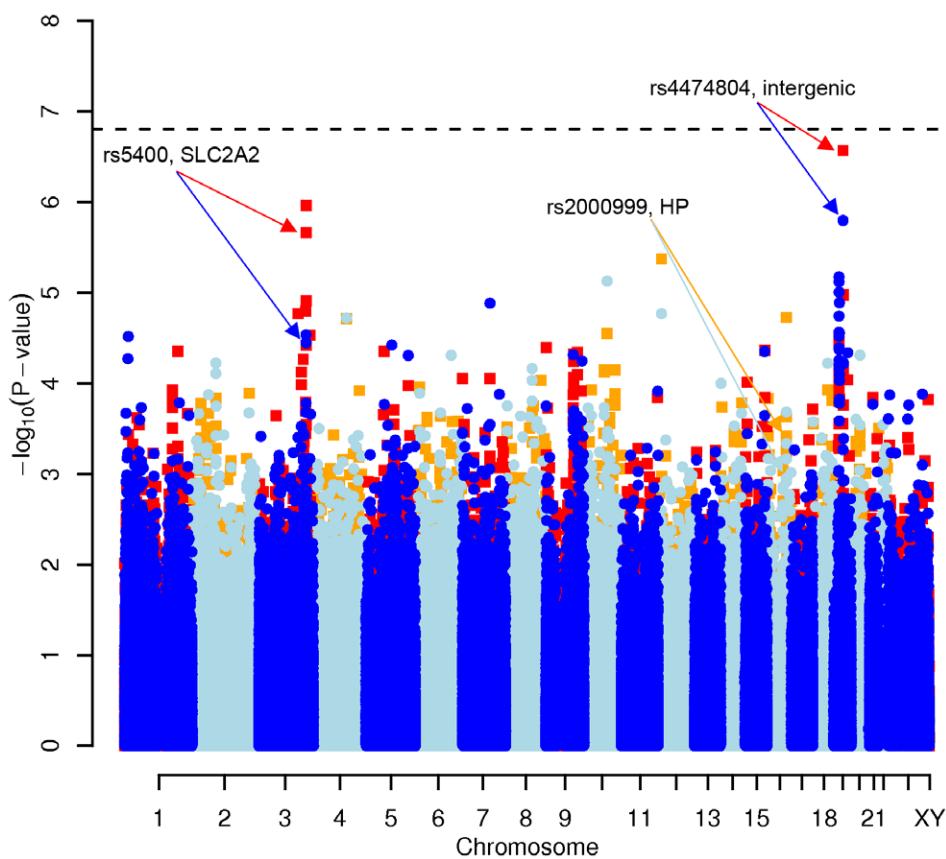


Figure 1. Manhattan plot of genome-wide effects on total cholesterol levels in the Swedish discovery cohort. Results for two GWAS analysis models are presented. The unadjusted model (dark blue and light blue circles) included only sex and age as covariates. The adjusted model (red and orange squares) additionally contained food intake and physical activity as predictors. The dashed line indicates the local Bonferroni-adjusted α error = 1.6×10^{-7} .

doi:10.1371/journal.pgen.1000798.g001

dl–222.19 mg/dl, $ES_{SE,\text{unadj}} = 0.57$, $ES_{SE,\text{adj}} = 0.66$) in the Swedish Cohort and a small total effect in all non-Swedish samples ($M_{NS,\text{unadj}}(\text{TC}|\text{A/A}) - M_{NS,\text{unadj}}(\text{TC}|\text{G/G}) = 236.69 \text{ mg/dl} - 223.34 \text{ mg/dl} = 13.35 \text{ mg/dl}$, $ES_{NS,\text{unadj}} = 0.30$).

No other associations, including LDL cholesterol or triglycerides levels, were replicated (all $p > 0.05$). The genome-wide significant SNP rs2292883 in the Melanophilin (*MLPH*) gene found in the Swedish cohort was not confirmed.

Discussion

Environmental covariates may either act as moderators, mediators or even suppressors, thereby affecting the discovery of genetic susceptibility loci [8,9]. Therefore, we conducted a GWAS, modeling genetic and important environmental effects, such as food intake and physical activity, on serum levels of classical lipids. To our knowledge, this is the first GWAS on blood lipid levels modeling environmental factors, in particular major food categories and physical activity, in international cohorts. Our analysis replicated one known locus in the *CETP* gene [1] and identified two other gene loci in the *SLC2A2* and *HP* gene, respectively, involved in energy metabolism but not previously reported to be associated with cholesterol levels.

SLC2A2 encodes the facilitated glucose transporter member 2 (GLUT-2, Solute carrier family 2) and is predominantly expressed in the liver. Mice deficient in GLUT-2 are hyperglycemic and have elevated plasma levels of glucagon and free fatty acids [10].

Mutations in GLUT-2 cause the Fanconi-Bickel syndrome (FBS) characterized by hypercholesterolemia and hyperlipidemia [11,12]. Cerf [13] argued that a high-fat diet causes a decreased expression of the GLUT-2 glucose receptor on β -cell islets. As a result, glucose stimulation of insulin exocytosis is impaired causing hyperglycemia, a clinical hallmark of type 2 diabetes. In addition, Kilpeläinen et al. [14] found that physical activity moderates the genetic effect of *SLC2A2* on type 2 diabetes. These studies suggest that these lifestyle factors could have masked genetic effects in previous, unadjusted GWAS. This is emphasized by the strong increase in statistical significance of the *SLC2A2* polymorphisms after adjusting for diet and physical activity, indicating that the examined lifestyle factors modified the effect of this gene. Our supplemental results show that physical activity markedly moderated the genetic effect on total cholesterol.

The *HP* gene encodes the Haptoglobin-related Protein Precursor (Hp), which binds hemoglobin (Hb) to form a stable Hp-Hb complex and, thereby, prevents Hb-induced oxidative tissue damage. Asheh et al. [15] identified severe impairment in the ability of Hp to prevent oxidation caused by glycosylated Hb. Diabetes is also associated with an increase in the non-enzymatic glycation of serum proteins, so these authors suggested that there is a specific interaction between diabetes, cardiovascular disease and the Hp genotype. It results from the increased need of rapidly clearing glycosylated Hb-Hp complexes from the subendothelial space before they oxidatively modify low-density lipoprotein to form the atherogenic oxidized low-density lipopro-

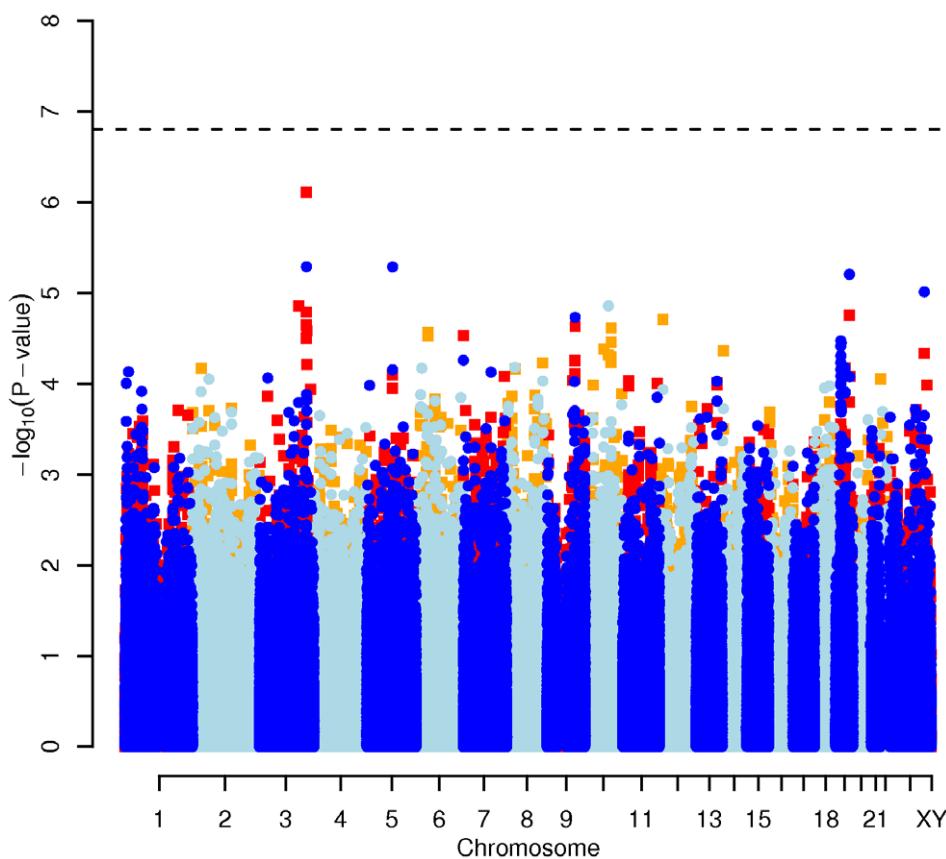


Figure 2. Manhattan plot of genome-wide effects on LDL cholesterol levels in the Swedish discovery cohort. Results for two GWAS analysis models are presented. The unadjusted model (dark blue and light blue circles) included only sex and age as covariates. The adjusted model (red and orange squares) additionally contained food intake and physical activity as predictors. The dashed line indicates the local Bonferroni-adjusted α error = 1.6×10^{-7} .

doi:10.1371/journal.pgen.1000798.g002

tein. The *p*-value for association between the *HP* SNP rs2000999 and total serum cholesterol concentration decreased in the model adjusted for diet and physical activity, suggesting that the genetic effect is moderated by diet and physical activity. Our supporting material points out the moderating role of physical activity in particular.

We also observed a highly significant association between rs1532624 in *CETP* and HDL-C levels. The *CETP* protein catalyzes the transfer of insoluble cholesteryl esters among lipoprotein particles. Variation in *CETP* is known to affect the susceptibility to atherosclerosis and other cardiovascular diseases [16]. Adjustment for diet and physical activity in our model caused an increase of the *p*-value of this SNP. Our supporting results indicate that the genetic effect is mediated by diet or by physical activity in a similar way.

This study also has some limitations. First, we are aware that our candidate gene association approach covers only a very small fraction of all genomic loci, which is one of the potential reasons why some classical lipid-influencing genes, such as *APOE*, are not represented in our candidate SNP list. Therefore, our approach is not comprehensive and may have failed to identify other relevant lifestyle-sensitive genetic variants. Nonetheless, we decided to apply this approach to make the best out of the available lifestyle data. Second, our study provides only limited information on the role of individual lifestyle factors for a genetic variant. However, in this study we aimed at amplifying genetic effects by adjusting for a maximum amount of environmental variance in a single model and,

therefore, we neglected some of these aspects here. Third, we did not model genetic covariates in known lipid-relevant genes which may also moderate the effect of other genetic predictors. This is due to the focus of this paper on gene-environment relationships.

In summary, we have demonstrated that modeling environmental factors, in particular major food categories and physical activity, can improve statistical power and lead to the discovery of novel susceptibility loci. Such models also provide an understanding of the complex interplay of genetic and environmental factors affecting human quantitative traits. Inclusion of environmental covariates represents a much needed next step in the quest to model the complete environmental and genetic architecture of complex traits.

Methods

Ethics statement

All EUROSPAN studies were approved by the appropriate research ethics committees according to the Declaration of Helsinki [17]. The Northern Swedish Population Health Study (NSPHS) was approved by the local ethics committee at the University of Uppsala (Regionala Etikprövningsnämnden, Uppsala). The Scottish ORCADES study was approved by the NHS Orkney Research Ethics Committee and the North of Scotland REC. The Croatian VIS study was approved by the ethics committee of the medical faculty in Zagreb and the Multi-Centre Research Ethics Committee for Scotland. The Dutch ERF study was approved by the Erasmus institutional medical ethics

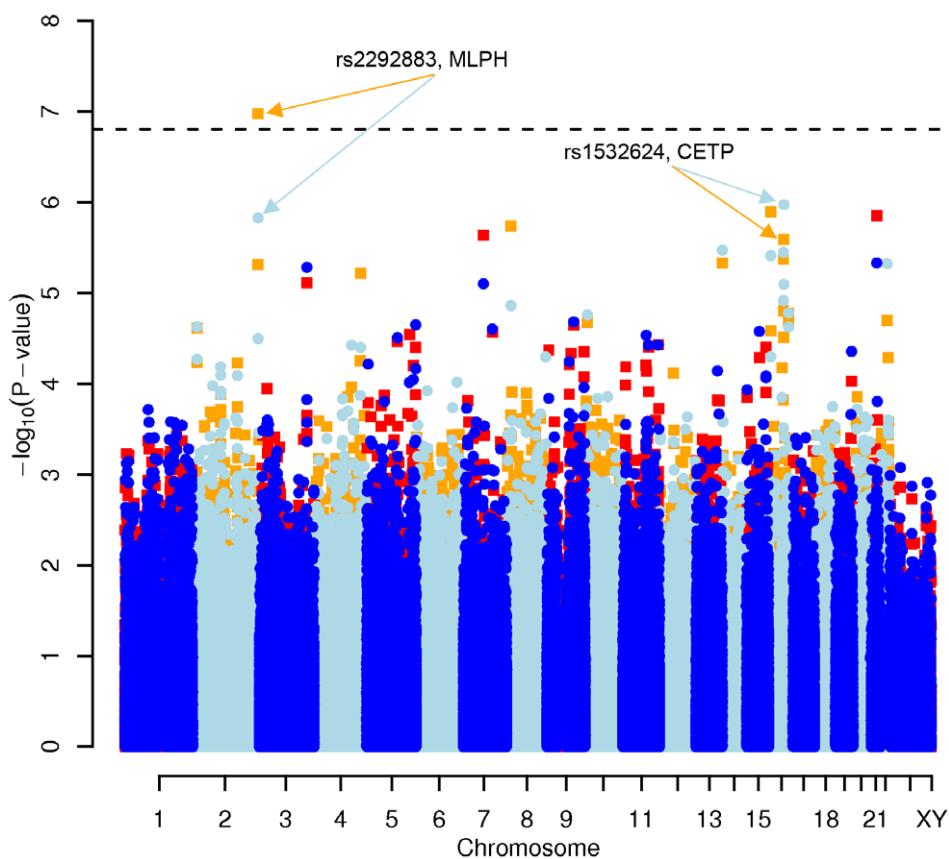


Figure 3. Manhattan plot of genome-wide effects on HDL cholesterol levels in the Swedish discovery cohort. Results for two GWAS analysis models are presented. The unadjusted model (dark blue and light blue circles) included only sex and age as covariates. The adjusted model (red and orange squares) additionally contained food intake and physical activity as predictors. The dashed line indicates the local Bonferroni-adjusted α error = 1.6×10^{-7} .
doi:10.1371/journal.pgen.1000798.g003

committee in Rotterdam, The Netherlands. The Italian MICROS study was approved by the ethical committee of the Autonomous Province of Bolzano, Italy.

Participants

The examined subjects stem from five different population-representative, pedigree-based cohorts from the EUROSPAN consortium (<http://www.eurospan.org>). All studies include a comprehensive collection of data on family structure, lifestyle, blood samples for clinical chemistry, RNA and DNA analyses, medical history, and current health status. All participants gave their written informed consent [18]. A brief description of each population is given below:

The *Northern Swedish Population Health Study* (NSPHS) represents a cross-sectional study conducted in the community of Karesuando in the subarctic region of the County of Norrbotten, Sweden, in 2006 [5]. This parish has about 1500 eligible inhabitants of whom 740 participated in the study. The final sample consisted of 309 men and 347 women who were aged between 14 and 91 years. The inclusion of diet and activity covariates in the analytical model and according missing values reduced the effective sample size by less than 5%.

The *Orkney Complex Disease Study* (ORCADES) is a longitudinal study in the isolated Scottish archipelago of Orkney [19]. Participants from a subgroup of ten islands ($N=719$) were used for the presented analysis. The sample comprised 334 men and 385 women aged between 18 and 100 years. The inclusion of diet

and activity covariates in the analytical model and according missing values reduced the effective sample size by less than 5%.

The VIS study is a cross-sectional study in the villages of Vis and Komiza on the Dalmatian island of Vis, Croatia, and was conducted between 2003 and 2004 [20–22]. 795 participants who had both genotype and phenotypic data available were analysed. This cohort included 328 men and 467 women with an age between 18 and 93 years.

The *Microisolates in South Tyrol Study* (MICROS) is a cross-sectional study carried out in the villages of Stelvio, Vallelunga, and Martello, Venosta valley, South Tyrol, Italy, from 2001 to 2003 [23]. The 1,097 participants (475 males, 622 females, age between 18 and 88 years) presented in this study are those for whom both relevant genotype and phenotype data were available.

The *Erasmus Rucphen Family Study* (ERF) is a longitudinal study on a population living in the Rucphen region, the Netherlands, in the 19th century [24]. Fasting total cholesterol, HDL cholesterol and triglyceride levels were available. LDL cholesterol was estimated using the Friedewald formula [25]. The 918 individuals included in this study consisted of the first series of participants with 354 men and 564 women aged between 18 and 92 years.

Genotyping

DNA samples were genotyped according to the manufacturer's instructions on Illumina Infinium HumanHap300v2 or HumanCNV370v1 SNP bead microarrays. Both arrays have

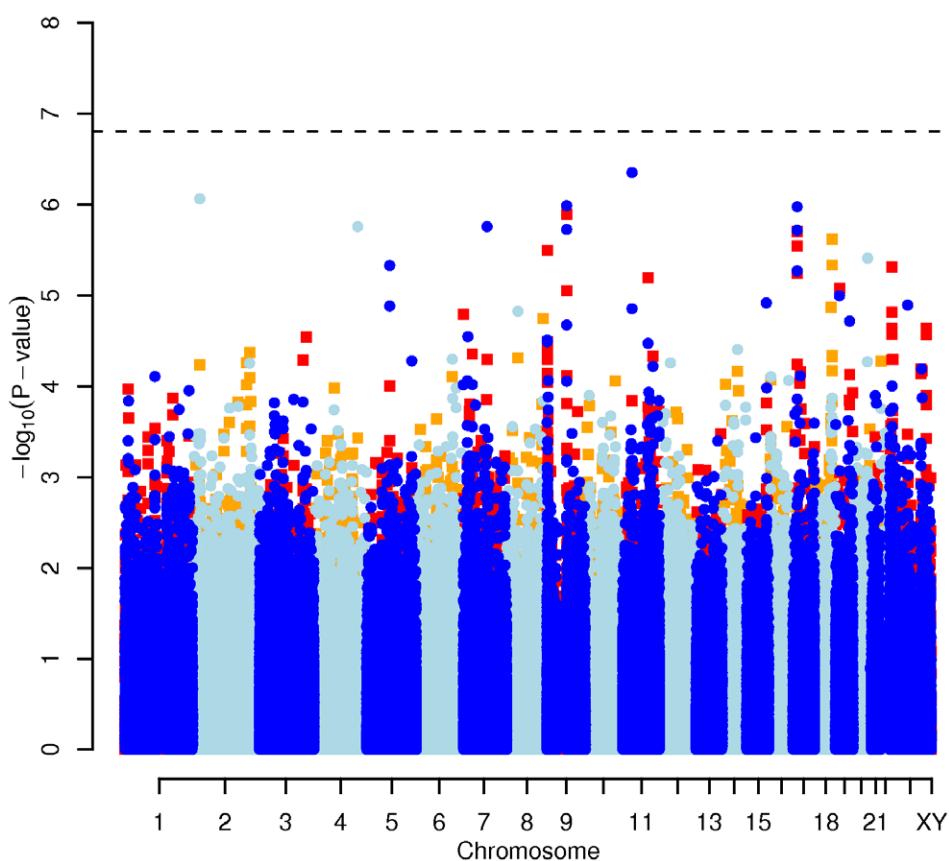


Figure 4. Manhattan plot of genome-wide effects on triglyceride levels in the Swedish discovery cohort. Results for two GWAS analysis models are presented. The unadjusted model (dark blue and light blue circles) included only sex and age as covariates. The adjusted model (red and orange squares) additionally contained food intake and physical activity as predictors. The dashed line indicates the local Bonferroni-adjusted α error = 1.6×10^{-7} .

doi:10.1371/journal.pgen.1000798.g004

311,388 SNP markers in common that are distributed across the human genome. Analysis of the raw data was done in the BeadStudio software with the recommended parameters for the Infinium assay and using the genotype cluster files provided by Illumina. Individuals with a call rate below 95% and SNPs with a call rate below 98%, deviating from Hard-Weinberg equilibrium

($p_{\text{HWE}} < 1 \times 10^{-6}$) or with a minor allele frequency of less than 1% were excluded from the analysis.

Lipids

Total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and

Table 2. SNPs ($n=3$) discovered in a Swedish and replicated in a non-Swedish EUROSPAN cohort.

SNP	Gene	Trait	Cohort	p -value, unadjusted ^a	p -value, adjusted ^b	Mean Difference, unadjusted ^c	Effect Size, unadjusted ^d	Effect Size, adjusted ^e
rs2000999	HP	TC	Discovery, SE	1.12E-03	3.84E-04	20.21 mg/dl	0.41	0.44
			Replication, SC	6.16E-03	4.33E-03	12.82 mg/dl	0.29	0.52
rs1532624	CETP	HDL-C	Discovery, SE	1.06E-06	2.55E-06	9.99 mg/dl	0.73	0.48
			Replication, SC	2.40E-09	1.96E-09	9.04 mg/dl	0.59	0.57
rs5400	SLC2A2	TC	Discovery, SE	3.57E-05	2.18E-06	27.11 mg/dl	0.57	0.66
			Replication, NS	4.68E-02	N.A.	13.35 mg/dl	0.30	N.A.

For all replicated SNPs p -values, mean differences, and effect sizes for unadjusted and adjusted lipid levels between homozygous genotypes are reported except for replication cohort NS. Discovery Cohort SE: Swedish EUROSPAN cohort ($N_{\text{SE}} = 656$), Replication Cohort SC: Scottish EUROSPAN cohort ($N_{\text{SC}} = 714$), Replication Cohort NS: Non-Swedish EUROSPAN cohorts (Scotland, Croatia, Italy, Netherlands, $N_{\text{NS}} = 3,282$), N.A.: not available; a) unadjusted: covariates include sex and age, b) adjusted: covariates include sex, age, game meat, non-game meat, fish, milk products, physical activity at work and at leisure, c) Genetic effect as mean difference of unadjusted lipid levels between homozygous genotypes: $M(A/A) - M(B/B)$, d) Genetic effect as standardized effect size of unadjusted lipid levels: $ES = (M_{A/A} - M_{B/B}) / SD_{\text{pooled}}$, e) Genetic effect as standardized effect size of adjusted lipid levels: $ES = (M_{A/A} - M_{B/B}) / SD_{\text{pooled}}$. doi:10.1371/journal.pgen.1000798.t002

triglycerides (TG) were quantified by enzymatic photometric assays using an ADVIA1650 clinical chemistry analyzer (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany) at the Institute for Clinical Chemistry and Laboratory Medicine, Regensburg University Medical Center, Germany.

Diet

In the NSPHS cohort, we collected data with a food frequency questionnaire based on the Northern Sweden 84-item Food Frequency Questionnaire (NoS-84-FFQ) [26]. We included in the questionnaire several items on foods specific for the lifestyle in this geographic region, in particular on game consumption (reindeer, moose). The answer options consisted of an 11-point format: 0 = “Never”, 1 = “less than 1 time per month”, 2 = “1 to 3 times per month”, 3 = “1 time per week”, 4 = “2 to 4 times per week”, 5 = “5 to 6 times per week”, 6 = “1 time per day”, 7 = “2 to 3 times per day”, 8 = “4 to 5 times per day”, 9 = “6 to 8 times per day”, 10 = “9 to 10 times per day”. The questionnaire was applied in electronic format by a trained study nurse as an interviewer. For each food item we calculated daily intake in gram per day as a standardized unit of measurement and aggregated the items to food categories, such game meat, non-game meat, fish, and dairy products. We evaluated the construct validity (known-groups validity) of the added items on game consumption in the NoS-84-FFQ questionnaire. We compared reindeer herders ($N=94$) versus non-reindeer herders ($N=505$). We observed highly significant, large effect sizes in men ($ES=1.25$, $p=9.7\times10^{-04}$) and women ($ES=1.15$, $p=2.9\times10^{-05}$) in the expected direction corresponding with an approximately three times higher consumption of absolute overall game intake in reindeer herders compared to others. A similar approach was used for the measurement and analysis of dietary data collected with a food frequency questionnaire in the Scottish cohort (Table S2).

Physical activity

In the NSPHS cohort, we used two self-report scales to measure overall physical activity at work and at leisure. The Work Activity Scale (WAS, 6 items) addresses typical occupational physical activities: sitting, standing, walking, lifting, and general indicators of physical activity, i. e. sweating and tiredness after work. The Leisure Activity Scale (LAS, 4 items) asks for various typical free-time activities such walking, cycling, other sporting activities, and sweating as a general indicator of physical activity. Participants reported the frequency of each activity on a 5-point rating scale (1 = “never”, 2 = “seldom”, 3 = “sometimes”, 4 = “often”, and 5 = “always”). Both scales showed satisfying internal consistency with Cronbach’s α (WAS) = 0.73 and Cronbach’s α (LAS) = 0.70. A similar approach was used for the measurement and analysis of data on physical activity collected with a self-report questionnaire in the Scottish cohort (Table S2).

Statistical analysis

Model selection. Sex and age are chosen as standard moderators of medical outcomes. Food and physical activity covariates have been selected based on findings on natural variation in lifestyle factors in this (data not presented) and other [7] northern Swedish populations between a modern, sedentary and a traditional, semi-nomadic lifestyle based on reindeer herding. Mostly significant associations between diet and activity covariates and lipid levels were found in the examined Swedish EUROSPAN cohort in the following ranges: $r=[-0.01;0.12]$ ($p=[1.28\times10^{-02};0.16]$) for game meat, $r=[-0.13;-0.05]$ ($p=[8.63\times10^{-04};0.74]$) for non-game meat, $r=[0.06;0.16]$ ($p=[2.12\times10^{-05};0.12]$) for fish, $r=[0.04;0.13]$ ($p=[2.51\times10^{-09}$;

$3.85\times10^{-06}]$) for physical activity at work, and $r=[-0.11;0.01]$ ($p=[5.05\times10^{-09};1.30\times10^{-06}]$) for physical activity at leisure (Table S3). We finally selected sex, age, game meat, non-game meat, fish, dairy products, physical activity at work, and physical activity at leisure as covariates in our diet- and activity-adjusted model (“adjusted” model) in the Swedish EUROSPAN sample. Sex and age were used as covariates in the “unadjusted” model.

We tested whether the inclusion of those covariates in the explanatory model led to a statistical significant improvement of the goodness of model fit compared to a restricted model by applying a maximum likelihood ratio (*MLR*) test. We inferred a significant better model fit of the full model if the difference of the χ^2 value between both models had an equal or lower probability than $p=0.05$ (one-sided, upper tail) on a χ^2 distribution with k degrees of freedom. The degrees of freedom k are equal to the difference of the number of parameters in each model. The difference of χ^2 values between both models is calculated according to the following formula with *MLE* indicating the maximum likelihood estimates per model: $\chi^2(\text{rest}-\text{full})=-2(\log_{10}(\text{MLE}_{\text{rest}})-\log_{10}(\text{MLE}_{\text{full}}))$. The comparison of the goodness of fit between the unadjusted and the diet- and activity-adjusted full model, using a *MLR* test, showed a statistically significant improvement for all four lipid traits (TC: $\chi^2_{\text{diff}}=59.69$, $df=6$, $p=5.21\times10^{-11}$; LDL-C: $\chi^2_{\text{diff}}=39.45$, $df=6$, $p=5.85\times10^{-07}$; HDL-C: $\chi^2_{\text{diff}}=29.57$, $df=6$, $p=4.75\times10^{-05}$; TG: $\chi^2_{\text{diff}}=69.32$, $df=6$, $p=5.65\times10^{-13}$). All included polygenic, anthropometric and lifestyle factors (with the effect of including only the polygenic, sex, and age effects in parentheses) explained 64.07% (58.02%) of the variation of TC, 59.47% (56.47%) of the variation of LDL-C, 83.73% (82.59%) of the variance of HDL-C and 58.68% (41.80%) of the variation of TG levels. Dietary measures accounted for 22% (TC), 40% (LDL-C), 74% (HDL-C), and 7% (TG), respectively, of the variance explained by lifestyle factors with physical activity being responsible for the rest. GWAS results for models adjusted for sex, age, and diet only (Figures S1A, S1B, S1C, S1D) or physical activity only (Figures S2A, S2B, S2C, S2D) are presented in the supporting figures.

The confounding effect of treatment with statins on total cholesterol level and LDL cholesterol level was adjusted for by imputing untreated lipid concentrations of medicated individuals using the *npsubtreated()* function of the *R/GenABEL* package which implements the algorithm of Tobin et al. [27]. Additionally, we conducted the same analysis in subsamples which did not receive any lipid-lowering treatment and found overall converging, but somewhat weaker results for rs2000999 ($p_{\text{SE,adj}}=2.55\times10^{-04}$; $p_{\text{SC,adj}}=2.07\times10^{-02}$, $p_{\text{NS,unadj}}=5.93\times10^{-02}$), rs1532624 ($p_{\text{SE,adj}}=2.26\times10^{-05}$; $p_{\text{SC,adj}}=2.28\times10^{-09}$, $p_{\text{NS,unadj}}=2.37\times10^{-19}$), and rs5400 ($p_{\text{SE,adj}}=5.34\times10^{-06}$; $p_{\text{SC,adj}}=2.23\times10^{-01}$, $p_{\text{NS,unadj}}=8.04\times10^{-02}$) (Table S4).

Genome-wide association analysis. First, deviations from normality for all quantitative traits (lipids, age, diet, and physical activity) were corrected by inverse-normal transformation without adjusting for covariates. Second, linear mixed effects models were fitted for the transformed outcomes (TC, LDL-C, HDL-C, TG) using the above mentioned covariates in the Swedish EUROSPAN sample and corresponding measures in the Scottish EUROSPAN sample (Table S2). The analysis was performed using the “polygenic” linear mixed effects model function *polygenic()* of the *R/GenABEL* package. Third, genome-wide association analysis was performed using a score test, a family-based association test [28], implemented in the *mmscore()* function of *R/GenABEL*. It uses the residuals and the variance-covariance matrix from the polygenic model and additional the SNP fixed effect coded under an additive model (0 = A/A, 1 = A/

$B_2 = B/B$). Fourth, genome-wide significance of a genetic loci was based on a local type I error of $\alpha = 0.05/311,388$ SNPs = 1.6×10^{-7} according to a Bonferroni adjustment.

Candidate gene association analysis. The same statistical approach was used for association analysis of candidate loci with a local type I error of $\alpha = 0.05$. No Bonferroni adjustment was applied to protect against α inflation since this method would be biased for the following reasons. The applied selection procedure for candidate loci makes the assumption of a global null hypothesis highly unlikely. Additionally, the phenotypes and some of the genotypes are highly correlated decreasing the number of independent tests. Instead all confirmatory tests are reported to allow the reader to evaluate the overall significance of the findings [29].

Relatedness. λ coefficients of lifestyle-adjusted genome-wide analysis varied in a low range between 1.00 and 1.04 in the Swedish cohort (see QQ-plots, Figures S3A, S3B, S3C, S3D, and Figure S4A, S4B, S4C, S4D) and between 1.00 and 1.01 in the Scottish cohort across all lipid traits. λ values for the unadjusted model used in the other three EUROSPAN cohorts did not exceed 1.01. These values indicate that our statistical model adequately handled relatedness in our pedigree-based samples since deflation of λ values is expected after correction for family structure.

Software and databases. We performed all analysis with the statistical analysis system *R* (V2.8.1) [30] mainly using the packages *GenABEL* (V1.4.2) [31] and *biomarRt* (V1.16.0) [32]. We accessed the following databases: *Ensembl* (<http://www.ensembl.org>) and *Online Mendelian Inheritance in Men* (<http://www.ncbi.nlm.nih.gov/omim/>).

Supporting Information

Figure S1 Manhattan plots of genome-wide effects on total cholesterol, LDL cholesterol, HDL cholesterol, and triglyceride levels in the Swedish discovery cohort. Results for two GWAS analysis models are presented. The unadjusted model (dark blue and light blue circles) included only sex and age as covariates. The adjusted model (red and orange squares) additionally contained dietary measures (game meat, non-game meat, fish, milk products) as predictors. The dashed line indicates the local Bonferroni-adjusted α error = 1.6×10^{-7} .

Found at: doi:10.1371/journal.pgen.1000798.s001 (0.31 MB DOC)

Figure S2 Manhattan plots of genome-wide effects on total cholesterol, LDL cholesterol, HDL cholesterol, and triglyceride levels in the Swedish discovery cohort. Results for two GWAS analysis models are presented. The unadjusted model (dark blue and light blue circles) included only sex and age as covariates. The adjusted model (red and orange squares) additionally contained physical activity measures (job, leisure) as predictors. The dashed line indicates the local Bonferroni-adjusted α error = 1.6×10^{-7} .

Found at: doi:10.1371/journal.pgen.1000798.s002 (0.31 MB DOC)

Figure S3 QQ-Plots for the unadjusted GWAS on total cholesterol, LDL cholesterol, HDL cholesterol, and triglyceride levels in the Swedish discovery cohort. The analysis model was only adjusted for sex and age, but not for diet and activity measures (black line = expected slope under no inflation, red line = slope fitted to observations).

Found at: doi:10.1371/journal.pgen.1000798.s003 (0.12 MB DOC)

Figure S4 QQ-Plots for the adjusted GWAS on total cholesterol, LDL cholesterol, HDL cholesterol, and triglyceride levels in the

Swedish discovery cohort. The analysis model was adjusted for sex, age, diet and activity measures (black line = expected slope under no inflation, red line = slope fitted to observations).

Found at: doi:10.1371/journal.pgen.1000798.s004 (0.12 MB DOC)

Table S1 GWAS results for all top candidate SNPs (0.05%) in the Swedish (SE) discovery cohort, the Scottish (SC), and all non-Swedish (NS) replication cohorts.

Found at: doi:10.1371/journal.pgen.1000798.s005 (0.41 MB XLS)

Table S2 Comparison of the diet- and activity-adjusted analysis model in the Swedish and the Scottish cohort.

Found at: doi:10.1371/journal.pgen.1000798.s006 (0.04 MB DOC)

Table S3 Pearson correlations, determination coefficients (explained variance), and p -values of the inverse-normal transformed lipid, dietary, and physical activity measures in the Swedish cohort.

Found at: doi:10.1371/journal.pgen.1000798.s007 (0.03 MB XLS)

Table S4 GWAS results for all top SNPs (0.05%) in the Swedish (SE) discovery cohort, and for all candidate SNPs in the Scottish (SC), and in the non-Swedish (NS) replication cohorts including only individuals without lipid-lowering treatment.

Found at: doi:10.1371/journal.pgen.1000798.s008 (0.34 MB XLS)

Acknowledgments

We would like to thank the many colleagues who contributed to collection and phenotypic characterization of the samples, genotyping and analysis of the GWAS data, as well as lipid species analysis. We would also like to acknowledge those who agreed to participate in these studies.

NSPHS: We are grateful for the contribution of samples from the Medical Biobank in Umeå and for the contribution of the district nurse Svea Hennix. **ORCADES:** We would like to acknowledge the invaluable contributions of Lorraine Anderson and the research nurses in Orkney, the administrative team in Edinburgh and the people of Orkney. **VIS:** We collectively thank a large number of individuals for their individual help in organizing, planning and carrying out the field work related to the project and data management: Professor Pavao Rudan and the staff of the Institute for Anthropological Research in Zagreb, Croatia (organization of the field work, anthropometric and physiological measurements, and DNA extraction); Professor Ariana Vorko-Jovic and the staff and medical students of the Andrija Štampar School of Public Health of the Faculty of Medicine, University of Zagreb, Croatia (questionnaires, genealogical reconstruction and data entry); Dr Branka Salzer from the biochemistry lab “Salzer”, Croatia (measurements of biochemical traits); local general practitioners and nurses (recruitment and communication with the study population); and the employees of several other Croatian institutions who participated in the field work, including but not limited to the University of Rijeka and Split, Croatia; Croatian Institute of Public Health; Institutes of Public Health in Split and Dubrovnik, Croatia. SNP Genotyping of the Vis samples was carried out by the Genetics Core Laboratory at the Wellcome Trust Clinical Research Facility, WGH, Edinburgh. **MICROS:** We thank the primary care practitioners Raffaela Stocker, Stefan Waldner, Toni Pizzocco, Josef Planger, Ugo Marcant and the personnel of the Hospital of Silandro (Department of Laboratory Medicine) for their participation and collaboration in the research project. **ERF:** We are grateful to all patients and their relatives, general practitioners, and neurologists for their contributions and to P. Veraart for her help in genealogy, Jeannette Vergeer for the supervision of the laboratory work and P. Snijders for his help in data collection. **UPPMAX:** The computations were performed on UPPMAX (<http://www.uppmx.uu.se>) resources under Project p2008027. Further information about The European Special Populations Research Network (EUROSPAN) consortium is available at <http://www.eurospan.org>.

Author Contributions

Conceived and designed the experiments: JFW NH PR TM PPP AAH BAO CMvD IR AW HC UG. Performed the experiments: ÅJ SHW OP

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- CH VV CG. Analyzed the data: WI. Contributed reagents/materials/analysis tools: GS. Wrote the paper: WI UG.
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Genome-wide significant predictors of metabolites in the one-carbon metabolism pathway

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Received May 21, 2009; Revised and Accepted September 4, 2009

Low plasma B-vitamin levels and elevated homocysteine have been associated with cancer, cardiovascular disease and neurodegenerative disorders. Common variants in *FUT2* on chromosome 19q13 were associated with plasma vitamin B₁₂ levels among women in a genome-wide association study in the Nurses' Health Study (NHS) NCI-Cancer Genetic Markers of Susceptibility (CGEMS) project. To identify additional loci associated with plasma vitamin B₁₂, homocysteine, folate and vitamin B₆ (active form pyridoxal 5'-phosphate, PLP), we conducted a meta-analysis of three GWA scans (total $n = 4763$, consisting of 1658 women in NHS-CGEMS, 1647 women in Framingham-SNP-Health Association Resource (SHARe) and 1458 men in SHARe). On chromosome 19q13, we confirm the association of plasma vitamin B₁₂ with rs602662 and rs492602 (P -value = 1.83×10^{-15} and 1.30×10^{-14} , respectively) in strong linkage disequilibrium (LD) with rs601338 ($P = 6.92 \times 10^{-15}$), the *FUT2* W143X nonsense mutation. We identified additional genome-wide significant loci for plasma vitamin B₁₂ on chromosomes 6p21 ($P = 4.05 \times 10^{-08}$), 10p12 (P -value = 2.87×10^{-9}) and 11q11 (P -value = 2.25×10^{-10}) in genes with biological relevance. We confirm the association of the well-studied functional candidate SNP 5,10-methylene tetrahydrofolate reductase (*MTHFR*) Ala222Val (dbSNP ID: rs1801133; P -value = 1.27×10^{-8}), on chromosome 1p36 with plasma homocysteine and identify an additional genome-wide significant locus on chromosome 9q22 (P -value = 2.06×10^{-8}) associated with plasma homocysteine. We also identified genome-wide associations with variants on chromosome 1p36 with plasma PLP (P -value = 1.40×10^{-15}). Genome-wide significant loci were not identified for plasma folate. These data reveal new biological candidates and confirm prior candidate genes for plasma homocysteine, plasma vitamin B₁₂ and plasma PLP.

INTRODUCTION

One-carbon metabolism comprises a network of biochemical reactions involved in the transfer of single-carbon moieties essential for purine and thymidylate synthesis necessary for maintenance of genomic integrity and remethylation of homocysteine for S-adenosylmethionine (SAM)-dependent DNA

methylation reactions (1,2). Folate, a B-complex nutrient from diet and supplements, donates methyl groups to generate methionine from homocysteine. Plasma homocysteine (Hcy), an integrated marker of one-carbon metabolism, is inversely related to folate, vitamin B₆ (3) and vitamin B₁₂ and positively related to high alcohol consumption (4,5). Vitamin B₁₂ is a necessary co-factor for the *methionine synthase* catalyzed

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remethylation of Hcy into methionine. The biologically active form of vitamin B₆, pyridoxal 5'-phosphate (PLP), catalyzes Hcy transulfuration (6) and is involved in over 100 enzyme reactions in the synthesis and catabolism of neurotransmitters (7), amino acids, glycogen and lipids (8).

Epidemiologic studies have shown that individuals with reduced intake of folate (found in fruits, vegetables and legumes) or vitamin B₆ (found in poultry, fish, meat, legumes, nuts and potatoes) or vitamin B₁₂ [found in liver, shellfish, fish, poultry, eggs and dairy products (9)] have an inverse relationship with Hcy levels. The accumulation of Hcy leads to an intracellular increase in S-adenosylhomocysteine (AdoHcy) and is an established risk predictor of cardiovascular diseases (6,10–16). In addition, individuals with inadequate intake of folate and vitamin B₁₂ are at an elevated risk of uracil misincorporation into DNA and aberrant DNA methylation (17–20). Vitamin B₁₂ deficiency (21), related to poor intestinal B₁₂ absorption (9) or dietary deficiency, can lead to inactivation of *methionine synthase* and is associated with pernicious anemia (22), cardiovascular disease (23–25), cancer (26–32) and neurodegenerative disorders (5,33–35). Plasma PLP levels are significantly inversely associated with risk of colorectal cancer in men (36) and women (37).

Plasma Hcy is the integrated marker of the one-carbon metabolism pathway, with a heritability estimate of 44% (38) [heritability estimate range 8–57% (38–43)]. Until recently, common genetic variations reported to influence plasma folate, vitamin PLP, vitamin B₁₂ and Hcy were focused on genes in the one-carbon metabolism pathway, specifically the *Ala222Val* polymorphism (23,44) (45) in *5,10* methylene-THF reductase (*MTHFR*), associated with reduced conversion of methylene-THF to methyl-THF (46,47). We previously identified a genome-wide significant association between common variants in *FUT2* and plasma vitamin B₁₂ levels in women (48). In a recent genome-wide analysis of data pooled from three genome-wide association study (GWASs) of 2931 persons and a replication study of 687 participants, Tanaka *et al.* (49) confirmed this loci and reported additional loci that met genome-wide significance criteria for PLP. In the current study, we report a meta-analysis of three GWASs with 4763 participants (1658 women in CGEMS, 1647 women in SHARe and 1458 men in SHARe) and test for the association between 2404675 genotyped or imputed SNPs with an R-sq > 0.3 (R-sq_hat estimates the squared correlation between imputed and true genotypes and allows us to assess imputation accuracy for markers with many different allele frequencies) in each study and plasma levels of Hcy, folate, vitamin B₁₂ and PLP.

RESULTS

Genome-wide significant associations of *P*-value < 5 × 10⁻⁸ [corresponding to *P* < 0.05 corrected *P*-value after adjusting for approximately 1 million independent loci (50)] were identified in meta-analyses for log-transformed plasma Hcy, vitamin B₁₂ and PLP. Age and the distributions of plasma vitamin B₁₂ and homocysteine in the study populations are described in Table 1. As expected, plasma Hcy was found to be inversely related to plasma levels of folate, vitamin B₁₂

Table 1. Mean and median plasma one-carbon metabolite levels in the NHS CGEMS, SHARe Women and SHARe Men study populations with GW data

	NHS CGEMS ^a , n = 1,658	SHARe Women, Exam 6 ^b , n = 1,647	SHARe Men, Exam 6 ^b , n = 1,458
Age, Mean, SD	58.92, 6.20	58.60, 9.71	58.57, 9.70
Plasma HCY ^c , Mean, SD	11.52, 5.41	9.06, 3.87	10.54, 3.99
Plasma HCY Median (IQR ^d)	10.62 (4.04)	8.39 (3.32)	9.82 (3.55)
Plasma Folate ^e , Mean, SD	10.02, 9.03	7.85, 5.32	6.54, 4.23
Plasma Folate Median (IQR ^d)	7.69, (7.44)	6.45 (5.80)	5.68 (4.22)
Plasma B ₁₂ ^f , Mean, SD	473.90, 249.53	435.10, 181.80	398.59, 148.26
Plasma B ₁₂ median (IQR ^d)	436.31 (224.0)	405.96 (211.91)	377.34 (172.68)
Plasma PLP ^g , Mean, SD	76.39, 90.55	83.14, 82.76	86.89, 84.05
Plasma PLP median (IQR ^d)	46.70 (47.77)	57.55 (56.87)	61.72 (52.61)

^aNHS blood collection was during 1989–1990.

^bExam 6 refers to Framingham Heart Study laboratory exam 6 measurements (1995–1998). For all plasma, one-carbon metabolites exam 6 measurements were used in this analysis because this exam had the largest sample size, and laboratory measurements were conducted using assays comparable to NHS CGEMS.

^cPlasma HCY measured in μmol/l; data were log-transformed for GWAS analysis.

^dIQR, interquartile range.

^ePlasma Folate measure in ng/ml; data were log-transformed for GWAS analysis.

^fPlasma Vitamin B₁₂ measured in pg/ml; data were log-transformed for GWAS analysis.

^gPlasma PLP measured in pmol/ml; data were log-transformed for GWAS analysis.

and vitamin B₆ in our study population (Supplementary Material, Table S1). No significant heterogeneity by sex or by study (Table 2) was detected for the genome-wide significant associations.

Plasma homocysteine

The well-studied functional polymorphism in *MTHFR Ala222Val* (also reported as *MTHFR C677T*), rs1801133, on chromosome 1 [position (bp): 11,790,644] was associated with plasma Hcy (*P*-value = 1.27 × 10⁻⁸; Fig. 1A; Table 2). Participants with the *Ala/Ala* (G/G) variant had lower plasma Hcy levels than variant carriers. Two other SNPs, rs12085006 [position (bp): 11,892,989] and rs1999594 [position (bp): 11,893,482], located 102 kb from *MTHFR*, had stronger associations with plasma Hcy (5.81 × 10⁻¹⁰ and 6.24 × 10⁻¹⁰, respectively; Fig. 1A; Table 2). Participants homozygous for the rs12085006 *G* and rs1999594 *A* alleles had higher Hcy levels compared with variant carriers for each SNP. The rs12085006 *A* allele is correlated with the *MTHFR 222Val* allele (composite *r*² = 0.53; *P* < 0.0001) (51). After mutual

Table 2. Genome-wide significant associations for plasma one-carbon metabolites

SNP	Chr	Position (bp)	Gene	Alleles	MAF ^d	NHS CGEMS n = 1658			SHARE Women n = 1647			SHARE Men n = 1458			Meta-analysis n = 4763				
						BETA ^a S.E.	P-value	BETA ^b S.E.	P-value	BETA ^b S.E.	P-value	BETA ^b S.E.	P-value	BETA ^b S.E.	P-value	BETA ^b S.E.	P-value		
Plasma homocysteine																			
rs12085006	1p36	11892989		G,A	0.43	0.04	0.01	1.21E-05	0.03	0.01	4.06E-03	0.04	0.01	6.49E-04	0.04	0.01	5.81E-10	0.72	
rs1199594	1p36	11893482		A,G	0.43	0.04	0.01	1.32E-05	0.03	0.01	4.06E-03	0.04	0.01	6.49E-04	0.04	0.01	6.24E-10	0.72	
rs1801133	1p36	11790644	MTHFR	G,A	0.33	0.04	0.01	5.12E-04	0.03	0.01	9.59E-03	0.04	0.01	1.51E-04	0.04	0.01	1.27E-08	0.65	
rs10986018	9q22	98202891	GPR51	C,T	0.22	-0.08	0.02	4.92E-05	-0.04	0.02	4.85E-02	-0.07	0.02	2.92E-04	-0.06	0.01	2.10	2.06E-08	0.36
Plasma Vitamin B ₁₂																			
rs622662	19q13	53898797	FUT2	A,G	0.44	-0.08	0.01	3.09E-10	-0.05	0.02	3.80E-04	-0.05	0.01	2.80E-04	-0.07	0.01	2.83	1.83E-15	0.23
rs601338	19q13	53898486	FUT2	G,A	0.45	0.09	0.01	4.25E-11	0.05	0.01	2.63E-03	0.05	0.01	4.02E-04	0.06	0.01	5.13	6.92E-15	0.08
rs492602	19q13	53898229	FUT2	A,G	0.44	0.09	0.01	5.39E-11	0.04	0.02	5.89E-03	0.05	0.01	2.36E-04	0.06	0.01	5.43	1.30E-14	0.06
rs1801222	10p12	17196157	CUBN	G,A	0.28	-0.05	0.01	9.04E-05	-0.04	0.02	6.32E-03	-0.05	0.02	3.56E-04	-0.05	0.01	0.42	2.87E-09	0.81
rs526934	11q11	59390069	TCNNI	A,G	0.27	-0.05	0.01	1.27E-03	-0.06	0.02	6.69E-05	-0.06	0.02	1.64E-04	-0.05	0.01	0.45	2.25E-10	0.82
rs9473558 ^e	6p21	49520392	MUT	C,T	0.35	-0.03	0.01	4.27E-02	-0.03	0.01	2.26E-02	-0.07	0.01	3.96E-07	-0.04	0.01	5.48	4.05E-08	0.08
rs9473555	6p21	49517446	MUT	G,C	0.35	-0.03	0.01	4.27E-02	-0.03	0.01	2.26E-02	-0.07	0.01	3.71E-07	-0.04	0.01	5.45	4.91E-08	0.08
Plasma PLP																			
rs1256335	1p36	21635692	ALPL	A,G	0.21	-0.15	0.03	6.44E-07	-0.11	0.03	2.37E-04	-0.16	0.03	2.36E-07	-0.14	0.02	1.56	1.40E-15	0.46
rs4554748	1p36	21531374	NBPF3	T,C	0.48	-0.12	0.03	1.40E-06	-0.05	0.03	7.32E-02	-0.12	0.02	2.21E-06	-0.10	0.01	5.63	4.30E-11	0.12

^aEstimates for NHS CGEMS data are from linear regression using log-transformed metabolite levels.^bEstimates SHARE data are from linear mixed-effects model using kinship information using log-transformed metabolite levels.^cMeta-analysis estimates and P-values for the NHS CGEMS, FHS SHARE Women and SHARE Men data sets were calculated using the fixed-effects model; between study variance is <0.001.^dMAF is averaged across the three GWAS studies.^edbSNP ID rs9473558 has been merged with rs1141321 Hs5324rg (97,98).

adjustment in a univariate regression model, both rs1285006 and *MTHFR Ala222Val* remained nominally statistically significantly associated with plasma Hcy ($P = 0.02$ and 0.002 , respectively). Variant carriers of both *MTHFR 222Val* and the *A* allele for rs12085006 had higher levels of plasma Hcy compared with non-carriers. Similar results were obtained for rs1999594. We also identified an association on chromosome 9 in the *gamma-aminobutyric acid B-type receptor G-protein coupled receptor 51 (GPR51)* gene, rs10986018, with plasma Hcy (P -value= 2.06×10^{-8} ; Fig. 1A, Table 2). A SNP, rs11041321 in *SYT9*, reported by Tanaka *et al.* (49) with a P -value of 1.11×10^{-4} in their joint analysis of GWAS meta-analysis and replication study, had a P -value=0.44 in our meta-analysis data. The SNPs in *CBS*, *CPS1*, *MUT* and *NOX4* reported in the Women's Genome Health Study by Paré *et al.* (52) were not genome-wide significant in our plasma homocysteine data (rs6586282 in *CBS*: P -value= 6.30×10^{-1} ; rs7422339 in *CPS1*: P -value= 4.18×10^{-4} ; rs4267943 in *MUT*: P -value 6.43×10^{-1} ; rs11018628 in *NOX4*: P -value= 4.35×10^{-3}).

Plasma folate

Overall, there were no genome-wide significant associations for plasma folate in the meta-analysis (Fig. 1B). The strongest associations were in chromosome 2 in the *FIGN* gene, rs982393 (P -value= 8.36×10^{-8} ; data not shown) and rs2119289 (P -value= 1.03×10^{-7} ; data not shown). Although not genome-wide significant, a moderate signal for SNPs in *MTHFR* was observed (rs3737965, P -value= 6.90×10^{-5} ; data not shown). As expected (53), the *MTHFR Ala222Val* variant carriers had lower plasma folate compared with the *Ala/Ala* variant (*MTHFR Ala222Val* P -value= 1.14×10^{-3} from meta-analysis, data not shown). The composite linkage disequilibrium (LD) with *MTHFR Ala222Val* and rs3737965 was 0.15 in our data. The rs1999594 SNP associated with plasma folate in the report by Tanaka *et al.* (49) had P -value= 3.01×10^{-3} in our analysis for plasma folate. The rs153734 SNP in the *PRICKLE* gene, reported by Tanaka *et al.* (49), was not associated with plasma folate in these data (P -value=0.44).

Plasma vitamin B₁₂

Genome-wide significant associations were identified on chromosomes 6, 10, 11 and 19 for plasma vitamin B₁₂ (Fig. 1C; Table 2). Two SNPs, in LD, in *Methylmalonyl-CoA mutase (MUT)* on chromosome 6p21—rs9473558 SNP and rs9473555 [chromosome position (bp): 49,520,392 and 49,517,446, respectively]—were genome-wide significant in the meta-analysis for plasma vitamin B₁₂ (P -meta= 4.91×10^{-8} , 4.05×10^{-8} respectively, Fig. 1; Table 2). Participants homozygous for the rs9473558 C and rs9473555 G alleles had higher B₁₂ levels compared with participants homozygous for the major allele. We identified an association ($P = 2.87 \times 10^{-9}$; Fig. 1C; Table 2) between rs1801222 in *CUBN* (*Cubilin*) on chromosome 10p12 and plasma vitamin B₁₂ levels in the meta-analysis of 4763 participants. The SNP is in exon 8 of *CUBN* and codes for an amino acid change *Ser253Phe* [chromosome 10p position (bp): 17,196,157].

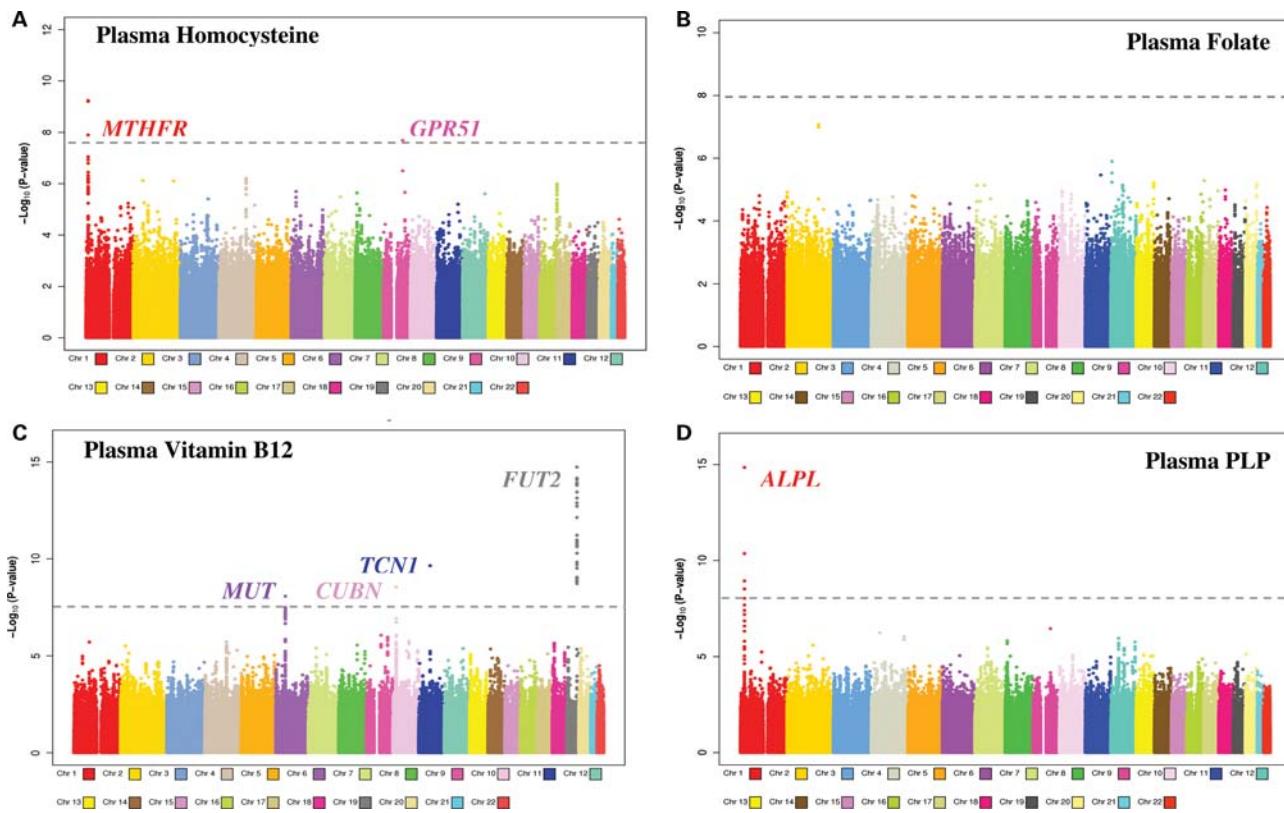


Figure 1. Meta-analysis of three GWA scans for plasma one-carbon metabolites. Manhattan plot of genome-significant associations (defined as $P = 5 \times 10^{-8}$ and depicted by the grey dashed line) for plasma homocysteine (A), plasma vitamin B₁₂ (C) and plasma PLP (D) in a meta-analysis of participants from NHS CGEMS, Framingham SHARe Women and Framingham SHARe Men.

Participants homozygous for the rs1801222 *G* allele had higher B₁₂ levels. The meta-analysis indicated several signals in the $P < 10^{-5}$ range for other SNPs in *CUBN* and on chromosome 10p15 in the neighboring *TRDMT1* gene (*DNMT2*), which may be explained by LD. A genome-wide significant association was also observed for rs526934 in the *transcobalamin I* (*TCNI*) gene ($P\text{-value} = 2.25 \times 10^{-10}$) located on chromosome 11q11 [position (bp): 59,390,069]. Variant carriers of the rs526934 *G* allele had lower vitamin B₁₂ levels when compared with individuals with the homozygous *AA* variant.

The most significant association in the meta-analysis data ($P\text{-value} = 1.83 \times 10^{-15}$; Fig. 1C; Table 2) was for the rs602662 SNP in *FUT2*. The *FUT2* nonsense SNP *W143X*, rs601338 [chromosome 19q13 position (bp): 53,898,486], the determinant of *FUT2* secretor status, was the second most significant association with a $P\text{-value} = 6.92 \times 10^{-15}$. Participants homozygous for non-secretor variants had higher B₁₂ levels than carriers of the secretor genotype. The rs492602 SNP reported to be in high LD ($D' = 1$, $r^2 = 0.76$) with *FUT2* *W143X* had a $P\text{-value} = 1.30 \times 10^{-14}$. We noted these associations despite the fact that the *FUT2* SNPs were not well tagged in the 347 458 genotyped SNPs that passed Quality Control filters in the SHARe data set; the MACH imputation *R*-sq value for rs602662: was 0.45, and for rs492602 it was 0.41 in SHARe.

Plasma PLP

SNPs in *alkaline phosphatase*, *ALPL*, had genome-wide significant associations with plasma PLP in the meta-analysis. We observed the strongest association for rs1256335 ($P\text{-value} = 1.40 \times 10^{-15}$; Fig. 1D; Table 2) in the *ALPL* gene. Participants homozygous for the *A* allele had higher plasma PLP levels compared with carriers of the *G* allele. The reported rs4654748 (49) SNP in *NBPF3*, upstream of *ALPL*, had a $P\text{-value} = 4.30 \times 10^{-11}$ with plasma PLP in these data (composite r^2 for rs1256335 and rs4654748 is 0.16, $P < 0.0001$).

DISCUSSION

We identified genome-wide significant associations for plasma homocysteine, plasma vitamin B₁₂ and plasma PLP. SNPs in chromosome 6p21, 10p12, 11q11 and 19q13 showed strong signals for plasma vitamin B₁₂. SNPs in chromosomes 1p36 and 9q22 were strongly associated with plasma homocysteine. We identified genome-wide significant associations between SNPs in chromosome 1p36 and plasma PLP.

Plasma homocysteine

Chromosome 1p36. *MTHFR* is a key enzyme required for DNA synthesis that catalyzes the irreversible transformation

of 5,10-MTHF into 5-MTHF (54). The *TT* variant alleles of the well-known *MTHFR* 677 (amino acid change: *MTHFR Ala222Val*) polymorphism are related to a 30% reduction in enzyme activity (55). A decrease in *MTHFR* activity associated with the 222 *Val/Val* (677 TT) polymorphism shifts the folate pool, leading to an elevation in 5,10-methyleneTHF. The *MTHFR Ala222Val* SNP results in a moderate (up to 15–19%) increase in mean Hcy levels between the *Ala/Ala* and *Val/Val* variants (23), and this effect is greatly attenuated in those individuals with mid-range to high folate levels (56). Rs12085006 and rs1999594, located 102 kb from *MTHFR*, had an even stronger association with plasma Hcy in our meta-analysis. RS1999594 had a genome-wide significant association ($P = 8.5 \times 10^{-12}$) with plasma Hcy in the Women's Genome Health Study (WGHS), but only rs180133 showed evidence for non-additive effects (52). The rs1999594 SNP was reported by Tanaka *et al.* (49) to be associated with plasma folate in a meta-analysis of 2931 participants ($P = 1.12 \times 10^{-7}$), although the association did not reach genome-wide significance in their report on plasma folate and was not reported for plasma Hcy. Given the strong correlation between rs1999594 (or rs12085006) and rs180133 and the modest evidence for association between rs1999594 (or rs12085006) and plasma Hcy after adjusting for *MTHFR Ala222Val*, we cannot conclude that these two SNPs mark distinct loci associated with plasma Hcy.

Chromosome 9q22. We identified a genome-wide significant association between plasma Hcy and a variant in *GPR51*. Carriers of the rs10986018 *T* allele had lower plasma vitamin B₁₂ compared with carriers of the *CC* variant. Expression of *GPR51* may be regulated by nicotine (57). Epidemiologic studies have reported increased homocysteine levels in smokers compared with non-smokers (58–62). The association with plasma Hcy and rs10986018 warrants future study on plasma Hcy and effect modification by smoking status.

Plasma folate

We did not identify any genome-wide significant association with plasma folate in this meta-analysis, although the *MTHFR Ala222Val* variant had the expected direction of association (53,56), with a modest *P*-value. FHS SHARe Exam 6 data available in dbGaP had the largest sample size for all four plasma one-carbon metabolites and laboratory assays comparable to the NHS CGEMS study population. All NHS CGEMS participants had plasma folate measured on blood drawn prior to folic acid fortification (1989–1990). However, the Framingham SHARe laboratory exam 6 measurements were taken during 1995–1998. While the majority of the SHARe participants included in this analysis had measurements prior to the US mandate for folic acid fortification in 1998, evidence suggests that the fortification may have started in 1996–1997. Pre-fortification plasma folate levels in the prior Framingham SHARe laboratory exam, available in dbGaP, were measured using a microbial assay and would not be equivalent to the HPLC assay used in the NHS CGEMS data. We also recognize that there may also be misclassification due to folic acid supplementation.

Plasma vitamin B₁₂

Chromosome 6. *MUT*, located on chromosome 6p21, encodes the mitochondrial enzyme methylmalonyl Coenzyme A (CoA) mutase. *MUT* requires 5-prime-deoxyadenosylcobalamin (AdoCbl, a coenzyme form of vitamin B₁₂) to catalyze the isomerization of *L*-methylmalonyl–coenzyme A (CoA) to succinyl-CoA, in the mitochondria, independent of folate status. Mutations in methylmalonyl-CoA mutase, especially in the C-terminus, result in methylmalonic aciduria (63–67). A patient with the mut(-) MMA phenotype due to the *MUT Gly717Val* variant was also homozygous for the *His532Arg* (rs9473558) polymorphism (68). The *T* variant of rs9473558 has reduced vitamin B₁₂ levels in our data and may be associated with decreased conversion of methylmalonyl CoA to succinyl CoA. The rs9473558 SNP in *MUT* was associated with plasma homocysteine ($P = 2.1 \times 10^{-7}$) in the Women's Genome Health Study (WGHS) participants (52). The rs9473558 SNP was positively associated with plasma homocysteine in the WGHS study ($\beta = 0.020$), but inversely associated with plasma vitamin B₁₂ in our meta-analysis ($\beta = -0.04$), which is consistent with the inverse relationship between homocysteine and vitamin B₁₂. The expression of *MUT* was associated with the rs9473555 SNP with a LOD score = 7.095 [enzyme activity for the *G*-allele is reduced by 0.42 standard deviation; mRNA by SNP browser 1-0-1 database (69)]. The preliminary association with rs9473558 or rs9473555 in the *MUT* gene and plasma vitamin B₁₂ should be replicated in other studies. Future studies on the relationship of rs9473555 or rs9473558 and *MUT* gene expression is warranted.

Chromosome 10. *Cubilin (CUBN)* is located on chromosome 10p12.31. The 460 kd multiligand hydrophobic *CUBN* protein binds to intrinsic factor-cobalamin (Cbl-IF) complexes with a high affinity and is expressed in both kidney and ileal epithelial cells. Mutations in *CUBN* are associated with megaloblastic anemia and characterized by selective intestinal vitamin B₁₂ malabsorption (70). The *P1297L* mutation in *CUBN* causes a 5-fold decrease of the binding domain affinity for Cbl-IF (the IF-Cbl binding region includes amino acids 928–1386). The rs1801222 (*Ser253Phe*) variant was associated with lower vitamin B₁₂ levels in this study and may decrease the binding and transport of vitamin B₁₂ in the ileum. Another SNP in *CUBN* reported by Tanaka *et al.* (49) with a *P*-value of 1.11×10^{-6} , rs11254363, had a *P*-value of 8.65×10^{-5} in our meta-analysis. The neighboring gene, *TRDMT1*, located on 10p15.1 is a methyltransferase that methylates the aspartic acid transfer RNA molecule (tRNA(Asp)) (71). A recent study on spina bifida and folate-related genes reported an association for different SNPs in *CUBN* and *TRDMT1* with increased serum RBC folate levels in controls (72). Further research is warranted to understand the LD pattern across the 305.3 kb *CUBN* gene and the *cis* relationships of the genome-wide significant SNPs with *CUBN* and *TRDMT1*.

Chromosome 11. *TCN1*, on chromosome 11q11, is a vitamin B₁₂ binding protein that promotes the cellular uptake of vitamin B₁₂ by receptor-mediated endocytosis. The protein

is a major constituent of secondary granules in neutrophils and facilitates the transport of cobalamin into cells (73). The meta-analysis and replication joint *P*-value for rs526934 from Tanaka *et al.*, was $P\text{-value}=1.51 \times 10^{-6}$. The *TCN1* rs526934 *G* variant may reduce transport of cobalamin, resulting in lower plasma vitamin B₁₂ levels.

Chromosome 19. We previously reported an association with variants in *FUT2* on chromosome 19q13 and plasma vitamin B₁₂ [rs602662: $P\text{-value}=3.52 \times 10^{-15}$; rs492602: $P\text{-value}=5.36 \times 10^{-17}$ in the combined NHS CGEMS and colorectal neoplasia data sets; (48)]. In the SHARe women and the SHARe men data sets, we replicated our genome-wide significant finding for rs602662 (SHARe combined $P=5.07 \times 10^{-8}$; Table 2 presents separate estimates and standard error, by SHARe Men and Women) and in this meta-analysis of CGEMS and SHARe data, we confirm the association of these SNPs with plasma vitamin B₁₂. In this study of plasma vitamin B₁₂, we confirm our previously reported hypothesis that *FUT2 W143X*, a nonsense mutation (74) and determinant of *FUT2* secretor status, is the causal variant for the association with plasma B₁₂ levels (rs601338: $P\text{-value}=6.92 \times 10^{-15}$). Individuals with the non-secretor status variant had higher plasma vitamin B₁₂ levels compared with carriers of the secretor status genotypes. Reduced vitamin B₁₂ absorption (75–79) in carriers of the secretor genotype may be a consequence of susceptibility to *H. pylori* infection compared with individuals with the non-secretor genotype. The association with rs602662 was replicated by an independent group (49) with a *P*-value of 2.43×10^{-12} in a meta-analysis of three GWASs ($n=2930$ participants).

Plasma PLP

Mutations in the *ALPL* gene, on chromosome 1p36, are associated with hypophosphatasia. Increased circulating concentrations of PLP in all clinical forms of hypophosphatasia have been reported by Whyte *et al.* (80,81). The findings suggested that tissue-non-specific alkaline phosphatase is involved in vitamin B₆ metabolism. The *ALPL* enzyme may function as an ectoenzyme to regulate extracellular concentrations of PLP (80). The rs1256335 *G* allele had lower plasma PLP levels compared with the homozygous *A* allele, possibly due to increased hydrolysis of PLP (82). The rs4654748 *C* allele, in the *neuroblastoma breakpoint family, member 3*, (*NBPF3*, alternate name *AE2*) gene had lower plasma PLP levels compared with the homozygous major allele.

Comparability of plasma phenotypes in the study population

The NHS and FHS study populations have comparable metabolite levels for conducting a meta-analysis of GWA data of plasma one-carbon metabolites and the biomarker measurement in these studies were all done in a single laboratory. However, one limitation is that the NHS CGEMS participants were all postmenopausal women, whereas SHARe Women participants included of both pre- and postmenopausal women. Plasma Hcy is lower in premenopausal women than

men, but after menopause, homocysteine levels in women increased to approach those in men, consistent with our data. All NHS and the majority of the FHS blood samples for participants in this analysis were collected prior to the introduction of folic acid fortification of breads, cereals, flours, corn meal, pasta products, rice and other cereal grain products sold in the USA (83,84). Therefore, the genetic variability signal should not be attenuated by this recent environmental influence.

In summary, for plasma vitamin B₁₂, we replicated our finding for *FUT2* (48) and identified genome-wide significant SNPs in biological candidate genes: *TCN1*, a vitamin B₁₂ binding protein; *MUT*, which converts methylmalonyl CoA to succinyl CoA; and *CUBN*, the receptor for intrinsic factor-vitamin B₁₂ complexes. For plasma homocysteine, we observed genome-wide significant associations with the *MTHFR* functional SNP *Ala222Val* and a possible new independent locus 102 kb upstream of *MTHFR*. For plasma PLP, we noted genome-wide significant associations in *ALPL*. These data reveal new biological candidates and confirm prior candidate genes for plasma homocysteine, plasma vitamin B₁₂ and plasma PLP.

MATERIALS AND METHODS

Ethics statement

All participants provided written informed consent, and local institutional review boards approved the study protocols.

Study populations

We conducted a meta-analysis of genome-wide data in 1658 women genotyped with the HumanHap500 as part of the Cancer Genetic Markers of Susceptibility Project (CGEMS) and in 1682 women genotyped with the Affymetrix 500K mapping array and the Affymetrix 50K Supplementary array as a part of the SNP Health Association Resource (SHARe data set, part of NHLBI FHS) to identify novel loci that influence plasma one-carbon metabolite levels. All participants were of self-reported European ancestry.

The Nurses' Health Study (NHS)

The NHS was initiated in 1976, when 121 700 US registered nurses between the ages of 30 and 55 returned an initial questionnaire reporting medical histories and baseline health-related exposures. Every 2 years, follow-up questionnaires are mailed to the participants (questionnaire response rate >90% for all follow-up cycles). Diet was assessed in 1986, 1990, 1994 and 1996 with a semiquantitative food frequency questionnaire. Between 1989 and 1990, blood samples and relevant sample collection information were collected from 32 826 women.

The CGEMS project (<http://www.cgems.cancer.gov>) is an NCI initiative to conduct genome-wide association studies (GWAS) to identify genes involved in breast and prostate cancer. The initial CGEMS scan, designed and funded only to study the main effect of SNP variants on breast cancer risk in postmenopausal women, has been completed (85).

A subset of the participants in the CGEMS project also has been assayed for plasma vitamin B₁₂. The NHS CGEMS participants had similar laboratory measurement of the plasma one-carbon metabolites as well as host characteristics and blood draw features (fasting status and year and month of blood draw).

Framingham Heart Study (FHS)

The FHS began in 1948 with the enrollment of two-thirds of the adult population of Framingham, Massachusetts (86), including 2873 women aged 28–62 years. In 1971, 5124 offspring of the original cohort members and offspring spouses were enrolled in the Framingham Heart Study, including 2641 women ranging in age from 12 to 60 years. A data set consisting of repeated and highly accurate measurements of many different phenotypes collected over multiple exam biennial cycles is available for each participant. The NHLBI Framingham SNP-Health Association Resource (SHArE) is a large-scale GWAS on the FHS original, offspring and third-generation cohorts with data accessible to the research community (87). In the Framingham cohort, the SNP Health Association Resource (SHArE) project genotyped 9274 Caucasian participants with the Affymetrix 500K mapping array and the Affymetrix 50K supplemental array (Affymetrix, Santa Clara, CA, USA). The 8508 samples from these participants had a sample call rate $\geq 97\%$ (88,89). Of the participants from the Framingham original and offspring cohorts, with measured plasma one-carbon metabolites at laboratory exam 6, between 1995 and 1998, the SHArE scan included 1647 (16) Caucasian women and 1458 Caucasian men. Exam 6 had the largest sample size for all four plasma one-carbon metabolites and laboratory assays comparable to the NHS CGEMS study population. Information on Framingham SHArE can be found at http://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000007.v1.p1.

Laboratory assays

Plasma levels of homocysteine for both the NHS CGEMS and FHS SHArE studies were measured by high-performance liquid chromatography, with fluorescence detection (90). Plasma levels of folate and vitamin B₁₂ for the NHS CGEMS and FHS SHArE participants were measured using by a radioassay kit (Bio-Rad, Richmond, CA, USA) according to the manufacturer's instructions. Plasma PLP levels were measured by an enzymatic procedure using radioactive tyrosine and the apo-enzyme tyrosine decarboxylase.

All assays were conducted at the Jean Mayer U.S. Department of Agriculture Human Nutrition Research Center on Aging at Tufts University. Plasma samples for matched case–control sets were always positioned next to each other, in random order, in boxes sent to the lab and were assessed in the same batch to minimize the impact of laboratory error due to batch drift. Quality control (QC) samples were also submitted with each batch and were randomly placed throughout the boxes (two assay batches were used for NHS CGEMS participants). Laboratory technicians were blinded to case, control and QC status of the samples. QC samples consisted of replicates of two pools of plasma. One QC sample was assayed per

10 study samples (27,37). No significant differences in mean levels of plasma homocysteine, folate, vitamin B₁₂ or PLP were detected by breast cancer case–control status in the NHS. The mean coefficient of variation for 75 pairs of replicate 6.5% for folate, 7.9% for homocysteine, 7.3% for B₁₂ and 7.2% for PLP (27).

Reliability of laboratory assays of plasma levels

In an analysis of 188 NHS participants, the correlation coefficients between folate intake calculated from the 1980 food frequency questionnaire and erythrocyte folate concentrations in 1987 were 0.55 for folate from foods and supplementation and 0.38 for folate from food only (18). Similar correlation coefficients were noted for plasma folate (0.55 for total folate; 0.35 for dietary folate) (18,27). The intraclass correlation coefficient over a 3-year period on 82 participants was 0.63 for plasma folate, suggesting reproducibility of these measurements over time.

Illumina SNP genotyping and quality-control analysis

Genotyping in the CGEMS project was carried out using the Illumina Infinium Sentrix HumanHap550 bead chip, which contains 556 566 SNPs derived from the HapMap phase I and II data (85). The polymorphisms on the bead chip are selected to be direct surrogates (R^2 , a measure of pair-wise SNP correlation, >0.8) of more than 75% of the common polymorphisms genotyped as part of the HapMap Phase II project. Overall, genotyping success was 98.45% of the possible genotypes in the first attempt and 99.27% after failed samples were repeated. Quality control of genotyping reproducibility was 99.99%. SNPs with significant deviations from Hardy–Weinberg (HWE) proportions (5% of SNPs at the level of $P = 0.05$ and 1.28% at $P = 0.01$) were not excluded from the analysis, as the tests for association applied to these data are valid in the presence of departure from HW proportions. The NHS cases and controls included in CGEMS are of self-reported European ancestry (85). Four samples (three cases and one control) were removed because of intercontinental admixture. In the CGEMS data, we saw no evidence for systematic bias in the distribution of P -values for analyses with and without further adjustment for residual population structure using four principal components of genetic variation compatible with no confounding of SNP-metabolite associations due to population stratification (85,91). A total of 1658 NHS participants had GWAS and plasma vitamin B₁₂ data.

Affymetrix SNP genotyping and quality-control analysis

Genotyping in the SHArE study (88,89) was carried out using the Affymetrix (Santa Clara, CA, USA) GeneChip Human Mapping 500K mapping array and supplemental 50K mapping array (HuGeneFocused50K) with gene-centric and coding SNPs. The Human Mapping 500K Array Set is comprised of two arrays, each capable of genotyping on average 250 000 SNPs (approximately 262 000 for Nsp arrays and 238 000 for Sty arrays).

Association analyses were restricted to individuals with $\geq 97\%$ genotyping call-rate. SNP results were filtered on Hardy–Weinberg equilibrium P -value of 1×10^{-6} , SNP call-rate of 95% and minor allele frequency (MAF) of 0.01. After cleaning the genotype data, 347 K SNPs were available for the analysis.

Statistical methods

We tested the association between log-transformed plasma one-carbon metabolite levels and each SNP using linear regression adjusted for age. SNP genotypes were coded as counts of minor alleles (additive coding) unless otherwise specified. We adopted an additive coding because it has nearly optimal power for a range of realistic genetic models, with the exception of rare recessive effects, which we have little power to detect (92). In CGEMS, we further adjusted for principal components of genetic variation, calculated with the EIGENSTRAT software (91).

The SHARE data were analyzed using linear mixed effects models to account for residual familial correlation; aside from fixed effects (age, SNP genotype), this model includes individual random effects with a correlation matrix equal to the kinship matrix times a scalar variance parameter. These analyses were implemented using the lme4 function in the R package kinship.

We imputed SNP genotypes using the hidden Markov model implemented in MACH 1.0 (<http://www.sph.umich.edu/csg/abecasis/MACH/index.html>) for each CGEMS and SHARE separately. For the SHARE data, the hidden Markov model was trained using unrelated founders (922 women and 692 biologically unrelated men); the fitted model was then used to infer missing genotypes for the remaining subjects. The certainty of the imputation is dependent on the stretches of haplotypes shared between the reference panel (HapMap Phase II CEU population) and the study population. The imputation result at each of the imputed SNPs, per study participant, can be expressed as a set of three genotype probabilities, that vary between 0 and 1 per genotype and sum to 1, or as an ‘allele dosage’, which is defined as the expected number of copies of the minor allele at each SNP and varies between 0 and 2. In the CGEMS data, we evaluated both methods and obtained comparable results for the genotype probabilities and allele dosage methods. In the SHARE data, we evaluated both methods for the genome-wide significant associations and obtained comparable results for the genotype probabilities and allele dosage methods.

Genotyped and imputed SNPs with an R^2 from MACH > 0.30 in both the CGEMS Illumina and SHARE Affymetrix data were used for a meta-analysis (93) to examine the association between ~ 2.5 million polymorphisms and the plasma metabolites. A fixed-effect model was used to pool the results for the NHS CGEMS, SHARE Women and SHARE Men studies. Cochran’s Q statistic and tau-squared were used to test for between-study heterogeneity, and the I^2 -squared statistic to quantify the proportion of total variation due to heterogeneity will be calculated (94). We saw no evidence for systematic bias in the distribution of P -values from the meta-analyses (Supplementary Material, Fig. S1). The P -for-heterogeneity is reported in Table 2. SAS PROC

GLM was used to test the independence of the estimates of multiple SNPs in a model. Composite LD was calculated according to the notation of Weir (51).

Our analyses do not explicitly account for the ascertainment of subjects on the basis of primary disease (breast cancer for the NHS CGEMS; Framingham SHARE data are from a cohort study and not ascertained on disease). This approach is appropriate, since our loci of interest are not associated with primary disease evaluated in the CGEMS NHS participants (85,95). All P -values are based on two-sided tests. All statistical analyses were performed with SAS (version 9.1; SAS Institute, Cary, NC, USA), R project (<http://www.r-project.org>) and PLINK (96).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

In this study, all plasma assays for CGEMS and SHARE were conducted at the Jean Mayer U.S. Department of Agriculture Human Nutrition Research Center on Aging at Tufts University. This manuscript does not necessarily reflect the opinions or views of Boston University or the NHLBI.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the National Institutes of Health [research grant numbers. U54 CA100971, P01 CA87969, P01 CA55075, U01 CA098233, R01 CA 065725, R01 CA070817 and R03 CA133937].

The Framingham Heart Study is conducted and supported by the National Heart, Lung and Blood Institute (NHLBI) in collaboration with Boston University.

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Diet and the evolution of human amylase gene copy number variation

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Starch consumption is a prominent characteristic of agricultural societies and hunter-gatherers in arid environments. In contrast, rainforest and circum-arctic hunter-gatherers and some pastoralists consume much less starch^{1–3}. This behavioral variation raises the possibility that different selective pressures have acted on amylase, the enzyme responsible for starch hydrolysis⁴. We found that copy number of the salivary amylase gene (*AMY1*) is correlated positively with salivary amylase protein level and that individuals from populations with high-starch diets have, on average, more *AMY1* copies than those with traditionally low-starch diets. Comparisons with other loci in a subset of these populations suggest that the extent of *AMY1* copy number differentiation is highly unusual. This example of positive selection on a copy number-variable gene is, to our knowledge, one of the first discovered in the human genome. Higher *AMY1* copy numbers and protein levels probably improve the digestion of starchy foods and may buffer against the fitness-reducing effects of intestinal disease.

Hominin evolution is characterized by significant dietary shifts, facilitated in part by the development of stone tool technology, the control of fire and, most recently, the domestication of plants and animals^{5–7}. Starch, for instance, has become an increasingly prominent component of the human diet, particularly among agricultural societies⁸. It stands to reason, therefore, that studies of the evolution of amylase in humans and our close primate relatives may provide insight into our ecological history. Because the human salivary amylase gene (*AMY1*) shows extensive variation in copy number^{9,10}, we first assessed whether a functional relationship exists between *AMY1* copy number and the amount of amylase protein in saliva. We then determined if *AMY1* copy number differs among modern human populations with contrasting amounts of dietary starch.

We estimated diploid *AMY1* gene copy number for 50 European Americans using an *AMY1*-specific real-time quantitative PCR (qPCR) assay. We observed extensive variation in *AMY1* copy number in this population sample (Fig. 1a and Supplementary Table 1 online), consistent with previous studies^{10,11}. Next, we performed protein blot experiments with saliva samples from the same individuals in order to estimate salivary amylase protein levels (Fig. 1b). These experiments showed a significant positive correlation between salivary amylase gene copy number and protein expression ($P < 0.001$; Fig. 1c).

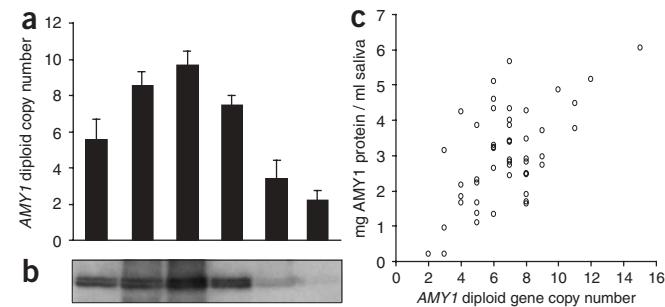
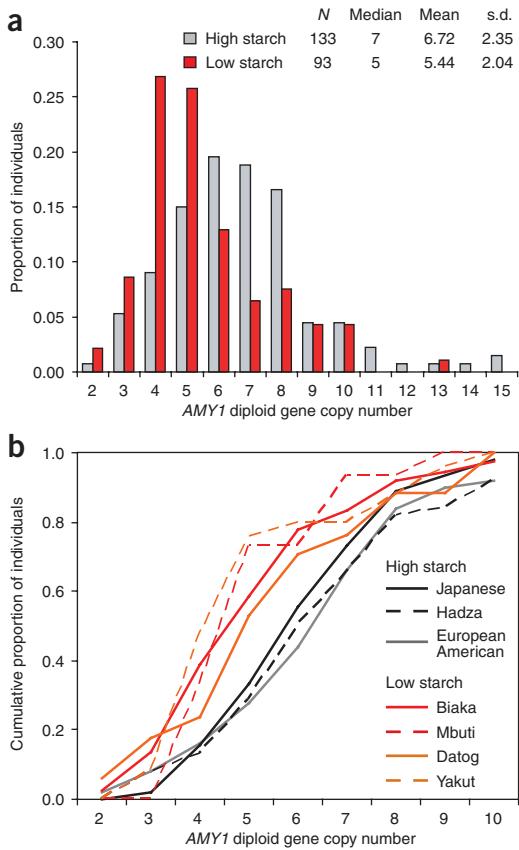


Figure 1 *AMY1* copy number variation and salivary amylase protein expression. (a,b) For the same European American individuals, we estimated diploid *AMY1* gene copy number with qPCR (a) and estimated amylase protein levels in saliva by protein blot (b). Error bars indicate s.d. (c) Relationship between *AMY1* diploid copy number and salivary amylase protein level ($n = 50$ European Americans). A considerable amount of variation in *AMY1* protein expression is not explained by copy number ($R^2 = 0.351$), which may reflect other genetic influences on *AMY1* expression, such as regulatory region SNPs or nongenetic factors that may include individual hydration status, stress level and short-term dietary habits.

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Received 9 May; accepted 3 August; published online 9 September 2007; doi:10.1038/ng2123



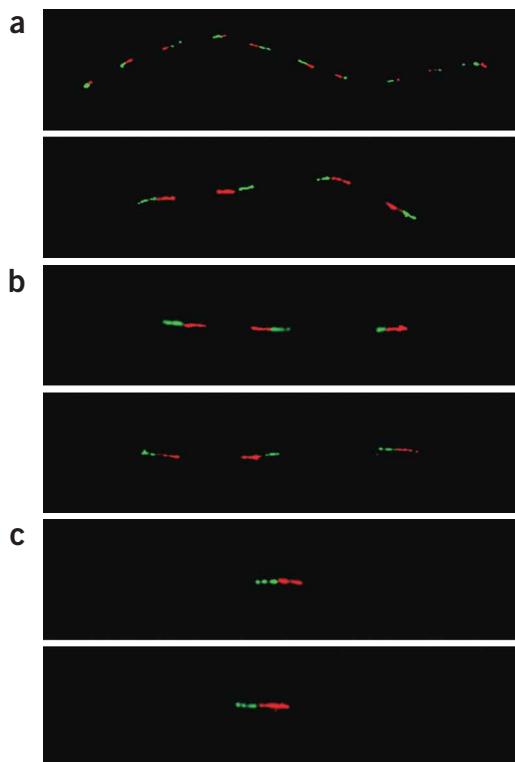
Although there is a considerable range of variation in dietary starch intake among human populations, a distinction can be made between ‘high-starch’ populations for which starchy food resources comprise a substantial portion of the diet and the small fraction of ‘low-starch’ populations with traditional diets that incorporate relatively few starchy foods. Such diets instead emphasize proteinaceous resources (for example, meats and blood) and simple saccharides (for example, from fruit, honey and milk). To determine if *AMY1* copy number differs among populations with high- and low-starch diets, we estimated *AMY1* copy number in three high-starch and four low-starch population samples. Our high-starch sample included two agricultural populations, European Americans ($n = 50$) and Japanese ($n = 45$), and Hadza hunter-gatherers who rely extensively on starch-rich roots and tubers ($n = 38$)¹². Low-starch populations included Biaka ($n = 36$) and Mbuti ($n = 15$) rainforest hunter-gatherers, Datog pastoralists ($n = 17$) and the Yakut, a pastoralist, fishing society ($n = 25$). Additional details on the diets of these populations are provided in **Supplementary Table 2** online. We found that mean diploid *AMY1* copy number was greater in high-starch populations (Fig. 2 and

Figure 3 High-resolution fiber FISH validation of *AMY1* copy number estimates. Red (~10 kb) and green (~8 kb) probes encompass the entire *AMY1* gene and a retrotransposon directly upstream of (and unique to) *AMY1*, respectively. (a) Japanese individual GM18972 was estimated by qPCR to have 14 (13.73 ± 0.93) diploid *AMY1* gene copies, consistent with fiber FISH results showing one allele with ten copies and the other with four copies. (b) Biaka individual GM10472 was estimated by qPCR to have six (6.11 ± 0.17) diploid *AMY1* gene copies, consistent with fiber FISH results. (c) The reference chimpanzee (Clint; S006006) was confirmed to have two diploid *AMY1* gene copies.

Figure 2 Diet and *AMY1* copy number variation. (a) Comparison of qPCR-estimated *AMY1* diploid copy number frequency distributions for populations with traditional diets that incorporate many starch-rich foods (high-starch) and populations with traditional diets that include little or no starch (low-starch). (b) Cumulative distribution plot of diploid *AMY1* copy number for each of the seven populations in the study.

Supplementary Fig. 1 online). Notably, the proportion of individuals from the combined high-starch sample with at least six *AMY1* copies (70%) was nearly two times greater than that for low-starch populations (37%). To visualize the allele-specific number and orientation of *AMY1* gene copies, we performed high-resolution FISH on stretched DNA fibers (fiber FISH); these results were consistent with diploid *AMY1* copy number estimates from our qPCR experiments (Fig. 3).

The among-population patterns of *AMY1* copy number variation do not fit expectations under a simple geographical region-based model of genetic drift: our high- and low-starch samples include both African and Asian populations, suggesting that diet more strongly predicts *AMY1* copy number than geographic proximity. Based on this observation, we hypothesized that natural selection may have influenced *AMY1* copy number in certain human populations. However, we cannot rigorously test such a hypothesis on the basis of our qPCR results alone, in part because we lack comparative data from other loci. Therefore, we next performed array-based comparative genomic hybridization (aCGH) on the Yakut population sample with a whole-genome tile path (WGTP) array platform that was previously used¹¹ to describe genome-wide patterns of copy number variation in 270 individuals (the HapMap collection), including the same Japanese population sample as in our study. For the Yakut aCGH experiments, we used the same reference DNA sample (NA10851) as in the previous study¹¹, facilitating comparisons of Japanese and Yakut relative intensity \log_2 ratios for the 26,574 BAC clones on the array, including two clones mapped to the *AMY1* locus.



Results from the two *AMY1*-mapped clones on the WGTP array supported our original observations: the \log_2 ratios were strongly correlated with the qPCR estimates of *AMY1* diploid copy number (**Supplementary Fig. 1**), and the population mean \log_2 ratios for both clones were greater for the Japanese sample (**Fig. 4a** and **Supplementary Fig. 1**). More importantly, with the WGTP data, we were able to compare the extent of population differentiation at the *AMY1* locus to other loci in the genome for the two Asian population samples in our study. We would expect the magnitude and direction of the Japanese-Yakut mean \log_2 ratio difference for the *AMY1*-mapped clones to be similar to those for other copy number-variable clones, if these CNVs have experienced similar evolutionary pressures. However, the two *AMY1*-mapped clones are significant outliers in this distribution (**Fig. 4b** and **Supplementary Fig. 2** online), leading us to reject this null hypothesis. In addition, we considered a database of genotypes for 783 genome-wide microsatellites for the same Yakut individuals and a different Japanese population sample¹³, because microsatellite loci are usually multiallelic (as is the *AMY1* locus). We found that the extent of Japanese-Yakut differentiation at the *AMY1* locus exceeded that for >97% of the microsatellite loci (**Supplementary Fig. 3** online). Although this result should be interpreted with caution because we do not know whether *AMY1* copy number and microsatellite mutation rates and patterns are similar, this finding is consistent with our results from the WGTP comparison.

These observations suggest that natural selection has shaped *AMY1* copy number variation in either the Japanese or the Yakut or in both populations. We cannot fully test the null hypothesis for the other high- and low-starch populations in our study, but the patterns of copy number variation we observed in these populations are similar to those for the Japanese and Yakut and thus may also reflect non-neutral evolution. We favor a model in which *AMY1* copy number has been subject to positive or directional selection in at least some high-starch populations but has evolved neutrally (that is, through genetic drift) in low-starch populations. Although it is possible that lower *AMY1* gene copy numbers have been favored by selection in low-starch populations, such an interpretation is less plausible for the simple reason that excessive amylase production is unlikely to have a significant negative effect on fitness.

Furthermore, several lines of evidence offer mechanisms by which higher salivary amylase protein expression may confer a fitness advantage for individuals with a high-starch diet. First, a significant amount of starch digestion occurs in the mouth during mastication¹⁴. For example, blood glucose has been shown to be significantly higher when high-starch foods such as corn, rice and potatoes (but not low-starch foods such as apples) are first chewed and then swallowed, rather than swallowed directly¹⁵. In addition, it has been suggested that oral digestion of starch is critically important for energy absorption during episodes of diarrhea⁴. Diarrheal diseases can have a significant effect on fitness; for example, such diseases caused 15% of worldwide deaths among children younger than 5 years as recently as 2001 (ref. 16). Last, salivary amylase persists in the stomach and intestines after swallowing¹⁷, thereby augmenting the enzymatic activity of pancreatic amylase in the small intestine. Higher *AMY1* copy number and a concomitant increase in salivary amylase protein thus are likely to improve the efficiency with which high-starch foods are digested in the mouth, stomach and intestines and may also buffer against the potential fitness-reducing effects of intestinal disease.

To understand better the evolutionary context of human *AMY1* copy number variation, we analyzed patterns of *AMY1* copy number variation in chimpanzees (*Pan troglodytes*) and bonobos (*Pan paniscus*). In contrast to the extensive copy number variation

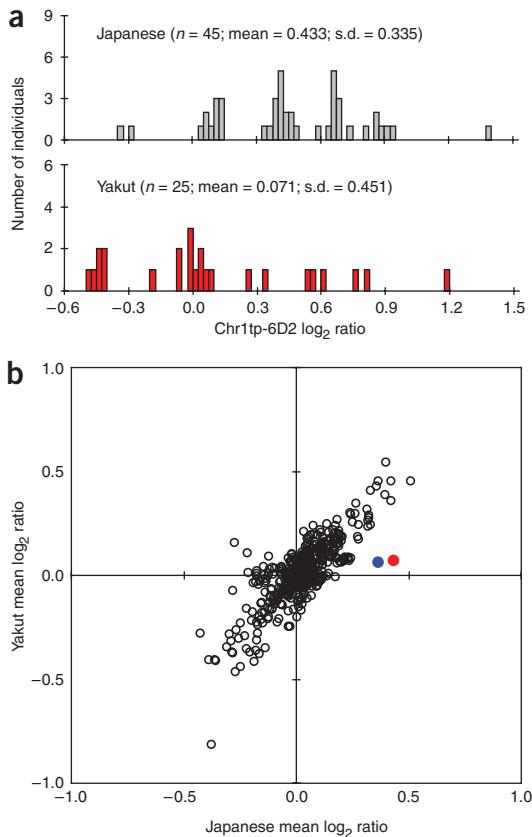


Figure 4 Japanese-Yakut copy number differentiation at *AMY1* versus other genome-wide loci. **(a)** Frequency distributions of WGTP aCGH relative intensity \log_2 ratios from *AMY1*-mapped clone Chr1tp-6D2 for Japanese and Yakut individuals. **(b)** Relationship between Japanese and Yakut mean \log_2 ratios for all autosomal WGTP clones that were copy number variable in both populations. *AMY1*-mapped clones Chr1tp-6D2 and Chr1tp-30C7 are depicted as filled red and blue circles, respectively.

we observed in humans, each of 15 wild-born western chimpanzees (*P. t. verus*) showed evidence of only two diploid *AMY1* copies (**Fig. 3c** and **Supplementary Fig. 4** online), consistent with previous findings^{18–21}. Although we observed evidence of a gain in *AMY1* copy number in bonobos relative to chimpanzees (**Supplementary Fig. 4**), our sequence-based analyses suggest that each of these *AMY1* copies has a disrupted coding sequence and may be nonfunctional (**Supplementary Fig. 5** online). Therefore, the average human has roughly three times more *AMY1* copies than chimpanzees, and bonobos may not have salivary amylase at all. Outgroup comparisons with other great apes suggest that *AMY1* copy number was probably gained in the human lineage, rather than lost in chimpanzees^{21,22}. Given that *AMY1* copy number is positively correlated with salivary amylase protein expression in humans, it stands to reason that the human-specific increase in copy number may explain, at least in part, why salivary amylase protein levels are approximately six to eight times higher in humans than in chimpanzees²³. These patterns are consistent with the general dietary characteristics of *Pan* and *Homo*; chimpanzees and bonobos are predominantly frugivorous and ingest little starch relative to most human populations²⁴.

Among other primates, New World monkeys do not produce salivary amylase and tend to consume little starch, but cercopithecines (a subfamily of Old World monkeys including macaques and

mangabeys) have relatively high salivary amylase expression, even compared to humans²³. Although the genetic mechanisms are unknown, this expression pattern may have evolved to facilitate the digestion of starchy foods (such as the seeds of unripe fruits) stowed in the cheek pouch, a trait that, among primates, is unique to cercopithecines²⁵.

The initial human-specific increase in *AMY1* copy number may have been coincident with a dietary shift early in hominin evolutionary history. For example, it is hypothesized that starch-rich plant underground storage organs (USOs) such as bulbs, corms and tubers were a critical food resource for early hominins^{26,27}. Changes in USO consumption may even have facilitated the initial emergence and spread of *Homo erectus* out of Africa^{5,28}. Yet such arguments are difficult to test, mainly because direct evidence for the use of USOs is difficult to obtain, particularly for more remote time periods. USOs themselves are perishable, as are many of the tools used to collect and process them. Therefore, understanding the timing and nature of the initial human-lineage *AMY1* duplications may provide insight into our ecological and evolutionary history. The low amount of nucleotide sequence divergence among the three *AMY1* gene copies found in the human genome reference sequence (hg18; $d = 0.00011$ to 0.00056) implies a relatively recent origin that may be within the time frame of modern human origins (that is, within the last $\sim 200,000$ years; based on human-chimpanzee *AMY1* $d = 0.027$ and using an estimate of 6 million years ago for divergence of the human and chimpanzee lineages). However, given the possibility for gene conversion, we do not necessarily consider this estimate to be reliable. The generation of *AMY1* sequences from multiple humans may ultimately help to shed light on this issue.

In summary, we have shown that the pattern of variation in copy number of the human *AMY1* gene is consistent with a history of diet-related selection pressures, demonstrating the importance of starchy foods in human evolution. Although the amylase locus is one of the most variable in the human genome with regard to copy number¹⁰, it is by no means unique; a recent genome-wide survey identified 1,447 copy number-variable regions among 270 phenotypically normal human individuals¹¹, and many more such regions are likely to be discovered with advances in copy number variation detection technology. It is reasonable to speculate that copy number variants other than *AMY1* are or have been subject to strong pressures of natural selection, particularly given their potential influence on transcriptional and translational levels (for example, see ref. 29). The characterization of copy number variation among humans and between humans and other primates promises considerable insight into our evolutionary history.

METHODS

Samples. Buccal swabs and saliva were collected under informed consent from 50 European Americans ages 18–30 (Arizona State University institutional review board (IRB) protocol number 0503002355). Saliva was collected for 3 min from under the tongue. Buccal swabs were collected from the Hadza ($n = 38$) and Datog ($n = 17$) from Tanzania (Stanford University IRB protocol number 9798-414). Genomic DNA samples from the Biaka (Central African Republic; $n = 32$), Mbuti (Democratic Republic of Congo; $n = 15$) and Yakut (Siberia; $n = 25$) are from the HGDP-CEPH Human Genome Diversity Cell Line Panel. Lymphoblastoid cell lines from 45 Japanese, 4 additional Biaka and the donor for the chimpanzee genome sequence (Clint) were obtained from the Coriell Institute for Medical Research. Whole blood samples were collected during routine veterinary examinations from chimpanzees and bonobos housed at various zoological and research facilities. Two additional bonobo samples were obtained from the Integrated Primate Biomaterials and Information Resource. DNA was isolated using standard methods.

Copy number estimation. Primers for qPCR (Supplementary Table 3 online) were designed to be specific to *AMY1* (that is, to have sequence mismatches with *AMY2A* and *AMY2B*) based on the human and chimpanzee reference genome sequences. A previous study reported a single (haploid) copy of *AMY1* for one chimpanzee¹⁸, and a recent analysis¹⁹ did not find any evidence of recent *AMY1* duplication for Clint. We used fiber FISH to confirm that Clint has two diploid copies of *AMY1* (Fig. 3c). Therefore, we were able to estimate diploid copy number based on relative *AMY1* quantity for human DNA compared to a standard curve constructed from the DNA of Clint. A fragment from the *TP53* gene was also amplified to adjust for DNA dilution quantity variation. Samples were run in triplicate and standards in duplicate. Experiments were performed and analyzed as described previously²⁰.

Protein blot analysis. Protein samples were prepared by solubilizing saliva samples in 2% SDS and heating at 100 °C for 5 min. These samples were analyzed on mini SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-Millipore). For quantification purposes, a human salivary amylase protein sample of known quantity (Sigma) was run on each gel, with 5 µL of saliva for each sample. After transfer, the membranes were incubated for 1.5 h with primary antibodies raised against human salivary amylase (Sigma). The membranes were washed and goat anti-rabbit alkaline phosphatase-conjugated IgG secondary antibodies (Pierce) were added for 1 h. The membranes were exposed to ECF substrate (Amersham Biosciences) for 5 min and then analyzed using a phosphorimager. Quantification of protein bands was performed using ImageQuant software (Molecular Dynamics).

Fiber FISH. DNA fibers were prepared by gently lysing cultured lymphoblast cells with 300 µL Cell Lysis Buffer (Gentra Systems) per 5 million cells. 10 µL of lysate was placed on a poly-L-lysine-coated slide (LabScientific) and mechanically stretched with the edge of a coverslip. After 30 s, 300 µL of 100% methanol was applied to fix the fibers. Slides were dried at 37 °C for 5 min and then stored at room temperature (22–25 °C).

PCR product probes were made from (i) the entire *AMY1* gene itself (~10 kb; red in images) and (ii) the retrotransposon found directly upstream of all *AMY1* copies but not of pancreatic amylase genes or amylase pseudogenes (~8 kb; green in images). The gene probe may not be specific to *AMY1* under all hybridization conditions (*AMY1* sequence divergence with *AMY2A* and *AMY2B* = 7.5% and 7.1%, respectively), but the upstream probe is. We used long-range PCR followed by nested PCR for each region (primers and conditions are provided in Supplementary Table 3). PCR products were purified with DNA Clean and Concentrator columns (Zymo).

For each nested PCR product, 750 ng was combined with 20 µL 2.5× random primer (BioPrime aCGH Labeling Module, Invitrogen) in a total volume of 39 µL. Samples were incubated at 100 °C for 5 min and were then placed on ice for 5 min. Next, 5 µL 10× dUTP and 1 µL Exo-Klenow Fragment (BioPrime Module) and either 5 µL (5 nmol) Biotin-16-dUTP (Roche; gene probe) or 5 µL (5 nmol) digoxigenin-11-dUTP (Roche; upstream probe) were added, and samples were incubated at 37 °C for 5 h. Labeled products were purified with Microcon Centrifugal Filter Devices (Millipore) using three washes of 300 µL 0.1× SSC, eluted with 50 µL H₂O. For each 1 µg of labeled DNA, we added 10 µg human Cot-1 DNA (Invitrogen).

For each experiment, 500 ng of labeled DNA from each of the nested PCR reactions was combined, lyophilized, reconstituted in 10 µL hybridization buffer (50% formamide, 20% dextran sulfate, 2× SSC) and added to the slide (18 × 18 mm cover glass; Fisher). Fibers and probes were denatured together (95 °C for 3 min) and hybridized in a humidified chamber (37 °C for 40 h). The slide was washed in 0.5× SSC at 75 °C for 5 min followed by three washes in 1× PBS at room temperature (22–25 °C) for 2 min each. Next, fibers were incubated with 200 µL CAS Block (Zymed) and 10% (vol/vol) normal goat serum (Zymed) for 20 min at room temperature (22–25 °C) under a HybriSlip (Invitrogen). We used a three-step detection and amplification (with reagents in 200 µL CAS Block with 10% (vol/vol) normal goat serum). Each step was conducted for 30 min at room temperature under a HybriSlip followed by three washes in 1× PBS for 2 min each at room temperature (22–25 °C). Reagents were as follows for each step: step (i): 1:500 anti-digoxigenin-fluorescein, Fab fragments (Roche) and 1:500 Streptavidin, Alexa Fluor 594

conjugate (Invitrogen); step (ii): 1:250 rabbit anti-FITC (Zymed) and 1:500 biotinylated anti-streptavidin (Vector Laboratories); step (iii): 1:100 goat anti-rabbit IgG-FITC (Zymed) and 1:500 streptavidin, Alexa Fluor 594 conjugate. Images were captured on an Olympus BX51 fluorescent microscope with an Applied Imaging camera and were analyzed with Applied Imaging's Genus software.

aCGH analysis. For aCGH experiments, we used a large-insert clone DNA microarray covering the human genome in tiling path resolution³⁰. Test genomic DNA samples (from Yakut individuals) and reference genomic DNA samples (from NA10851) were labeled with Cy3-dCTP and Cy5-dCTP, respectively (NEN Life Science Products) and were cohybridized to the array. For each sample, a duplicate experiment was performed in dye-swap to reduce false-positive error rates. Labeling, hybridization, washes and analyses were performed as described^{11,30}.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

We are grateful to all our study participants. We thank H. Cann and C. de Toma of the Fondation Jean Dausset (CEPH), the Cincinnati Zoo, the Lincoln Park Zoo, the New Iberia Research Center, the Primate Foundation of Arizona, the Southwest Foundation for Biomedical Research, the Coriell Institute for Medical Research and the Integrated Primate Biomaterials and Information Resource for samples. C. Tyler-Smith and Y. Gilad provided comments on a previous version of the manuscript. We would also like to thank the Wellcome Trust Sanger Institute Microarray Facility for printing the arrays and T. Fitzgerald and D. Rajan for technical support. This study was funded by grants from the L.S.B. Leakey Foundation and Wenner-Gren Foundation (to N.J.D.), the Department of Pathology, Brigham & Women's Hospital (to C.L.), the National Institutes of Health (to the University of Louisiana at Lafayette New Iberia Research Center; numbers RR015087, RR014491 and RR016483) and the Wellcome Trust (H.F., R.R. and N.P.C.).

AUTHOR CONTRIBUTIONS

G.H.P. and N.J.D. contributed equally to this work. G.H.P., N.J.D., C.L. and A.C.S. designed the study; G.H.P., F.A.V., J.L.M. and A.C.S. collected the samples; G.H.P. and A.S.L. performed qPCR experiments; J.W. performed protein blot experiments; G.H.P. performed fiber FISH experiments; H.F. and R.R. performed and analyzed aCGH experiments; K.G.C. performed nucleotide sequencing experiments; G.H.P. performed data analyses; R.M., N.P.C., C.L. and A.C.S. supervised the experiments and analyses and G.H.P. and N.J.D. wrote the paper.

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METHYL GROUP DEFICIENCY IN HEPATOCARCINOGENESIS*

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I. INTRODUCTION

My interest in the role of dietary and physiological methyl insufficiency in hepatocarcinogenesis has its roots in research conducted during my

*Presented at the First Arkansas Toxicology Symposium, honoring Elizabeth C. Miller, Ph.D., and James A. Miller, Ph.D., December 10–11, 1992, at the Arkansas Excelsior Hotel, Little Rock, Arkansas.

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tenure as a graduate student with the Millers at the University of Wisconsin [1]. In the mid-1960s, we discovered that the compound *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene acted as a model ultimate carcinogen for the aromatic aminoazo dye carcinogens. The reaction with methionine of this compound *in vitro*, and of the presumptive *N*-hydroxy metabolite *in vivo*, laid the basis for the identification of the metabolic activation of aromatic aminoazo dye carcinogens [1, 2]. Earlier, for reasons wholly unrelated to the *N*-oxidation of aromatic amines, the Millers, in 1953, had suggested "a methyl deletion hypothesis" as a possible mechanism for aminoazo dye carcinogenesis [3]. Other professors who contributed to my interest in this area of research were: Dr. Charles Heidelberger through his studies on the synthesis and biochemical activity of 5-fluorodeoxyuridine; through its inhibition of the enzyme thymidylate synthetase, this compound upsets the labile 1-carbon pool in cells [4]. Finally, the influence of Dr. Carl A. Bauman, who was then a professor in the Department of Biochemistry at the University of Wisconsin, and who emphasized the importance of both dietary deficiency and antimetabolites as means of obtaining nutritional deficiencies, can be seen throughout this presentation.

II. HISTORICAL DEVELOPMENT PRIOR TO 1980

The major scientific developments showing possible causal links between methyl group insufficiency and hepatocarcinogenesis *in vivo* are summarized in Table 1 [5-15]. The hepatocarcinogenic activity of choline-deficient diets was first reported in 1946 [5]. These early observations were extended to other species and organs but were eventually called into question because of the discovery of aflatoxin contamination in the peanut meal diets used to achieve a low methyl content in the diet [16]. From 1955 to 1983, no major studies appeared in the literature describing the complete carcinogenic activity of choline-deficient diets. Yet, other studies continued to provide indications that hepatocarcinogenesis was frequently associated with or enhanced by methyl insufficiency (Table 1). Also, the administration to rats of the methionine antimetabolite ethionine [17], of the low-lipotrope diet of Newberne [18], and of the amino-acid-defined, methyl-deficient diet of Poirier [19], all provided evidence of hepatic methyl insufficiency either with low levels of *S*-adenosylmethionine (SAM) or of macromolecular hypomethylation [20]. Finally, the results of LaPeyre and Becker [15] clearly showed that the administration of the hepatocarcinogen 2-acetylaminofluorene, under conditions leading to liver tumor formation, also led to the hypomethylation of hepatic DNA.

TABLE 1

Major Developments Prior to 1980 Showing Links Between Methyl Group Insufficiency and Hepatocarcinogenesis

Year(s)	Group	Findings
1946–1954	Salmon and Copeland [5, 6]	Choline-deficient diets, containing peanut meal or soybean meal, caused liver cancer in rats and mice. These diets were later shown to be contaminated with the liver carcinogen aflatoxin.
1956–1963	E. Farber [7, 8]	The methionine antagonist ethionine caused liver cancer in rats.
1972–1980	L. Poirier et al. [9, 10]	Chronic administration of liver carcinogens or of methyl-deficient diets to rats led to similar biochemical changes in the liver, including decreased hepatic SAM.
1974–1980	Newberne, Rogers et al. [11, 12]	A low-lipotrope (methyl-deficient) diet enhanced the activities of several hepatocarcinogens in rats.
1978–1980	Lombardi, Shinozuka et al. [13, 14]	A choline-devoid diet exerted both cocarcinogenic and tumor-promoting activities toward the livers of rats.
1979	LaPeyre and Becker [15]	Feeding a carcinogenic regime of 2-acetylaminofluorene to rats led to DNA hypomethylation in the liver.

III. HEPATOCARCINOGENESIS AND BIOCHEMICAL CHANGES

Beginning in 1983, three publications clearly showed that carcinogen-free methyl-deficient diets caused liver cancer in rats [21–23]. In the first study, Mikol et al. [21], using an amino acid-defined diet, showed that a

diet lacking methionine and choline produced liver cancer by itself and enhanced liver tumor formation in rats given a single initiating dose of diethylnitrosamine. Ghoshal and Farber [22] and Yokoyama et al. [23], using alcohol-extracted, peanut-meal-containing diets, obtained similar tumor yields in the livers of male rats. Thus, the hepatocarcinogenicity of choline or methyl deprivation seemed to be clearly established. In fact, most of the biochemical changes commonly associated with liver tumor formation by chemical carcinogens were also seen during hepatocarcinogenesis by methyl deprivation. These included: the accumulation of lipid peroxides [24, 25], increased levels of ornithine and SAM decarboxylases [19], increased thymidine incorporation into liver DNA [26], decreased levels of histidase and urocanase [27], and finally, increased formation of altered hepatic foci [28, 29]. The latter findings were particularly interesting from a potentially mechanistic standpoint. In the absence of diethylnitrosamine initiation, the chronic feeding of the amino-acid-defined diet, lacking both methionine and choline, led to the formation of altered hepatic foci which continuously increased in volume but not in number; this observation is consistent with the hypothesis that methyl deprivation is not a continuously initiating stress, but rather is a highly effective promoter of preexisting, initiated hepatocytes [28]. The strong liver-tumor-promoting activity of choline deficiency had been previously described [30].

While the effects of dietary methyl deficiency on hepatocarcinogenesis were being explored, other investigations were conducted on the interactions between hepatocarcinogens and liver tumor promoters and labile methyl groups. In particular, the effects of such interactions in liver tumor formation, on the hepatic levels of SAM, and on the DNA content of 5-methyldeoxycytidine (5-MC) were determined. The results are summarized in Table 2 [8, 9, 12, 15, 18, 21, 31-39]. The hepatocarcinogenic activities of three of the four carcinogens examined—ethionine, diethylnitrosamine, and 2-acetylaminofluorene—were shown to be enhanced by dietary lipotrope deficiency or to be inhibited by high dietary levels of methionine and/or choline (Table 2). In one study, lipotrope deficiency was not shown to exert any significant effect upon the hepatocarcinogenic activity of dimethylnitrosamine [12]. Further, the liver-tumor-promoting activity of phenobarbital and of DDT was suppressed in rodents by the feeding of high dietary levels of methionine and choline (Table 2) [31]. All six of the hepatocarcinogenic stressors whose activity was inhibited by methyl donors were also shown to decrease the hepatic content of SAM or the ratio of SAM to S-adenosylhomocysteine (SAH) in the livers of chronically fed rats (Table 2). SAH is both a product of and a strong inhibitor of SAM-dependent methylations [40]. Similarly, four such carcinogenic regimes led to decreased 5-MC in hepatic DNA under chronic feeding conditions (Table 2) [15, 35-

TABLE 2

Hepatocarcinogens and Liver Tumor Promoters Whose Activities Are Altered by Methyl Donors or Which Decrease Hepatic SAM or 5-Methyldeoxycytidine in Total Hepatic DNA of Rats

Compound or diet	Antagonism by methyl donors ^a	SAM	5-MC	Ref.
Methyl deficiency	+	+	+	[21, 32, 35]
Ethionine	+	+	+	[8, 33, 36, 37]
Diethylnitrosamine	+	+		[12, 21, 9]
2-Acetylaminofluorene	+	+	+	[12, 18, 15]
Phenobarbital	+	+	+	[31, 34, 38]
DDT	+	+		[31, 34]
Dimethylnitrosamine	-			[12]
Methapyrilene		- ^b	- ^b	[39]

^aOr enhancement by dietary methyl deficiency.

^bIncreased.

38]. An exception to the above generalization is the compound methapyrilene. The chronic administration of this hepatocarcinogen to rats was shown to increase ratios of SAM to SAH and to increase the extent of DNA methylation in the liver [39].

IV. ALTERATIONS IN ONCOGENES DURING HEPATOCARCINOGENESIS BY METHYL DEPRIVATION

The early association between decreased hepatic SAM and DNA hypomethylation led to the proposal that increased oncogene expression caused by such hypomethylation was a causative factor in hepatocarcinogenesis by methyl deficiency. This hypothesis was supported by a number of related observations. Specific gene hypomethylation has been commonly associated with increased expression [41]. Two DNA hypomethylating agents, the antimetabolites ethionine and 5-azacytidine, altered differentiation of Friend erythroleukemia cells *in vitro* [42]. 5-Azacytidine transformed cells *in vitro* [43] and enhanced liver tumor formation *in vivo* [44, 45]. SAM-dependent DNA methylation inhibited the transforming activity of the Moloney sarcoma virus *in vitro* [46]. Generalized DNA hypomethylation was

commonly seen in the tumors and transformed cells compared to the tissues and cells of origin [47]. Finally, the H-ras oncogene of colon carcinomas was hypomethylated compared to the adjacent normal tissue [48]. Our own studies showed that the 18-month feeding of any of several methyl-deficient amino-acid-defined diets led to hypomethylation of the c-H-ras and c-K-ras oncogenes in both neoplastic and preneoplastic liver [49]. More recent results showed that 1- to 32-week feeding of the carcinogenic methyl-deficient amino-acid-defined diet resulted in the hypomethylation of CCGG sequences in the c-H-ras, the c-K-ras, and the c-fos protooncogenes at all times investigated [50]. In fact, the methylation changes in the c-H-ras gene increased in intensity throughout the experimental period until at 32 weeks they resembled those seen in the livers of rats fed the same diet for 18 months. Slot-blot analyses of the same livers showed increased expression of the same three protooncogenes correlating with decreased methylation [51]. These results demonstrate early selective hypomethylation of some CCGG sites, accompanied by increased gene expression, in rats undergoing hepatocarcinogenesis by dietary methyl deprivation. All of the oncogene changes described above were independent of carcinogen administration.

Several other laboratories have investigated the correlations between DNA methylation and oncogene expression during hepatocarcinogenesis. Wainfan, Christman, and their collaborators [52, 53] showed that the chronic administration to rats of an amino-acid-defined, methyl-deficient diet for 1–4 weeks led to the following: (1) DNA hypomethylation as measured by SAM-dependent, methyl acceptor activity; (2) increased DNA methylase activity; (3) increased expression and decreased methylation of the protooncogenes c-myc, c-fos, and c-H-ras; and (4) no change in the expression of methylation of the c-K-ras oncogene. All of the changes observed by this group could be reversed by placing the animals on the corresponding methyl-adequate diet for 1–2 weeks [52, 53]. The group of Locker and Lombardi has examined the hepatocarcinogenic effects of a choline-devoid diet on the amplification and methylation of the c-myc protooncogene [54]. They found that: (1) c-myc was amplified 2- to 70-fold in all tumors produced by the diet; (2) relative c-myc transcript levels increased with the stages of neoplasia; (3) hypomethylation of c-myc was observed in the livers of rats fed the choline-devoid diet for 12 or more months; and (4) the c-myc gene in tumors showed varying degrees both of hypermethylation and of specific site hypomethylation. In related studies, Feo and his collaborators [55] investigated SAM/SAH ratios as well as oncogene methylation and expression in the persistent nodules produced in the livers of rats treated by a modified Solt-Farber regime [56]. They found the following changes in the persistent nodules: (1) decreased SAM/SAH ratios to 40% of controls;

TABLE 3

Effects of SAM on the Incidence of Hepatocellular Carcinomas in Rats Subjected to the Initiation/Selection/Promotion Regime^a

Time of sacrifice (months)	Incidence of liver carcinomas	
	Control	SAM-treated
6	0/10	0/10
14	11/12	1/12
24–28	10/10	3/11

^aDerived from Ref. 58.

(2) decreased 5-MC content to 50% of control values; (3) 4- to 6-fold increases in the c-K-ras, c-H-ras, and c-myc RNA transcripts; and (4) hypomethylation of all three protooncogenes investigated [55]. Chronic treatment of such animals with SAM led to partial, but marked, reversal of all molecular changes noted [57]. In an extension of these studies, the same group showed that the chronic administration of SAM itself markedly inhibited the tumor-promoting activity of phenobarbital in rats previously treated by the Solt-Farber regimen (Table 3) [58].

While the changes described above essentially describe correlations between oncogene hypomethylation and activation, other results recently showed a high frequency of mutations in the *p53* gene in the DNA from tumors caused by choline deprivation [59]. In preliminary studies, DNA from tumors derived from the livers of rats fed amino-acid-defined, methyl-deficient diets transformed NIH-3T3 cells [49]. However, in this study, only 4 of 28 tumor DNA samples examined exhibited transfecting activity; all showed amplified H-ras in the transfecants. However, all of the positive DNA samples came from diethylnitrosamine-initiated rats; none of the DNA samples from the uninitiated rats fed the severely methyl-deficient diets transformed NIH-3T3 cells [49]. In more recent studies, DNA samples from liver tumors developing in rats fed the methionine- and choline-deficient diet, as well as from cells obtained from these tumors, transformed NIH-3T3 cells; the cells displayed c-myc amplification [60].

In summary, the molecular biological studies conducted to date on hepatocarcinogenesis by dietary methyl deprivation indicate that while hypomethylation and increased expression of c-H-ras, c-K-ras, and c-fos have all been observed, their role in carcinogenesis, in the absence of an initiating agent, remains to be established. On the other hand, the presence of activated *myc* and mutated *p53* genes in the tumors of animals fed the

methyl-deficient diets without any further carcinogen treatment suggests that such changes are likely to play a causative role in carcinogenesis in this system.

V. ALTERNATE MECHANISMS

While the prospective mechanism of carcinogenesis by methyl insufficiency emphasized in this presentation is enhanced oncogene expression caused by gene hypomethylation, other mechanisms may be responsible for such biological effects. Chief among the hypotheses proposed are mutations produced by varying mechanisms such as oxidative damage [61, 62], abnormal DNA methylation [63, 64], and altered deoxynucleotide pool sizes [65]. Consistent with a hypothesis that oxidative damage to DNA results in the mutations leading to cancer are the observations that the chronic administration of choline-deficient diets leads to: (1) single-strand breaks [61]; (2) high hepatic levels of peroxidized lipids [61, 66]; (3) the inhibition of altered hepatic foci induced by choline deprivation through feeding of antioxidants [67]; and (4) the formation of 8-hydroxyguanine in hepatic DNA [62, 68]. On the other hand, a recent elegant study of Shen et al. [64] has shown that the transfection of the Hpa II methylase gene into bacteria lacking uracil-DNA glycosylase results in a high frequency of C to U transition mutations in the absence of the substrate SAM. Such mutations were reduced to background levels by high levels of SAM or SAH [64]. These results are consistent with previous findings that a high proportion of inactivating mutations in the *p53* gene in colorectal cancer occur at methylation sites on DNA [69] and that the cytosine complex with methylase is chemically susceptible to deamination unless it is rapidly methylated with SAM [70]. Finally, the control exerted by SAM on folate distribution is expected to alter the deoxynucleotide pool sizes in target tissues [71]. This is clearly the case in the spleens of rats fed diets deficient in folate, methionine, and choline (Table 4) [72]. Significant decreases in the dGTP, dATP, and dTMP were noted in the spleens of rats fed a folate-deficient or a methionine/choline-deficient diet.

Extensions to other organs of such studies indicate that physiological methyl insufficiency may contribute to carcinogenesis to biological systems other than the livers of choline-deficient rats. For example, a number of observations have linked the abnormal differentiation of pancreatic acinar cells with an inadequate supply of methyl donors. Thus, pancreatic degeneration is a common feature of ethionine toxicity in rodents [8]. Pancreatic

TABLE 4
Deoxyribonucleotide Pools in the Spleens of Rats
Chronically Fed Methyl-Deficient Diets^a

Dietary group	Deoxyribonucleotide level (pmol/10 ⁶ cells) ^b					
	dCTP	dTTP	dGTP	dATP	dUMP	dTMP
Control	23.1 ±3.7	20.9 ±5.9	20.1 ±2.2	4.2 ±1.5	4.7 ±0.6	9.6 ±1.8
FD ^c	19.2 ±3.8	13.6 ±6.1	7.5 ^d ±8.5	2.5 ^d ±0.3	4.1 ±0.9	5.2 ^d ±2.1
MCD ^c	18.0 ±5.5	13.9 ±4.2	13.6 ^d ±3.9	2.5 ^d ±0.6	3.2 ±1.1	6.8 ^d ±2.4
MCFC ^c	17.3 ±4.6	13.3 ^d ±3.8	2.0 ^d ±3.5	2.3 ^d ±0.8	6.4 ^d ±1.2	5.7 ^d ±1.3

^aCompiled from Ref. 72.

^bMean ± SD.

^cFD = folate deficient; MCD = methionine and choline deficient; MCFC = methionine, choline, and folate deficient.

^dSignificantly different from corresponding control value.

acinar cells from hamster embryos differentiate normally only in the presence of such methyl group sources as methionine, choline, and SAM [73]. Further, hepatization of pancreatic acinar cells occurs in hamsters treated sequentially with ethionine and a choline-devoid diet [74]. Finally, such hepatization of pancreatic acinar cells is seen in rats chronically fed an amino-acid-defined methyl-deficient diet [75]. Recent results from the studies by Lyn-Cook at NCTR [76-79] have tended to increase the number of associations found between methyl insufficiency, DNA hypomethylation, and susceptibility to transformation by pancreatic acinar cells (Table 5). For example, pancreatic acinar cells *in vitro* acquire hepatocyte-like properties following treatment with the DNA-hypomethylating agent azacytidine (Table 5). Also, pancreatic acinar cells derived from rats undergoing hepatocarcinogenesis by methyl deprivation seem to transform more readily *in vitro* than do the corresponding cells from control animals [79].

The evidence currently available indicates that in humans, folate deprivation may contribute strongly to a methyl insufficiency *in vivo*. Our own interests in this area occurred with the discovery that HIV-infected patients had low levels of both SAM and *N*⁵-methyltetrahydrofolate in their cere-

TABLE 5
Major Findings on Hypomethylation and the Pancreas^a

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1. The DNA hypomethylating agent azacytidine induces transdifferentiation of pancreatic acinar cells *in vitro*.
 2. Azacytidine treatment of pancreatic acinar cells *in vitro* produces biochemical changes similar to those caused by H-ras transfection.
 3. The pancreatic carcinogen azaserine induces hypomethylation and increased expression of c-H-ras oncogene in the pancreas of rats.
 4. Pancreatic acinar cells derived from rats fed an amino-acid-defined, methyl-deficient diet transform more readily than do the corresponding cells from control animals.
-

^aCompiled from Refs. 76–79.

brospinal fluid [80, 81]. Normally, low SAM arising from dietary methyl insufficiency is accompanied by an increased proportion of tissue N^5 -methyltetrahydrofolate [71]. Thus, the prospect arose that the low SAM in these patients was a consequence and not a cause of the one-carbon deficiency noted in these patients [82]. Other studies have increased the number of associations between folate deficiency, physiological methyl group insufficiency *in vivo*, and cancer formation in humans (Table 6) [83–94]. At present, however, we are unaware of any clear evidence indicating that in humans folate deficiency results in methyl insufficiency in any organ.

In rats, however, the situation is different. Within the last few years, Walzem and Clifford [95] developed an amino-acid-defined, folate-deficient diet permitting the long-term survival of rats. The chronic administration of these diets to rats has resulted in: (1) significant general hypomethylation of

TABLE 6
Folate Deficiency, Choline Deficiency, and Cancer in Humans

Folate deficiency is relatively common and choline deficiency is fairly uncommon in the human population [83, 84].

Alcohol induces both hepatotoxicity and folate deficiency in humans and experimental animals; its hepatotoxic effects can be inhibited by SAM [85–90].

Clinical studies have shown an association between folate deficiency and dysplasia in the cervical, bronchial, and colonic epithelium, as well as with colon tumor formation [91–94].

liver DNA [96]; (2) 57–88% decreases in the pancreatic ratios of SAM to SAH, chiefly by large increases in SAH [97]; and (3) enhanced formation of dysplasia and of neoplastic lesions in the colon of dimethylhydrazine-treated rats [98]. Thus, it appears reasonable to propose that the physiological insufficiency of methyl donors that occurs in rats as a consequence of either choline or methionine deficiency may also be seen in humans, especially under conditions of toxic stress.

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Prevention and Epidemiology**Multivitamins, Folate, and Green Vegetables Protect against Gene Promoter Methylation in the Aerodigestive Tract of Smokers**

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Abstract

One promising approach for early detection of lung cancer is by monitoring gene promoter hypermethylation events in sputum. Epidemiologic studies suggest that dietary fruits and vegetables and the micronutrients they contain may reduce risk of lung cancer. In this study, we evaluated whether diet and multivitamin use influenced the prevalence of gene promoter methylation in cells exfoliated from the aerodigestive tract of current and former smokers. Members ($N = 1,101$) of the Lovelace Smokers Cohort completed the Harvard Food Frequency Questionnaire and provided a sputum sample that was assessed for promoter methylation of eight genes commonly silenced in lung cancer and associated with risk for this disease. Methylation status was categorized as low (fewer than two genes methylated) or high (two or more genes methylated). Logistic regression models were used to identify associations between methylation status and 21 dietary variables hypothesized to affect the acquisition of gene methylation. Significant protection against methylation was observed for leafy green vegetables [odds ratio (OR) = 0.83 per 12 monthly servings; 95% confidence interval (95% CI), 0.74–0.93] and folate (OR, 0.84 per 750 µg/d; 95% CI, 0.72–0.99). Protection against gene methylation was also seen with current use of multivitamins (OR, 0.57; 95% CI, 0.40–0.83). This is the first cohort-based study to identify dietary factors associated with reduced promoter methylation in cells exfoliated from the airway epithelium of smokers. Novel interventions to prevent lung cancer should be developed based on the ability of diet and dietary supplements to affect reprogramming of the epigenome. *Cancer Res.* 70(2): 568–74. ©2010 AACR.

Introduction

Lung cancer, the leading cause of cancer-related death in the United States, occurs largely from chronic exposure to tobacco carcinogens (1). The development of this disease over 30 to 40 years involves field cancerization, characterized as the acquisition of genetic and epigenetic changes throughout the respiratory epithelium (2, 3). The silencing of genes through promoter hypermethylation is now recognized as a major and causal epigenetic event that occurs during lung cancer initiation and progression to affect the function of hundreds of genes. Gene silencing involves methylation of cy-

tosines in the gene promoter region, recruitment of transcriptional corepressors, and modification of histone tails that culminate in the establishment of chromatin modifications that block transcription (4, 5). Genes involved in all aspects of normal cell function, such as regulating the cell cycle, differentiation, adhesion, and death, are silenced in lung tumors (3). Importantly, the tumor suppressor gene *p16*, which plays a critical role in regulating the cell cycle, is not only commonly silenced by methylation in lung cancer but also inactivated early in the development of this disease. Silencing of *p16* and other genes is detected in bronchial epithelium of smokers and in precursor lesions to adenocarcinoma and squamous cell carcinoma and increases during disease progression, substantiating a vital role for gene silencing in lung cancer etiology (6–8).

Based on the silencing of key tumor suppressor genes in the lungs of smokers, we hypothesized that the detection of gene-specific promoter hypermethylation in exfoliated cells in sputum would provide an assessment of the extent of field cancerization that in turn may predict early lung cancer. This hypothesis has been validated in two studies, the first detecting methylation of the *p16* and *MGMT* genes in sputum up to 3 years before clinical diagnosis of squamous cell carcinoma (9). The second study, a nested, case-control study of incident lung cancer cases from a high-risk cohort, identified six genes associated with >50% increased lung cancer risk. Importantly,

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi: 10.1158/0008-5472.CAN-09-3410

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concomitant methylation of three or more of these six genes was associated with a 6.5-fold increased risk of incident lung cancer and sensitivity and specificity both at 64% (10). These studies suggested that gene promoter hypermethylation in sputum could be used as a molecular marker for identifying people at high risk for cancer incidence (11). However, the precise mechanism by which carcinogens disrupt the capacity of cells to maintain the epigenetic code during DNA replication and repair is largely unknown.

The fact that gene promoter methylation is a promising marker for lung cancer makes understanding factors that influence the propensity for this epigenetic process throughout the respiratory epithelium a high priority because such knowledge could be used not only for early detection but also to identify persons who would benefit most from chemoprevention. The precise mechanism by which carcinogens disrupt the capacity of cells to maintain the epigenetic code during DNA replication and repair is largely unknown. Carcinogens within tobacco induce single- and double-strand breaks in DNA, and reduced DNA repair capacity (DRC) has been associated with lung cancer (12). Accumulating evidence from our group suggests that extensive DNA damage could be responsible for acquisition of gene promoter hypermethylation during lung carcinogenesis (3, 13). Strong support for this supposition was provided through a recent community-based study in which a highly significant association was seen between DRC and sequence variants within specific DNA repair genes and the propensity for methylation of genes detected in sputum from cancer-free smokers from the Lovelace Smokers Cohort (14). Specifically, smokers with a high methylation index (defined by having three or more genes methylated from an eight-gene panel in sputum) had a 50% reduction in DRC compared with smokers with no genes methylated in sputum. Single nucleotide polymorphisms within five double-strand break DNA repair genes were also highly associated with methylation index. This study suggests that chronic DNA damage coupled with reduced DRC could be an important determinant for inducing gene promoter hypermethylation.

Epidemiologic studies suggest that select dietary nutrients and vitamin supplements might protect against lung cancer (15). Fruits, vegetables, and multivitamins all possess antioxidant activity that should reduce tobacco-induced DNA damage. In addition, folate, a B vitamin, is metabolized to 5-methyltetrahydrofolate that provides methyl groups for *S*-adenosylmethionine (SAM), a universal donor for reactions that include methylation of DNA (16). Low folate has been associated with reduced DRC and an increase in prevalence for gene promoter methylation (17, 18). The purpose of the current investigation was to determine whether diet and multivitamin use influence the presence of methylation in cells exfoliated from the aerodigestive tract of current and former smokers. Composite variables were selected based on our hypotheses that fruits, tomatoes, cruciferous vegetables, leafy vegetables, yellow vegetables, and vitamin intake will be associated with a reduction in number of genes methylated in sputum, whereas animal fat and red and processed meat will be associated with increased methylation.

Materials and Methods

Study population. The Lovelace Smokers Cohort began recruitment of female smokers in 2001 and expanded to include male smokers in 2004 (14). Enrollment, which is still ongoing, is restricted to current and former smokers ages 40 to 75 y with a minimum of 15 pack-years of smoking. Participants primarily are residents of the Albuquerque, New Mexico metropolitan area. Participants complete a standard questionnaire covering demographics, smoking history, personal and family health, and a food frequency questionnaire. Weight and height are measured. Participants provide both blood and sputum samples and undergo standard pulmonary function testing. A total of 1,145 people completed a food frequency questionnaire and were assessed for prevalence for methylation of eight genes in sputum. Those with caloric intake outside of gender-specific bounds ($n = 44$) were excluded, resulting in a total of 1,101 participants (845 women and 256 men) in this study. All participants signed a consent form, and the Western Institutional Review Board approved this project.

Dietary questionnaire. Participants completed the adult version of the Harvard University Food Frequency Questionnaire Dietary Assessment form, a self-administered instrument that includes ~150 food items (19). The participant indicates consumption frequency for most food items by choosing 1 of 5 to 10 categories that vary depending on the food item and can range from never to six or more servings per day. A food group analysis was conducted to combine food items to obtain estimates of intake of macronutrients and micronutrients. We focused on factors known or suspected of being associated with lung cancer or methylation. Thus, the macronutrients animal fat and total fat and the micronutrients vitamin C, vitamin E, folate (that included supplements and fortified foods), carotene, α -carotene, β -carotene, lycopene, lutein and zeaxanthin, and retinol were examined. Alcohol, multivitamins, and cod liver oil intake were also assessed as categorical variables. In a study of eating patterns, Fung and colleagues (20) created a set of 38 composite variables using the food frequency questionnaire. Six of these composite variables, red and processed meats, fruit, tomatoes, cruciferous vegetables, leafy green vegetables, and yellow vegetables, were related to our hypotheses, so we calculated these variables.

Total caloric intake was assessed. People with either extremely low ($n = 8$) or extremely high ($n = 36$) intake were excluded. Cutoffs for extremely low intake were <500 and <800 calories for women and men, respectively. Extremely high intake was defined as >3,500 and >4,200 calories for women and men, respectively. It is standard to exclude participants with at least 70 missing items on the food frequency questionnaire, but none of our participants met this criterion. Participants with missing data on individual food items were excluded from analyses of these items.

Methylation-specific PCR. Eight genes (*p16*, *MGMT*, *DAPK*, *RASSF1A*, *PAX5 α* , *PAX5 β* , *GATA4*, and *GATA5*) were selected for analysis of methylation in sputum based on our previous studies establishing their association with risk for lung

cancer (10, 21). DNA was isolated from sputum and modified with bisulfite as described (10). Nested methylation-specific PCR was used to detect methylated alleles from individual genes in DNA recovered from the sputum samples as described (21). Methylation index, the number of genes methylated in a sputum sample, was also defined. Sputum from males and females was randomly selected and included in batches of 96 samples for assessment of gene methylation. A Hamilton robot was used to assemble PCRs in 96-well plates.

Covariates. The questionnaire included questions on gender, age, ethnicity, and smoking. Cigarette smoking history included current status (former or current), pack-years, and duration of smoking. Body mass index (BMI) was calculated from measured height and weight and categorized as normal ($<25 \text{ kg/m}^2$), overweight ($25\text{--}29.9 \text{ kg/m}^2$), and obese ($\geq 30 \text{ kg/m}^2$). Age was categorized as 40 to 54, 55 to 64, and ≥ 65 y. Pack-years of smoking were categorized as light (<29), moderate (30–49), and heavy (≥ 50).

Statistical methods. Demographic, dietary, and methylation variables were summarized overall and by gender. Proportions were used for categorical variables and medians with the interquartile range (IQR) for continuous variables. Differences between men and women in clinical covariates and categorical dietary variables were assessed with Fisher's exact test. For continuous dietary variables, a two-step linear regression analysis was used to account for differences in total caloric intake between genders. In the first step, the dietary variable was regressed on the total caloric intake. In the second step, the residuals from the first analysis were regressed on gender, which resulted in an estimate of the differences in the mean dietary variable after adjustment for total caloric intake, along with 95% confidence intervals (95% CI).

The total number of methylated genes in the eight-gene panel was dichotomized into low (fewer than two genes methylated) and high (two or more genes methylated). This binary outcome, methylation status, was modeled with logistic regression. Initially, only the clinical covariates gender, age, BMI, and three smoking variables (status, pack-years, and duration) were assessed. Interactions among the covariates, including interactions with gender, were evaluated. After the development of a model with only clinical covariates, individual dietary variables were included, along with adjustment for total caloric intake. Continuous variables, such as total fat intake, were included as a continuous variable or were categorized into quartiles, with the quartiles defined by gender and the quartile medians used as the predictor values. Interactions between dietary variables and clinical covariates were assessed. Only the 21 dietary variables specific to our hypothesis were examined to reduce the potential for false-positive results. In addition, no formal adjustment for multiple comparisons was made to reduce the chance of false-negative results because this is one of the first studies to examine the association between dietary factors and methylation. However, the issue of examining multiple predictor variables is considered in the interpretation of the results. Methylation index was also used as the outcome

variable to further assess the association between methylation and dietary factors. Because the methylation index could theoretically take on nine values, but actually took on only seven, it was unclear that linear regression would be appropriate. Thus, results obtained from linear and ordinal logistic regression were compared. The association between significant dietary factors and each of the individual genes was explored using logistic regression models but viewed as secondary analyses to reduce the issue of multiple comparisons. All statistical analyses were conducted in Statistical Analysis System 9.2.

Results

Population characteristics. The demographics and smoking history of the 1,101 participants are described in Table 1. The Lovelace Smokers Cohort is largely composed of females (76.7%) and non-Hispanic whites (77.8%). Median age was 56 years, with males slightly older than females. More than half of the participants currently smoked, and median duration of smoking was 33 years. There was no difference between men and women with regard to smoking status or duration, but men had significantly higher pack-years of smoking (median = 39 pack-years for men versus 34 pack-years for women).

Summary of dietary intake. The completion rate of the food frequency questionnaire was excellent, as only 2.7% of

Table 1. Characteristics of study participants overall and by gender: frequencies (%)

Demographic characteristic	Overall			Females	Males	P*
	Sample size	1,101	845	256		
Age (y)						
	40–54	46.0	46.7	43.4	0.06	
	55–64	31.8	32.7	28.9		
	65+	22.3	20.6	27.5		
Ethnicity [†]	NHW	77.8	77.5	78.7	0.36	
	Hispanic	16.8	17.5	14.6		
	Other	5.4	5.0	6.7		
Smoking status	Current	56.7	56.7	56.6	1.00	
Duration of smoking	<30	35.0	35.9	32.0	0.53	
	30–40	38.7	38.1	40.6		
	40+	26.3	26.0	27.3		
Pack-years of smoking	15–29	35.6	38.7	25.4	<0.001	
	30–49	40.7	40.4	41.8		
	50+	23.7	20.9	32.8		
BMI	<25	31.7	32.5	28.9	0.40	
	25–29.9	37.7	36.7	41.0		
	30.0+	30.6	30.8	30.1		

Abbreviation: NHW, non-Hispanic white.

*Comparison of females and males, from Fisher's exact test.

[†]Ethnicity is not reported for six participants.

Table 2. Summary and differences of dietary intake by gender

Dietary factor	Females (n = 845)*	Males (n = 256)*	Adjusted difference (95% CI)†	P‡
Macronutrients§				
Total caloric intake (kcal)	1,688 (1,312–2,121)	1,975 (1,517–2,600)		<0.001
Total fat (g)	69 (52–91)	78 (60–104)	3.5 (1.1–5.9)	0.004
Animal fat (g)	33 (23–45)	38 (26–53)	0.9 (−0.9 to 2.6)	0.33
Micronutrients§				
Vitamin C (mg)	185 (88–668)	181 (84–623)	71 (3–140)	0.04
Folate (μg)	985 (423–1,298)	1,038 (479–1,408)	28 (57–112)	0.52
Vitamin E (mg)	23 (8–191)	21 (8–40)	23 (8–38.5)	0.003
Carotene (IU)	5,599 (3,419–9,921)	5,334 (3,026–8,510)	1,568 (761–2,374)	<0.001
α-Carotene (μg)	357 (176–641)	370 (146–585)	127 (50–1,349)	0.001
β-Carotene (μg)	3,095 (1,912–5,518)	2,925 (1,592–4,621)	891 (432–1,349)	<0.001
Lycopene (μg)	4,991 (3,242–8,283)	5,968 (3,655–9,665)	−320 (−968 to 328)	0.33
Lutein and Zeaxanthin (μg)	2,315 (1,326–3,612)	2,059 (1,138–3,097)	844 (476–1,213)	<0.001
Retinol (IU)	3,736 (1,512–6,072)	3,683 (1,613–6,106)	505 (−120 to 1,129)	0.11
Food groups¶				
Red and processed meats	20 (12–34)	28 (16–50)	−4.1 (−6.4 to −1.9)	<0.001
Fruit	19 (9–37)	19 (8–38)	5.3 (1.1–9.6)	0.01
Tomatoes	12 (5–17)	13 (5–17)	0.5 (−1.0 to 1.9)	0.54
Cruciferous vegetables	5 (3–9)	5 (3–9)	1.5 (0.3–2.8)	0.01
Leafy green vegetables	13 (5–25)	10 (5–18)	5.7 (3.6–7.9)	<0.001
Yellow vegetables	5 (3–12)	4 (2–9)	3.1 (1.8–4.4)	<0.001
Alcohol				
Drinks per day (%)				<0.001
None	30	22		
0.1–1.0	57	48		
>1	13	31		
Multivitamin use (%)				
Never	13	18		
Past	24	21		
Current	63	61		
Current cod liver oil use (%)	1.7	2.0		0.78

*Summary statistics are medians (IQRs) for continuous variables and percents for categorical variables.

†Differences are calculated as mean for females minus mean for males after adjustment for total caloric intake.

‡P values are obtained from linear regression after adjustment for total caloric intake or, for those variables without adjustment, from linear regression for total caloric intake and from Fisher's exact test for categorical variables.

§Daily intake.

¶Servings per month.

participants failed to respond to >10 items. Dietary information was compiled for the 21 items of particular interest to this study and included three macronutrients, nine micronutrients, six serving variables, and three categorical variables (Table 2). The selection of these variables was based on our hypotheses that fruits, tomatoes, cruciferous vegetables, leafy vegetables, yellow vegetables, and vitamin intake will be associated with a reduction in number of genes methylated in sputum, whereas animal fat and red and processed meat will be associated with increased methylation. On average, men had higher intake of total calories, but after adjustment for caloric intake, women generally had higher consumption of micronutrients and men had higher consumption of red meat. There was no difference in vitamin usage, but men

were more likely to report having at least one alcoholic drink per day.

Prevalence for gene promoter hypermethylation in sputum. Methylation of an eight-gene panel that included *p16*, *MGMT*, *DAPK*, *RASSF1A*, *GATA4*, *GATA5*, *PAX5α*, and *PAX5β* was evaluated. Methylation of these genes is associated with increased risk for lung cancer (10, 21). The prevalence of methylation ranged from 1.1% and 0.0% for *RASSF1A* to 33.8% and 51.2% for *GATA4* for women and men, respectively (Table 3). Three genes, *MGMT*, *GATA4*, and *PAX5α*, were more frequently methylated among men than women ($P < 0.001$). Methylation index, the number of methylated genes in each sputum sample, was higher in men than women (median = 2 and 1, respectively; $P < 0.001$). Our previous study was

Table 3. Prevalence of gene methylation in sputum

Gene	Overall (N = 1,101)	Females (n = 845)	Males (n = 256)	P*
	(% positive)			
P16	16.6	16.2	18.0	0.50
MGMT	26.3	24.4	32.8	0.009
RASSF1A	0.8	1.1	0.0	0.13
DAPK	17.8	16.7	21.5	0.09
GATA4	37.9	33.8	51.2	<0.001
GATA5	18.0	17.0	21.1	0.14
PAX5 α	15.3	13.1	22.3	<0.001
PAX5 β	9.6	9.5	10.2	0.72
≥ 2 genes methylated	39.8	36.6	50.4	<0.001

*Comparison of females and males, from Fisher's exact test.

composed largely of male participants and dichotomized methylation index as fewer than three or three or more genes methylated in sputum (10). However, because this study is composed of 77% women who have a lower prevalence for gene methylation, high methylation index was defined as two or more genes methylated and was present in sputum from 36.6% of women and 50.4% of men ($P < 0.001$).

Association of clinical covariates with gene methylation. Gender, age, BMI, and cigarette smoking history were assessed in a multivariate model for association with methylation status. Meaningful interactions, including interactions between gender and all of the other covariates, were assessed. There were significant interactions between gender and both BMI ($P = 0.04$) and pack-years of smoking ($P = 0.05$). There were relatively more women with high methylation among obese participants than among both normal weight and overweight individuals, whereas for men the association was less consistent. The odds of high methylation increased with pack-years among men, but there was no association among women. Age and duration of smoking were

not associated with methylation, after adjustment for the other clinical covariates, but age was retained in the model. With two other smoking variables included in the model (pack-years and current status), duration was excluded. Thus, the clinical covariates that were included in the modeling with the nutritional variables were gender, pack-years of smoking, current smoking status, BMI, age, and interactions between gender and both pack-years and BMI.

Association of dietary factors with gene methylation. Each dietary factor was assessed for association with methylation status (two or more genes methylated versus fewer than two genes) using logistic regression. A total of 21 variables were examined: three macronutrients, nine micronutrients, six serving variables, and three categorical variables (consumption of alcohol, vitamins, and cod liver oil; Table 4; Supplementary Table S1). Leafy green vegetable consumption was significantly associated with reduced risk for high methylation status [odds ratio (OR), 0.83; 95% CI, 0.74–0.93], as was higher folate (OR, 0.84; 95% CI, 0.72–0.99; Table 4). The most striking effect seen was the association between current multivitamin use and methylation status (OR, 0.57; 95% CI, 0.40–0.83; Table 4). The duration of vitamin use was not associated with methylation (data not shown). Moreover, because folate levels were higher for participants taking multivitamins, stratification by vitamin use was also conducted and folate remained significantly associated with methylation. There was a marginal increase in odds for methylation associated with total fat and animal fat that did not reach statistical significance (Supplementary Table S1). None of the other dietary or nutrient predictor variables analyzed were associated with methylation (Supplementary Table S1). Interactions between each dietary variable and gender were assessed, but none was significant. The association between these dietary variables and methylation of the individual genes also was examined, but because of the number of tests (8 genes \times 3 dietary variables = 24 tests), these results were viewed as exploratory. Associations with at least one of the three significant dietary variables were observed for DAPK, GATA4, PAX5 α , and PAX5 β (Supplementary Table S2).

A further analysis was conducted to assess whether any extreme outliers influenced the observed results for

Table 4. Dietary variables significantly associated with methylation status in the Lovelace Smokers Cohort

Dietary predictor variable	OR* (95% CI)	P
Leafy green vegetables (per 12 monthly servings)	0.83 (0.74–0.93)	<0.001
Folate (per 750 μ g/d)	0.84 (0.72–0.99)	0.04
Multivitamin use versus never		
Current	0.57 (0.40–0.83)	0.01
Past	0.68 (0.45–1.03)	

*ORs are obtained from models with a single dietary variable but including adjustment for gender, age, BMI, pack-years of smoking, current smoking status, total caloric intake, and interactions between gender and both BMI and pack-years of smoking.

continuous variables by defining quartiles by gender and then using the medians within the quartiles as predictors in logistic regression modeling. Similar results were obtained as in the initial analysis, except that total fat was associated with marginally increased methylation (OR, 1.43 per 40 g/d; 95% CI, 1.03–1.99). The same dietary variables were identified to be significant in analyses that used the continuous methylation index as the outcome. In addition, vitamin E showed a protective effect for methylation per gene (OR, 0.98 per 20 mg/d; 95% CI, 0.96–1.00; $P = 0.04$).

Discussion

This is the first cohort-based study to systematically evaluate the association between dietary factors and risk for methylation in cells exfoliated from the aerodigestive tract of smokers and former smokers. Our findings support a significant, biologically plausible role for leafy green vegetables, folate, and multivitamin use in protection against the acquisition of gene promoter methylation.

There has been considerable interest and debate for decades about the effect of diet and vitamins on the risk for cancer. Recent large epidemiologic studies along with functional investigations are beginning to provide a clearer picture as to the dietary variables that may influence risk for cancers such as lung where a clear causative environmental exposure in the form of smoking has been established. Reduced folate intake has been associated with increased risk for lung cancer in current and former smokers (22). A link between folate and gene methylation exists through the role of 5-methyltetrahydrofolate in providing methyl groups for SAM, a key methyl donor in the methylation of DNA. Higher folate has been associated with a lower prevalence for methylation of individual and total number of genes in colorectal tumors (17). This finding was validated in a second study of colorectal tumors in which folate was inversely associated with gene-specific promoter hypermethylation (23). Our study shows for the first time that the acquisition of gene promoter methylation throughout the airway epithelium is influenced by folate. The biological mechanisms related to low folate and hypermethylation are still unclear; however, Jhaveri and colleagues (24) suggested that folate deficiency leads to increased levels of SAM and S-adenosylhomocysteine (SAH), an inhibitor of SAM. The increase in free intracellular SAM could contribute to gene-specific hypermethylation if an absolute level of SAH needed to regulate SAM is not maintained. Folate is also involved in DNA repair through *de novo* synthesis of purines and pyrimidines, and low dietary folate has been associated with reduced DRC (18). This observation, combined with our recent study identifying reduced DRC as a determinant for gene promoter methylation in sputum (14), suggests that folate may directly, through affecting the one-carbon pool, and indirectly, by affecting DRC, affect the propensity for methylation.

Green leafy vegetables were the only food item in this analysis to exhibit protection against methylation status. Leafy vegetables are rich in phytochemicals such as vitamin C, carotenoids, lutein, and folic acid in addition to vitamins A and K. A comprehensive and systematic review of the litera-

ture up to 2007 by the World Cancer Research Fund (WCRF) concluded that probable evidence existed for reduction of lung cancer risk among persons with higher intake of fruits, whereas evidence was inconclusive about green leafy vegetables (15). However, a recent hospital-based case-control study of lung cancer (25) showed a strong protective effect of green leafy vegetables (OR, 0.5; 95% CI, 0.3–0.81). The lack of an effect of cruciferous vegetable on methylation status in our study is not surprising because lung cancer observational studies report only modest effects that may be influenced by genetic variation (26). In addition, the lack of association with red meat and processed meat intake is consistent with the inconclusive evidence, as summarized by the WCRF (15). However, a recent cohort study has observed increased risk for lung cancer among those consuming meats prepared in ways that would be expected to have increased their carcinogenicity (27).

In our study, strong protection against gene methylation was also associated with the use of multivitamins that contain some of the same agents as leafy green vegetables. Although a clear connection between vitamin supplements and risk of lung cancer has not been established (28), vitamin supplementation has been associated with reduction in DNA damage by benzo(a)pyrene, a major tobacco carcinogen (29–32).

The silencing of genes by promoter hypermethylation is now well established as a major component of lung cancer initiation and progression and has emerged as a potential disease marker for early detection. The ability to affect reprogramming of the epigenome through diet and chemopreventive supplements could significantly affect mortality from lung cancer. This study has identified two dietary variables, leafy green vegetables and folate, along with multivitamin use that could help reduce the incidence of lung cancer by reducing the induction of methylation in the aerodigestive tract of smokers.

Disclosure of Potential Conflicts of Interest

S.A. Belinsky is a consultant and has licensed intellectual property with Oncomethylome Sciences. The other authors disclosed no potential conflicts of interest.

Acknowledgments

We thank Kieu Do and Amanda Bernauer for assistance in the conduct of gene methylation assays in the sputum specimens.

Grant Support

NIH grant U01 CA097356 and the State of New Mexico as a direct appropriation from the Tobacco Settlement Fund, and in part by the New Mexico VA Health Care System.

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Received 9/15/09; revised 10/28/09; accepted 11/4/09; published OnlineFirst 1/12/10.

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Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Multivitamins, Folate, and Green Vegetables Protect against Gene Promoter Methylation in the Aerodigestive Tract of Smokers

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Cancer Res 2010;70:568–574. Published OnlineFirst January 12, 2010.

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