



UMEÅ UNIVERSITY

Metabolic consequences of a Paleolithic diet  
in obese postmenopausal women

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*Till min älskade familj* 



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# Abstract

## Background

Obesity, in particular abdominal adiposity, is associated with elevated fatty acids and pro-inflammatory adipokines, which are linked to ectopic fat storage and insulin resistance. During menopause, there is a redistribution of fat from the peripheral to abdominal depots. This transition is associated with an increased risk of type 2 diabetes and cardiovascular diseases. We hypothesized that a Paleolithic diet, with high proportions of lean meat, fish, vegetables, fruits, and oils, but devoid of dairy products and cereals, might have long-term beneficial effects on inflammation, fat metabolism, and circulating fatty acids. These effects might potentially reduce the risk of metabolic complications in postmenopausal women that are obese.

## Methods

Postmenopausal women with obesity were studied before, after six months, and after 24 months of one of two specified *ad libitum* diets. One diet was a Paleolithic diet, in which approximately 30% of the total energy (E%) was protein, 30 E% was fat, and 40 E% was carbohydrate. The other diet was a prudent control diet, consistent with Nordic Nutrition recommendations of 15 E% protein, 25 E% fat, and 55 E% carbohydrate. Dietary intakes of polyunsaturated fatty acids and protein were validated objectively by measuring circulating and urinary biomarkers. Anthropometrics and diet reports were analyzed, and abdominal subcutaneous fat samples were evaluated for the expression of proteins key in inflammation and fat metabolism and for lipoprotein lipase mass and activity. In addition, blood samples were analyzed to determine concentrations of specific serum proteins, serum lipids, and the fatty acids carried in cholesterol esters.

## Results

The Paleolithic diet group reported reduced intakes of saturated fatty acids and carbohydrates and elevated intakes of protein and unsaturated fatty acids, compared to baseline. The elevated intakes of polyunsaturated fatty acids and protein were objectively verified for this group. After 24 months, both diets were found to have beneficial effects on the expression of inflammation-related genes in adipose tissue and pro-inflammatory factors in the circulation. Compared to the control group, the Paleolithic diet group exhibited more pronounced reductions of circulating cardiometabolic risk factors, including the ratio of triglycerides to high density lipoprotein, lipogenic index, specific fatty acids, and

indices of desaturase activities. After six months, the Paleolithic group also exhibited more pronounced reductions in lipogenesis-promoting factors, including the expression of key proteins in fat synthesis, the activity of lipoprotein lipase, and the activity of stearoyl-CoA desaturase 1, compared to the control group.

## **Conclusion**

Long-term weight loss in postmenopausal obese women was accompanied by reductions in low-grade inflammation in adipose tissue and in the circulation. In addition, a Paleolithic diet, with a high content of unsaturated fatty acids and a low content of refined carbohydrates, appeared to provide greater reductions in cardiometabolic risk factors associated with insulin resistance and lipogenesis, compared to a prudent control diet.



# Abbreviations

CD, prudent control diet

CE, cholesterol ester

CRP, C-reactive protein

DGAT2, diglyceride acyltransferase 2

DHA, docosahexaenoic acid

DNL, *de novo* lipogenesis

E%, energy percent

FAS, fatty acid synthase

HDL, high density lipoprotein

HOMA, homeostasis model assessment

IL, interleukin

LPL, lipoprotein lipase

LPS, lipopolysaccharide

M1, pro-inflammatory macrophages

M2, anti-inflammatory macrophages

MCP-1, monocyte chemoattractant protein 1

MIF, macrophage migration inhibitory factor

MUFA, monounsaturated fatty acid

OPLS-EP, orthogonal partial least squares analysis effect projections

PAEE, physical activity energy expenditure

PD, Paleolithic diet

PUFA, polyunsaturated fatty acid

SCD-1, stearoyl-CoA desaturase -1

SFA, saturated fatty acid

TLR, toll-like receptor

TNF- $\alpha$ , tumor necrosis factor-alpha

# Original papers

This thesis is based on the following papers, which are referred to in the text by the corresponding Roman numerals (I-III)

- I. **Caroline Blomquist**, Elin Chorell, Mats Ryberg, Caroline Mellberg, Evelina Worrjö, Elena Makoveichuk, Christel Larsson, Bernt Lindahl, Gunilla Olivecrona, and Tommy Olsson.  
Decreased lipogenesis-promoting factors in adipose tissue in postmenopausal women with overweight on a Paleolithic-type diet  
*European Journal of Nutrition*. 2017. doi 10.1007/s00394-017-1558-0  
*Epub ahead of print*
- II. **Caroline Blomquist**, Malin Alvehus, Jonas Burén, Mats Ryberg, Christel Larsson, Bernt Lindahl, Caroline Mellberg, Ingegerd Söderström, Elin Chorell, and Tommy Olsson.  
Attenuated Low-Grade Inflammation Following Long-Term Dietary intervention in Postmenopausal Women with Obesity  
*Obesity*. 2017; 25: 892-900
- III. **Caroline Blomquist**, Elin Chorell, Mats Ryberg, Caroline Mellberg, Christel Larsson, Bernt Lindahl, Tommy Olsson and Ulf Riserus.  
Long-term influences of a Paleolithic diet on fatty acid composition in postmenopausal women with obesity: a randomized trial  
*Manuscript*

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# Sammanfattning på svenska

## Bakgrund

De senaste decennierna har förekomsten av övervikt och fetma ökat kraftigt i stora delar av världen. Detta beror på en kombination av olika faktorer såsom specifika gener vilka främjar fettinlagring, men kanske främst ett överintag av energirik mat i kombination med minskad fysisk aktivitet. Fetma och specifikt bukfetma, vilket tilltar hos kvinnor efter klimakteriet (postmenopausala), ökar risken för höjda blodfettsnivåer och lågradig inflammation, vilket kan leda till utveckling av typ 2-diabetes samt hjärt- och kärlsjukdomar. Kost och viktnedgång är avgörande för bibehållen hälsa och av stort intresse är att befolkningar runt om i världen har låg förekomst av fetma, diabetes, hjärt- och kärlsjukdom, troligtvis kopplat till olika livsstilsfaktorer som högre fysisk aktivitet samt kostfaktorer.

## Syfte

Vårt syfte var att undersöka metabola förändringar i fettväv och cirkulation hos postmenopausala kvinnor med fetma kopplat till en 24 månaders paleolitisk kostintervention. Den paleolitiska kosten, som ingick i studien består av en hög andel magert kött, fisk, grönsaker, frukt, nötter, oliv- och rapsolja och där mjölkprodukter och spannmål är uteslutna. Vår hypotes var att en paleolitisk kost med hög andel protein och omättade fettsyror har fördelaktiga långtidseffekter på inflammation, fettmetabolism och cirkulerande fettsyror jämfört med en kost baserad på Nordiska näringsrekommendationer med ett högt intag av kolhydrater.

## Metoder

Postmenopausala kvinnor med fetma studerades före, vid sex månader och efter 24 månaders intag, utan energirestriktioner, av antingen en paleolitisk kost eller en kost enligt Nordiska näringsrekommendationer. Kroppsmätningar, kostregistreringar, genuttryck av nyckelproteiner i inflammation och fettmetabolism i fettväv samt koncentrationer av blodfetter, specifika proteiner och fettsyror bestämdes i plasma.

## Resultat

I linje med rekommendationerna så rapporterade gruppen som åt den paleolitiska kosten ett minskat intag av mättat fett och kolhydrater samt ett ökat intag av protein och omättat fett jämfört med baslinjenivåerna. Det ökade

intaget av fleromättade fettsyror och protein bekräftades med objektiva mätmetoder. Efter 24 månaders intervention uppvisade båda grupperna en jämförbar viktnedgång och en minskning av flertalet proinflammatoriska faktorer i såväl fettväv som i cirkulation. Den grupp som åt paleolitisk kost uppvisade en kraftigare reduktion av cirkulerande kardiometabola riskfaktorer som index för fettsyntes och desaturaser, specifika fettsyror samt kvoten triglycerider till HDL (high density lipoprotein). Efter sex månader bidrog den paleolitiska kosten också till en mer påtaglig minskning av faktorer involverade i fetthinlagring, som uttryck av specifika nyckelproteiner i fettsyntes, aktivitet för lipoprotein lipas och stearyl-CoA desaturase 1 index jämfört med kontrollkosten.

## **Slutsatser**

En långvarig viktminskning hos postmenopausala kvinnor med fetma åtföljs av en minskad låggradig inflammation i fettväv och i cirkulation. En paleolitisk kost med hög andel omättade fettsyror och låga halt kolhydrater är kopplat till en kraftigare minskning av riskparametrar för insulinresistens och nyckelfaktorer för fetthinlagring jämfört med en kontrollkost enligt Nordiska näringsrekommendationer.



# Introduction

Since 1980, the prevalence of obesity ( $\text{BMI} > 30 \text{ kg/m}^2$ ) worldwide has more than doubled to 600 million adults, and the number of overweight adults ( $\text{BMI} > 25$ ) has reached 1.9 billion (1). Although trends in the overall growth of obesity seem to have stabilized in most developed countries, morbid obesity in many of these countries continues to climb (2). In addition, the prevalence of obesity in developing countries has continued to rise (2). The primary risk factors for diseases and deaths worldwide have shifted from malnutrition and infections to obesity and non-communicable diseases. Non-communicable diseases, such as cancer, type 2 diabetes, and cardiovascular diseases, affect obese individuals, particularly those with abdominal adiposity, more frequently than lean individuals (3). Notably, the transition into menopause is closely affiliated with an increased accumulation of abdominal fat. Indeed, in northern Sweden, the prevalence of abdominal obesity in postmenopausal women (55-74 years) is twice as high as the prevalence among men in the same age group (4).

The worldwide epidemic of excess body weight and obesity is due to a combination of energy-conserving genes (thrifty genotype) and a rapidly changing environment, which offers abundant energy sources and a sedentary life-style (5). Energy intake has increased in the last 50 years by over 500 kcal per person per day. This increase is linked to rising incomes and urbanization, which has led to a change from traditional diets to diets with higher content of refined sugars, oils, and meats (6). Moreover, physical inactivity has increased, due to sedentary forms of work, affordable transportation, and urbanization. Currently, over 75% of adults are not sufficiently active, according to the World Health Organization (7).

Elevated BMIs cause both harm to the individual and an economic burden to entire health care systems. Therefore, it is of great importance to find effective strategies for weight loss. Currently, the foundation of obesity care is assisting patients in making healthier dietary and physical activity choices that will lead to weight reduction (8). Reducing caloric intake seems to be the most important component of weight loss, and increased physical activity is particularly important for weight maintenance. Dietary strategies to reduce fat mass have changed over time. Dietary recommendations have varied regarding macronutrient composition, fiber content, and the practice of intermittent fasting. However, experts continue to debate intensely over which dietary regime might be most beneficial for weight loss and long-term weight maintenance. This ongoing debate partly arises from the fact that there remains a lack of knowledge about the long-term physiological changes associated with different diet regimes. Therefore, there is a major need for validated, long-term

(over 12 months) studies that include analyses on tissue-specific metabolic changes related to dietary interventions. (8, 9)

This thesis includes a randomized trial that compared a Paleolithic diet (PD) to a prudent control diet (CD) in postmenopausal obese women for 24 months. We aimed to analyze the effects of these interventions on adipose tissue-specific and circulating markers on the risk of type 2 diabetes and cardiovascular diseases. We validated dietary intake via by combining self-reported dietary intake with objective measurements. Notably, the PD had high contents of protein, unsaturated fatty acids, and fiber; these diet components have been shown to be beneficial for weight maintenance and metabolic balance, as discussed in further detail below (10).

## **Insulin resistance**

As mentioned above, obesity is strongly associated with insulin resistance, which may develop into type 2 diabetes and increase the risk of cardiovascular diseases. Insulin is a hormone that provides an integrated set of signals that act to balance the availability of nutrients with the demand for energy. When organs fail to respond normally to insulin (i.e., insulin resistance), due to insensitivity in the insulin receptor and/or internal signal pathways, alterations in carbohydrate and fat metabolism ensue. In adipose tissue, insulin resistance mainly causes increased lipolysis and fatty acid secretion, which can provoke ectopic fat storage in muscles and liver. In turn, ectopic fat accumulation in muscles and liver impairs insulin signaling, and cells become insulin resistant. In muscles, insulin resistance mainly causes reductions in glucose uptake (by the GLUT4 transporter), glycolysis, and glycogen synthesis. Consequently, the lack of glucose utilization leads to increased blood glucose levels. In the liver, insulin resistance causes a reduction in glycogen synthesis and increased gluconeogenesis, which also increase the blood glucose concentration. (11)

## **Energy balance**

The central nervous system plays a key role in sensing and controlling energy status. In particular, the hypothalamus has emerged as a key regulator of whole-body energy homeostasis. The hypothalamus controls both energy consumption and energy expenditure. Food intake is regulated in the hypothalamus by signals that arise from physiological processes in the gastrointestinal tract (food quantity), blood (circulating nutrients), muscles (metabolic rate), and fat mass (energy storage). In addition, the central nervous system receives external cues, including eating habits, food visibility, and social norms, which elicit subjective feelings of hunger and satiety through psychological processes. The appetite control system in the hypothalamus appears to be adapted to respond to energy



shortages, which presumably occurred during evolution. Therefore, it is less effective in situations where food is abundant. Moreover, subjective hunger, which arises from psychological processes, increases the risk of weight gain. (12, 13)

The appetite control center in the hypothalamus senses food quantity and nutrient content after a meal through hormones secreted from the gastrointestinal tract and pancreas. These hormones from the gut enter the brain to affect the hypothalamus. Hypothalamic signaling decreases energy intake and increases energy expenditure by activating sympathetic nerves. In addition, as adipose tissue expands, it increases its secretion of the hormone leptin, which also has physiological effects on the hypothalamus, leading to reduced energy intake and increased energy expenditure. The circulating levels of leptin are closely associated with whole-body fat stores. In contrast, ghrelin is a hormone secreted from the empty stomach which signals hunger. The hypothalamus responds to ghrelin by transmitting signals to decrease energy expenditure (13). In addition, distinct tissues have different energy demands and muscle mass has a higher metabolic rate than other tissues including fat mass with a more pronounced impact on resting metabolic rate, that represent a physiological signal for hunger (14).

Satiety is influenced by both the quantity and composition of food, particularly the amounts of protein, fiber, and omega-3 fatty acids. Protein intake was shown to increase thermogenesis and elicit gastrointestinal hormonal signaling. Humans seem to regulate the intake of protein energy more tightly than non-protein energy. Consequently, diets with low protein energy lead to overconsumption. Randomized trials have also supported the observation that weight loss could be maintained for longer periods with high protein diets. Fiber is another component that is particularly effective at signaling satiety and promoting weight management, due to its low energy density. Fiber was shown to slow the rate of gastric emptying, and stimulate the release of satiety hormones from the gastrointestinal tract. Notably, viscous fibers from fruit and vegetables seem to be more satiating than fermentable fibers from cereals. (15)

Studies in humans have supported findings in animal experiments, which showed that omega-3 polyunsaturated fatty acids (PUFA), like eicosapentaenoic acid and docosahexaenoic acid (DHA), could promote less hunger and more satiety than other nutrients. However, different studies have published conflicting results. The discrepancies are probably due to differences in the intakes of omega-6 PUFAs among different studies. The ratio of omega-3 to omega-6 PUFAs is essential, because these substrates compete for many of the same enzymes. (16)

## **Adipose tissue**

### ***Fat distribution***

Although obesity has a negative impact on health, not all obese individuals have health problems. One major factor that appears to affect the risk of metabolic diseases in obesity is body shape, due to the regional distribution of adipose tissue. Metabolic dysfunction is highly associated with high accumulations of fat in upper body depots, particularly in the abdominal region. These depots include both subcutaneous (under the skin) and visceral (in-between organs) spaces. Conversely, lower-body adipose tissue (gluteal/femoral) is associated with reduced metabolic risk; indeed, this fat distribution may be protective against the adverse health effects of obesity. (17)

Subcutaneous adipose tissue is the largest fat depot. Subcutaneous adipose tissue sequesters triglycerides in periods of excess energy intake and supplies the organism with free fatty acids in periods of fasting, starvation, or exercise. Moreover, subcutaneous adipose tissue takes up circulating fatty acids more rapidly in the abdominal region than in lower-body fat depots. This feature serves to reduce long-term fat storage in organs outside the adipose tissue, known as ectopic fat storage. Thus, a reduced ability to store fat in subcutaneous adipose tissue during times of positive energy balance can lead to an increase in visceral adipose tissue expansion and ectopic fat storage in organs. For example, excess fat stored in muscle, liver, pancreas, and heart can induce organ failure and lead to insulin resistance. (17) High volumes of visceral adipose tissue may lead to increased ectopic triglyceride accumulation in liver, which is associated with insulin resistance. The link between visceral adipose tissue and liver fat storage may be due to the high rate of lipolysis in visceral adipose tissue and the fact that the circulation from visceral adipose drains through the portal vein, which delivers free fatty acids directly to the liver. (18)

The tendency to accumulate triglycerides in a particular depot during excess energy intake differs from one individual to another. Up to 70 percent of the inter-individual variation in body weight variability may be due to genetic differences between individual according to twin and family studies. A large proportion of the heritability of BMI and fat distribution has been associated with common DNA polymorphisms. The BMI-associated loci are associated to synaptic functions and neurotransmitter signaling in the central nervous system including the hypothalamus and pituitary gland. Waist-to-hip ratio associated loci point to pathways in adipogenesis, angiogenesis, insulin resistance and processes that affect fat distribution. In contrast, epidemiological data has indicated that genetics may be responsible for 20-25 percent of inter-individual variability in body weight. Nevertheless, genetic and epigenetic effects are the

most likely explanations for the observed pronounced ethnic differences in regional fat distribution. Indeed, it is thought that ethnic differences in fat distribution may have arisen from genetic adaptations that served to optimize defenses against local pathogens. (18-20)

Another factor that effects fat distribution is age. With age, fat deposition increases in visceral adipose tissues.

Gender also affects fat distribution. Men are more likely to accumulate upper-body and visceral fat than women. This observation suggests that the regulation of fat distribution may depend on sex hormones. Estrogens have a significant influence on adipose tissue metabolism. Indeed, estrogen may be involved in determining sex differences in body composition, even though fat storage in visceral adipose tissue seems to be stimulated mainly by testosterone. Reduced estrogen levels after menopause may induce an increase in visceral adipose tissue fat storage, and decrease in subcutaneous adipose tissue observed in postmenopausal women, compared to similar-aged men, which is associated with an increased prevalence of cardiometabolic diseases. (18)

These differences in body fat distribution indicate that different thresholds of abdominal obesity are needed to evaluate individuals of different ethnicities, genders, and ages.

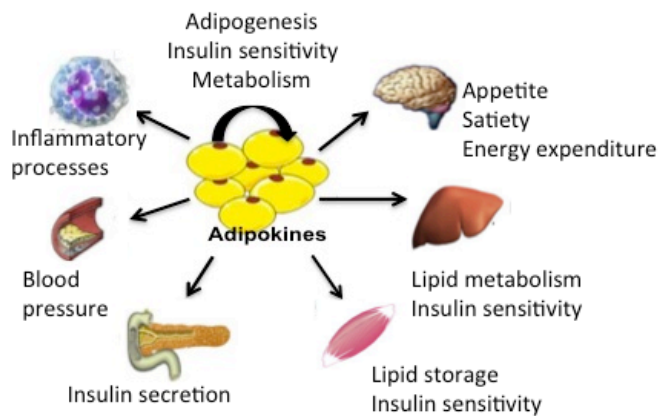
### ***The structure of adipose tissue***

Adipose tissue responds rapidly and dynamically to alterations in nutrient availability (deprivation and excess) to fulfill its major role in whole-body energy homeostasis. Adipose tissue consists of a large number of different cells, including adipocytes, pre-adipocytes (precursor fat cells), fibroblasts, and immune cells. The latter group includes macrophages, neutrophils, and lymphocytes. All these cells are surrounded by a connective tissue matrix, blood vessels, and nerves.

Adipocytes are the characteristic cells of adipose tissue. Adipocytes store triglycerides in one large intracellular lipid droplet. Then, when energy is needed, the lipid droplet is degraded into its fatty acid and glycerol components. During times of energy surplus, triglyceride storage increases in adipose tissue. Adipose expansion can occur in two ways; existing adipocytes can increase in size (hypertrophy) or new fat cells can be generated (hyperplasia). Hypertrophy is considered a pathological expansion, because it is associated with high levels of fatty acid secretion and inflammation, which ultimately results in the development of systemic insulin resistance. In contrast, hyperplasia is considered healthy in both visceral and subcutaneous adipose regions, because

it appears to be protective against lipid and glucose/insulin abnormalities associated with obesity. However, the underlying mechanisms that influence these means of expansion have not been elucidated. (21)

Mechanisms of adipose tissue expansion differ between genders and between different fat depots. Women exhibit a higher proportion of early differentiated adipocytes than men (22). Visceral adipose tissue predominantly expands by hypertrophy, and subcutaneous adipose tissue predominantly expands by hyperplasia. Moreover, abdominal subcutaneous adipose tissue has a higher risk of undergoing hypertrophy than femoral subcutaneous adipose tissue (21, 23).



*Figure 1.*

*Adipokine secretion is associated with adipocyte cell size. Adipokines can affect many different organs. Thus, adipokines contribute to the regulation of lipid metabolism, inflammatory processes, insulin secretion, insulin sensitivity, energy balance, and blood pressure.*

In addition to providing fuel for metabolism, adipose tissue is key in maintaining a viable immune system. Moreover, adipose tissue secretes regulatory peptides, known as adipokines, which have autocrine, paracrine, and endocrine functions (Figure 1). Adipokines reflect the functional status of adipose tissue, and it signals this status to many organs. Adipokines have highly diverse physiological roles. They can regulate appetite, satiety, and energy expenditure in the brain; they affect lipid metabolism and insulin sensitivity in muscles and liver; they modulate insulin secretion from the pancreas; they monitor blood pressure, and they control inflammatory processes. Within adipose tissues, adipokines affect adipocyte growth and differentiation, lipid storage, insulin sensitivity, immune cell migration, and adipocyte ‘browning’ (the process by which white adipocytes acquire brown adipose-like properties, with increased thermogenesis). (24)

In parallel with adipose tissue expansion and the development of obesity, adipokine secretion is significantly altered. In a majority of obese subjects, adipokines tend towards diabetogenic and atherogenic patterns, which can be partly reversed with weight reduction. Notably, individuals that remain metabolically healthy and insulin sensitive, despite obesity, tend to have normal adipokine patterns. (24)

### ***Hypertrophic adipocytes and inflammation***

Adipose expansion during obesity influences adipocyte biology. For example, the hypertrophic fat cell may exhibit elevated triglyceride degradation (lipolysis), followed by elevated fatty acid secretion and reduced triglyceride uptake from triglyceride-rich lipoproteins. These activities may lead to increased plasma concentrations of fatty acids and triglycerides, which is associated with ectopic fat storage. Increased fat storage (e.g., diglycerides in the plasma membrane and cytosolic compartments of muscles and liver) can impair insulin signaling and induce insulin resistance. (11) However, in some cases, ectopic fat storage is not associated with insulin resistance. For example, fat stored in the muscles of endurance athletes does not impair insulin signaling ("the athletic paradox"). Endurance-trained muscles may have different lipid compartmentation and an increased percentage of triglycerides relative to lipotoxic lipids (e.g., diglycerides and ceramides). The endurance athletes have also a faster fat turnover and increased fat oxidation due to higher energy demands. In addition, the coatings of lipid droplets seem to determine functionality; different coatings seem to have different effects on the association between intracellular lipid storage and insulin sensitivity. (11, 25)

Hypertrophic fat cells are also associated with an adipokine secretion pattern of a more pro-inflammatory nature, due to hypoxia and/or disturbances in the endoplasmic reticulum (Figure 2, next page). The diffusional limit of oxygen restricts its perfusion in expanding fat cells, which results in hypoxia. Hypoxic cells up-regulate their secretion of adipokines related to inflammation, including plasminogen activator inhibitor 1, leptin, and macrophage migration inhibitory factor (MIF) (26). Moreover, physical expansion may cause disturbances in the function of the endoplasmic reticulum, which lead to a conserved cell stress response, i.e., the unfolded protein response. This response initially aims to compensate for damage, but it can eventually trigger cell death (necrosis), when the disturbances are prolonged (27). The unfolded protein response is linked to the production of reactive oxygen species and the activation of inflammatory pathways related to adipokine expression, like leptin and the cytokines, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin (IL)-6 (27). In addition to inflammatory effects, TNF- $\alpha$  and IL-6 can increase lipolysis, reduce fat storage, and reduce adipogenesis in adipose tissue (28). Thus,

inflammation may be an adaptive response to excess nutrition, which evolved to limit fat storage in cells, by inducing insulin resistance, and to prevent hypoxia, by promoting angiogenesis (29).

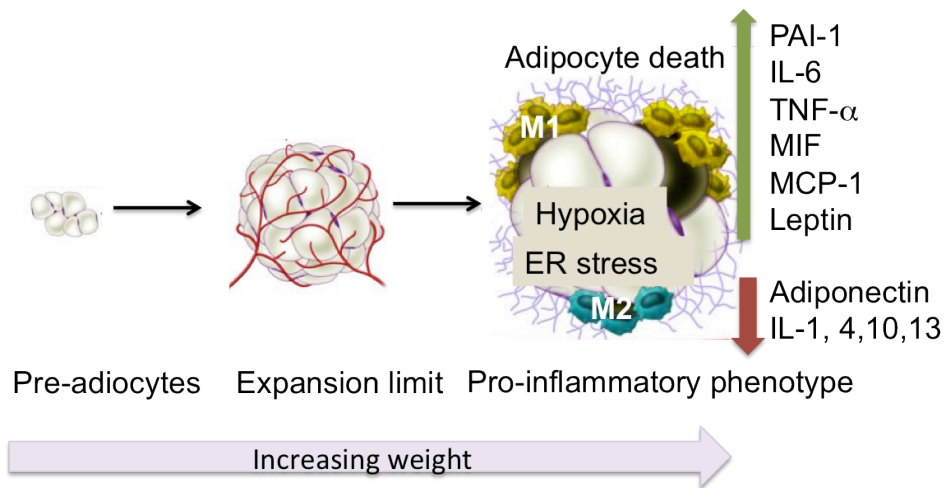
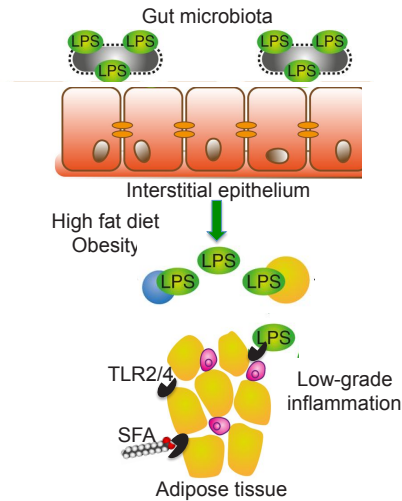


Figure 2.

Adipocyte expansion is limited by hypoxia. Hypertrophic fat cells that grow beyond the expansion limit increase their secretion of pro-inflammatory adipokines and chemokines, e.g. plasminogen activator inhibitor-1 (PAI-1), interleukin 6 (IL-6), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and chemokines, like macrophage migration inhibitory factor (MIF) and monocyte chemoattractant protein-1 (MCP-1). These factors increase the infiltration of macrophages into adipose tissue and induce macrophage polarization. Concomitantly, they inhibit the secretion of anti-inflammatory adipokines; e.g., adiponectin and some interleukins.

It has been reported that adults that are overweight and obese have elevated circulating levels of inflammatory cytokines, such as TNF- $\alpha$ , IL-6, monocyte chemoattractant protein-1 (MCP-1), MIF, and C-reactive protein (CRP). Obese individuals also have reduced levels of anti-inflammatory adipokines, including adiponectin and the cytokines, IL-4, IL-10, and IL-13. These changes are associated with reduced insulin signaling and reduced  $\beta$ -cell function, which are linked to insulin deficiency. (28)

Immune cells constitute the second largest cellular component in adipose tissue, after adipocytes. Thus, immune cells play important roles in maintaining adipose tissue homeostasis. Obesity-induced changes, including enhanced adipokine and fatty acid secretion, hypoxia, and necrosis, may cause immune cell infiltration and altered immune cell activity (most notably macrophages). These changes could lead to a chronic low-grade inflammatory state. Notably, chemotactic adipokines (chemokines), like MCP-1 and MIF, play a crucial role in attracting circulating monocytes (macrophages) to adipose tissue. (28)



*Figure 3.*

*Obesity or high fat diets affect the diversity of microorganisms in the gastrointestinal tract. These factors cause parallel increases in short-chain saturated fatty acid (SFA) synthesis and lipopolysaccharide (LPS) leakage. Both these changes can act systemically to activate toll-like receptors (TLR) 2 and 4 and promote an inflammatory state. The increased leakage of LPS may be a consequence of increased permeability of the intestine.*

The macrophages that infiltrate adipose tissue can switch from an anti-inflammatory phenotype (M2) to a more pro-inflammatory phenotype (M1). Polarization to M1 increases with adiposity, and it can be induced by pro-inflammatory cytokines, including MIF, TNF- $\alpha$ , and IL-6 (28). The shift towards the M1 phenotype can also be induced by an interaction between toll-like receptors (TLR) 2/4 on macrophages and elevated circulating levels of saturated fatty acids (SFA) and lipopolysaccharides (LPS), which are associated with obesity (Figure 3). In obesity, LPS secretion from the gut is elevated, due to increased intestinal permeability and changes in microbial diversity that are linked to changes in dietary fiber and fat contents. When SFAs and LPS interact with TLR2/4, they initiate the activation of nuclear factor kappa-light-chain-enhancer of activated B cells; this activation is required for the production of inflammatory cytokines, such as MCP-1, TNF- $\alpha$ , IL-6 and IL-8. (30)

The M1 polarization state in adipose tissue plays a central role in the development of insulin resistance. This probably involves either the secretion of TNF- $\alpha$ , which mediates inhibition of insulin signaling, or the down-regulation of the insulin-sensitive glucose transporter, GLUT-4 (28). In contrast to SFAs, unsaturated fatty acids drive macrophages to shift towards the M2 phenotype by binding to peroxisome proliferator-activated receptor gamma and inhibiting TLR signaling (30, 31).



## Lipogenesis

In addition to the influences of fat distribution and inflammation, impaired fat metabolism in obese subjects increases the susceptibility to metabolic disease. Impairments in fat metabolism are mainly due to changes in triglyceride synthesis (lipogenesis) and oxidation (lipolysis).

Lipoprotein lipase (LPL) is the gatekeeper of triglyceride storage in adipose tissue. Triglycerides in adipocytes originate primarily from fatty acids carried in triglyceride-rich lipoproteins in the circulation, including chylomicrons and very low-density lipoproteins. LPL hydrolyzes fatty acids from these lipoproteins to generate free fatty acids, which can be taken up by cells (Figure 4). (32)

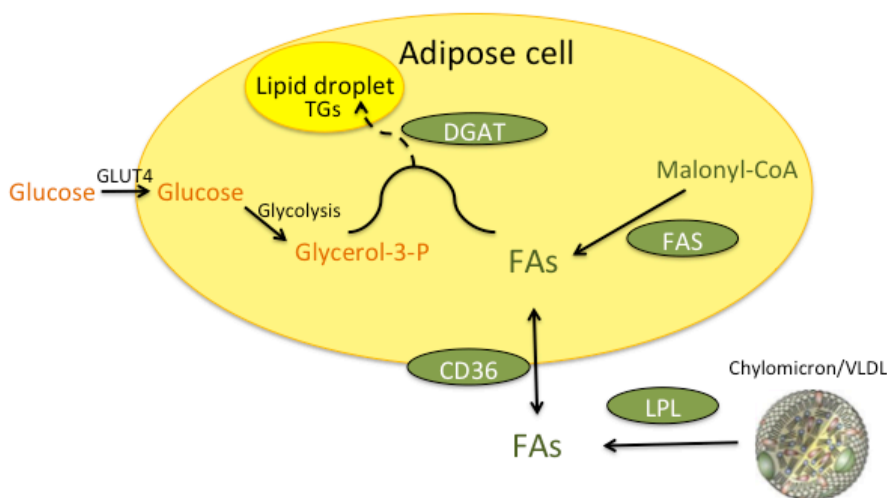


Figure 4.

*Lipogenesis. 1. Fatty acids (FAs) are bound to glycerol to form triglycerides (TGs) in lipid droplets. Most of these FAs originate from triglyceride-rich lipoproteins, like chylomicrons and very low-density lipoprotein (VLDL), in the circulation. Triglycerides in the lipoproteins are hydrolyzed by lipoprotein lipase (LPL) to release free glycerol and fatty acids, which can be transported to cells and taken up by CD36 into the adipocyte cytosol. 2. Endogenous fatty acids can be synthesized from malonyl-CoA by the multi-enzyme, fatty acid synthase (FAS). 3. Glycolysis produces glycerol-3-phosphate (glycerol-3-P) from glucose. Then, glycerol-3-P and fatty acids are bound to synthesize triglyceride. This process is regulated by several enzymes, but the last, and probably rate-limiting, step is catalyzed by diglyceride acyltransferase (DGAT).*

Physiological variations in the LPL activity in various tissues are regulated by transcription factors. However, during feeding and fasting, LPL regulation is primarily achieved via post-translational mechanisms involving extracellular



proteins, hormones, and nutrients. There are two types of proteins that modulate LPL: apolipoproteins, associated with triglyceride-rich lipoproteins, and angiopoietin-like proteins. Of these extracellular proteins, angiopoietin-like protein 4 seems to be the key regulator of LPL. During fasting and exercise, angiopoietin-like protein 4 dissociates the active LPL dimer in adipose tissue into inactive monomers to redirect dietary fat for oxidative tissues. In contrast, the postprandial hormone, insulin, and the stress hormone cortisol increase LPL activity, which leads to increased fat storage in adipose tissue. In addition, diet composition can affect postprandial LPL activity and fat storage in adipose tissue. Noteworthy is that dietary carbohydrates elicit a greater effect on LPL activity than fats. (33)

Free fatty acids can enter the adipocyte cytoplasm, either by passive diffusion or by a transport protein that facilitates diffusion. The major transport protein for fatty acid uptake is CD36. The importance of CD36 for fatty acid transport seems to increase when fatty acid concentrations are low. In addition, CD 36 content is associated with increased plasma levels of insulin and glucose, rate of fat storage, fat cell size, abdominal adiposity, and metabolic diseases. Therefore, low CD36 expression is considered metabolically protective; however, both a complete deficiency and overexpression of CD36 are linked to metabolic complications. Thus, there may be a range of CD36 expression that is metabolically favorable. (34)

Within fat cells, fatty acids undergo a series of enzymatic reactions that lead to their storage as triglycerides in large lipid droplets. The final, and most likely the rate-limiting, step in triglyceride synthesis is catalyzed by diglyceride acyltransferase 2 (DGAT2). DGAT2 activity is increased with increasing circulating levels of glucose and insulin. DGAT2 activity is also associated with the storage rate of triglycerides and adipocyte size. DGAT mRNA levels are decreased during fasting and increased with weight gain. (35)

The expression of *LPL*, *DGAT2*, and *CD36* genes are regulated by the adipogenic transcription factor, peroxisome proliferator-activated receptor- $\gamma$ , which is expressed in the late stage of adipocyte differentiation. Peroxisome proliferator-activated receptor- $\gamma$  can be activated by PUFAs. The activated receptor reduces insulin resistance, probably by increasing adipogenesis or/and lipogenesis in adipose tissue, which is followed by reduced levels of fatty acids and triglycerides in plasma. (36)

## De novo lipogenesis

*De novo* lipogenesis (DNL) is a complex, tightly regulated pathway in adipose tissue and liver. DNL converts excess carbohydrates into fatty acids, which are either secreted or esterified to form triglycerides. An important enzyme involved in DNL is fatty acid synthase (FAS), which synthesizes the 16-carbon saturated palmitic acid (16:0) from malonyl-CoA (37). FAS expression is upregulated in the liver by elevations in circulating levels of glucose, fructose, and insulin. FAS expression is downregulated by high-fat diets and probably also by dietary intake of PUFAs. FAS expression in response to dietary factors seems to be less pronounced in adipose tissue than in the liver (37). In addition, visceral and subcutaneous expressions of FAS are correlated with FAS protein levels in both depots, impaired insulin sensitivity and increased proinflammatory adipokines (38).

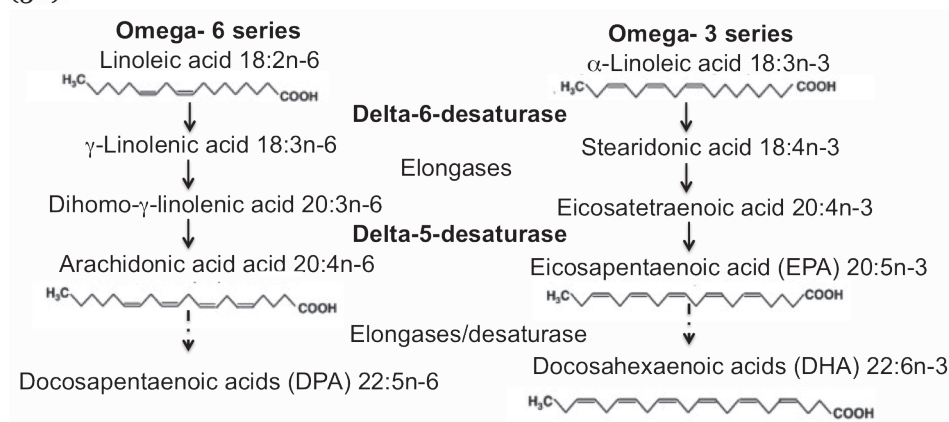


Figure 5.

*Endogenous synthesis of polyunsaturated fatty acids. Two essential fatty acids, linoleic acid (18:2 n-6) and α-linolenic acid (18:3 n-3), can be elongated with elongases. They can become more unsaturated with the desaturase activities of the delta-5 and delta-6 desaturases.*

The end-product of DNL, palmitic acid, can be further elongated to form stearic acid (18:0), and these can be desaturated to form palmitoleic acid (16:1 n-7) and oleic acid (18:1 n-9) by stearoyl-CoA desaturase 1 (SCD-1). Two PUFAs cannot be produced endogenously in humans, and thus, they are classified as essential: linoleic acid (18:2 n-6; omega-6 family) and α-linolenic acid (18:3 n-3; omega-3 family) (Figure 5). These PUFAs can be further elongated and desaturated to form other fatty acids of the same families by delta-6 desaturase and delta-5 desaturase. High SCD-1 and delta-6 desaturase activities and low delta-5 desaturase activity are associated with an increased risk of developing insulin resistance. (39, 40)

## Lipolysis

Lipolysis is the breakdown of triglycerides into glycerol and fatty acids in adipose tissue (Figure 6). The lipolytic pathway is regulated by hormonal and nutritional factors. Lipolysis is stimulated under conditions of negative energy balance; it produces energy by generating fatty acids for oxidation (41). Lipolysis is facilitated by stress hormones, such as catecholamines and cortisol, by natriuretic peptides, by pro-inflammatory factors (including IL-6 and TNF- $\alpha$ ), and by dietary intake of SFAs. The main inhibitor of lipolysis is insulin, but estrogen and dietary PUFAs can also inhibit lipolysis, to a lesser extent (41, 42).

The basal lipolytic activity in adipocytes depends on the fat depot location and on the individual's sex, age, and genetic variance. Lipolysis is elevated in upper-body compared to lower-body fat depots. Lipolysis is higher in women than in men. Lipolysis is reduced in older individuals compared to younger individuals. In general, dysregulated adipose tissue lipolysis in obese subjects is considered a major contributor to the development of metabolic disease. (43)

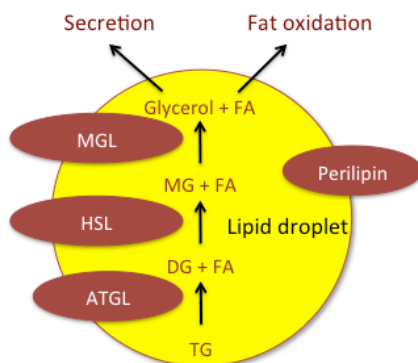


Figure 6.

*Lipolysis. The steps of triglyceride (TG) hydrolysis to glycerol and fatty acids (FA). This process produces diglycerides (DG) and monoglycerides (MG) along the way. The first, rate-limiting step is catalyzed by adipose triglyceride lipase (ATGL). The next steps require hormone sensitive lipase (HSL) and monoglyceride lipase (MGL).*

Triglycerides can be hydrolyzed by different lipases, but inside fat cells, adipose triglyceride lipase (ATGL), which produces diglycerides, plays the most important role. Hormone sensitive lipase catalyzes the second step, which produces monoglycerides. Finally, monoglyceride lipase hydrolyzes the last fatty acid from glycerol. The free fatty acids can be either secreted or sent to the mitochondria for oxidation. In addition, perilipin and other proteins on the surface of the lipid droplet are potent regulators of lipolysis. These proteins regulate the access of lipases to the triglycerides. (41)

## Lifestyle interventions

A high BMI is a risk factor for morbidity and mortality. A high BMI causes harm to the individual's health and it also represents an economic burden to health care systems. Therefore, it is of great importance to find effective strategies for weight loss. Substantial weight loss can reduce the health risks associated with being overweight. The foundation of obesity care is currently assisting patients in making healthier dietary and physical activity choices that will lead to a net negative energy balance. When lifestyle treatments are unsuccessful, bariatric surgery can be performed in selected individuals to achieve substantial weight reduction. (8, 9)

Lifestyle therapy can include recommendations for changes in the diet, in the amount of physical activity, or both. Diet-only interventions and combined interventions that include both diet and physical activity lead to similar degrees of short-term weight loss. However, combined interventions produce the highest degree of long-term (over 12 months) weight loss. Caloric reduction seems to be the most important component in achieving weight loss, while increased and sustained physical activity is particularly important in maintaining the lower weight. (8, 9)

Certainly, energy-restriction diet programs are effective, but they can be difficult to follow for long time periods. Their limited success may be due to increasing hunger and adaptations in energy expenditure. Adaptive changes occur in response to increases in hunger hormones secreted from the gut and reductions in circulating anorexic hormones secreted from the intestine and adipose tissue. Thus, appetite regulation is a major determinant of weight reduction, because it affects adherence to the weight loss program. Notably, *ad libitum* programs reduce the feeling of being restricted to a diet, which may improve adherence and weight loss on a long-term basis. (44, 45)

Moreover, weight maintenance is challenging. Reductions in fat cell size increase insulin sensitivity. However, these changes are followed by increased lipogenesis, reduced lipolysis, and reduced circulating leptin levels. The consequences are a reduction in energy expenditure, elevated energy intake, and increases in fat storage. About 50% of dieters return to their original weights after one year, and almost all dieters return to their original weights in three to five years (44).

Weight loss programs can include behavioral strategies, such as encouraging individuals to self-monitor dietary and physical activities. There is strong evidence for a consistent, significantly positive relationship between self-

monitoring the diet or physical activity and successful outcomes related to weight management. (46)

### ***Diet effect on weight loss***

Dietary strategies to reduce fat mass have changed over time. Specifically, dietary recommendations have varied regarding macronutrient composition and fiber content. There is an ongoing debate over which dietary regime might be the most beneficial for weight loss and long-term weight maintenance. (44)

In a systematic review of randomized control trials, Tobias et al suggested that following any low-carbohydrate or low-fat diet for twelve months could lead to substantial weight loss. The effect of a diet on body weight depended more on the level of caloric restriction than on macronutrient content (47). However, earlier data has supported the hypothesis that increasing the consumption of dietary protein (ranging from 1.2 to 1.6 g protein/kg per day) is a successful long-term weight loss strategy, also preserving lean mass (48). Moreover, diets high in protein and low in carbohydrate content were associated with higher adherence and weight loss maintenance. Therefore, those diets appear to be ideal for preventing weight regain (49). The effectiveness of high-protein diets may, in part, be due to modulations in thermogenesis and appetitive signaling, which lead to lower overall energy intakes (15).

In addition, high intakes of monounsaturated fatty acids (MUFA), chromium, isoflavones, vitamin B6, and vitamin B12 in a diet with a low glycemic index seem to promote weight maintenance. Favorable food choices for both weight loss and maintenance may include nuts, canola and olive oils, fruits, vegetables, whole grain products, and reduced meat intake. (50, 51)

According to epidemiological studies, weight reduction and obesity prevention are facilitated with a high fiber diet. These diets are equally satiating, but provide fewer calories than a low fiber diet. The weight-reducing effect of dietary fiber might be due to its ability to increase the viscosity of the diet, which slows down digestion and the absorption of nutrients. Dietary fiber is associated with increased fat oxidation and improved insulin sensitivity. In addition, fiber increases the secretion of gut hormones that regulate the appetite. These features of fiber should benefit weight control, as suggested by epidemiological data, but less consistent results were reported in intervention studies. In addition, acute fiber intake was weakly correlated to satiety and energy intake. This weak link may indicate that either the period of intervention was too short to detect changes or that the effect of fiber was not caused by appetite modulation. Other potential mechanisms could be that fat metabolism was

altered by insulin regulation or that increased satiation led to early meal cessation. (52-54)

### ***Diet effects on health***

Although energy intake is the major determinant of body weight and composition, the differential health effects of diets are determined by macronutrient contents. Pooled data have provided evidence that low glycemic index diets had long-term beneficial effects on the fasting levels of insulin and pro-inflammatory markers, such as CRP. These effects may reduce the risk of metabolic diseases (55). Evidence has also pointed to the potential benefits of consuming PUFAs in place of SFAs. For example, high-PUFA diets reduced the risk of cardiovascular diseases by reducing the ratio of triglycerides to high density lipoprotein (HDL), reducing blood pressure, lowering the resting heart rate, and ameliorating systemic inflammation, fatty liver, and insulin resistance (56). High-protein diets showed small improvements in blood pressure and triglyceride levels, in both healthy individuals and individuals with type 2 diabetes (57, 58).

Many of these favorable elements are included in a Paleolithic diet. Variants of this diet consumed by hunter and gathering societies today with a comparable lifestyle, e.g. food sources and high physical activity as the Paleolithic era have a very low prevalence of cardiometabolic diseases. One society in Kitava that follows a traditional lifestyle with a Paleolithic diet were shown to have low circulating insulin levels and a very low incidence of cardiovascular diseases, compared to individuals in modern Western societies. (59, 60)

The Paleolithic diet contains lean meat, fish, vegetables, fruits, nuts, and seeds. These food sources supply 25 percent of the total energy (E%) in the modern Western diet. The remaining energy of the modern Western diet originates from cereals, dairy products, refined fat, sugar, and legumes. These food sources have lower contents of minerals, vitamins, fibers, and unsaturated fatty acids compared to the Paleolithic diet. These differences are partly due to the fact that wild vegetables and fruits have higher fiber and mineral contents, and wild animal meat is leaner with higher unsaturated fatty acid content, compared to the cultivated fruits and meat available in modern Western societies. In addition, refined fat and sugar have relatively low mineral and vitamin contents. (61-63)

### ***Dietary intake validations***

It is known that dietary surveys often fail to measure food and nutrient intakes adequately. There is a major problem with under- and over-reporting food intake; therefore, validating dietary intake is of great importance.

Reported energy intakes, based on dietary records, food frequency questionnaires, and diet histories, can be validated to the total energy expenditure during weight maintenance. The most reliable method for validation of energy intake, but also the most expensive and time-consuming method, is to estimate total energy expenditure, based on the recovery of biomarkers, such as double-marked water. Total energy expenditure can also be easily calculated by multiplying a theoretical BMI (dependent on sex, weight, and age) by a given activity factor (to represent the physical activity level). Another method, which is both accurate and relatively cost-effective, is to measure physical activity with an accelerometer. Accelerometers detect accelerating movements, sometimes in combination with heart rate. (64)

Protein intake can be estimated by analyzing 24-h urinary nitrogen levels. This method depends on two assumptions: first, that the subject is in nitrogen balance, and second, that the ratio between dietary protein intake and nitrogen excretion is equal to 0.8. Several days are needed to measure protein intake and nitrogen output to avoid daily variations and to establish nitrogen balance. Eight days of collection will estimate nitrogen output within 5%. For this method, para-aminobenzoic acid is administered in conjunction with the urinary nitrogen measurements, to verify that urine collection is complete. (65)

Fat intake is particularly difficult to estimate with traditional methods. Moreover, the degree of under-reporting of dietary fat increases with increasing BMIs of the participants. Measuring fatty acids in the plasma or in cell membranes are accurate methods for estimating fatty acid intake. Fatty acids are transported bound to albumin or lipoproteins in the blood. The lipoproteins have an outer layer of phospholipids and apolipoproteins; the inner core comprises cholesteryl esters (CEs) and triglycerides, together with cholesterol. The fatty acid compositions of the different fractions of the lipoprotein, e.g. the outer phospholipids and the inner CEs, reflect the dietary intake of fatty acids over the prior days and weeks. (66)

Fatty acids can be endogenously synthesized, elongated, desaturated, or oxidized. These processes can complicate the use of fatty acids as biomarkers of fat intake, because the correlation between dietary fatty acids and plasma fatty acids varies. The best biomarkers are fatty acids that cannot be endogenously synthesized, e.g., linoleic acid,  $\alpha$ -linoleic acid, and trans-fatty acids. Plasma fatty acids that are 15:0 and 17:0 serve as biomarkers for dairy product intake; the plasma DHA level is a biomarker for fish intake. (67)

# Aims

The overall aim of this thesis was to investigate the long-term metabolic consequences of a Paleolithic diet in postmenopausal obese women. The effects of this diet, with a high content of unsaturated fatty acids, were compared to the effects of a prudent control diet, with a high content of carbohydrates. We evaluated changes in adipose tissue metabolism and specific plasma protein and fatty acid concentrations.

Specific hypotheses:

- The hypothesis for paper I was that, compared to a prudent control diet, the Paleolithic diet, with a higher content of polyunsaturated fatty acids and a lower content of carbohydrates, would have stronger effects on fat metabolism, including reductions in lipogenesis, *de novo* lipogenesis, and lipolysis.
- The hypothesis for paper II was that, compared to a prudent control diet, the Paleolithic diet, with a higher content of polyunsaturated fatty acids, would more effectively reduce the expression of genes that encode pro-inflammatory factors in adipose tissue and circulating pro-inflammatory markers in serum.
- The hypothesis for paper III was that, compared to the prudent control diet, the Paleolithic diet, with a higher content of unsaturated fatty acids and a lower content of carbohydrates, would have beneficial effects on the fatty acid profile in plasma associated with improved metabolic health.



# Subjects and Methods

This study was a randomized control trial. The 70 participating postmenopausal women were randomized to undertake, *ad libitum* (no limitation to total caloric energy intake), either a Paleolithic diet (PD, n=35) or a prudent control diet (CD, n=35). The randomization was stratified by BMI to ensure the groups were balanced. The study period was 24 months. Both groups underwent the same measurements. Group sessions were organized that were specialized for each diet group. Women were recruited through advertisements in the local newspaper. Participants were randomized between September 2007 and February 2008 (Figure 7). The study was conducted in accordance with the Declaration of Helsinki. Written informed content was obtained from all included participants. The study was approved by the local Ethics Board of Umeå University, Umeå, Sweden. This trial was registered at [clinicaltrials.gov](http://clinicaltrials.gov), number: NCT00692536.

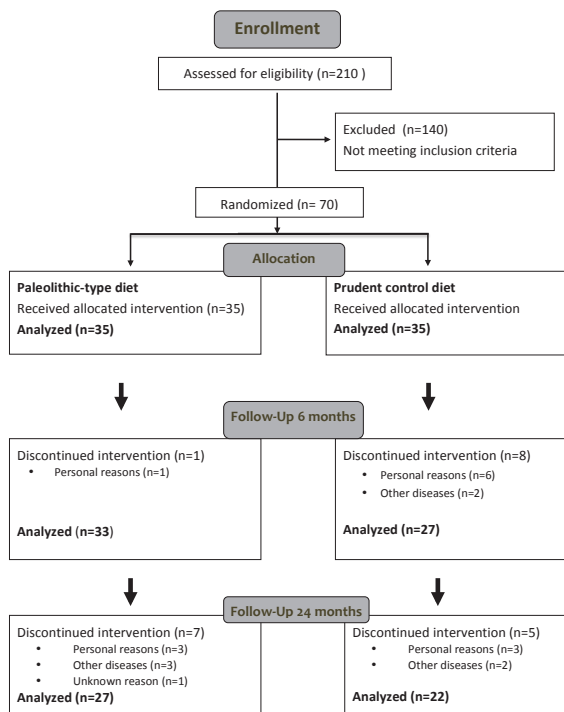


Figure 7.  
Flow diagram shows participant selection for the dietary interventions and analyses.

## Study participants

The participants were postmenopausal women with a mean age of  $60.5 \pm 5.6$  years. All subjects were overweight or obese with a mean BMI of  $33 \pm 3.4$ . All included women had experienced at least 12 consecutive months without menstruation, which is the definition of menopause according to the World Health Organization. The included women were non-smokers, healthy, and had normal fasting plasma glucose concentrations. In addition, they did not use any prescribed drugs, and they were not on any specific diet prior to the intervention.

## Diet intervention

The *ad libitum* PD targeted a macronutrient composition of 30 E% protein, 30 E% carbohydrates, and 40 E% fat, with a high content of unsaturated fatty acids (Figure 8). The PD included lean meat, fish, eggs, vegetables, fruits, berries, nuts, avocado, and vegetable oils. This diet excluded dairy products, cereals, added salt, and sugar.

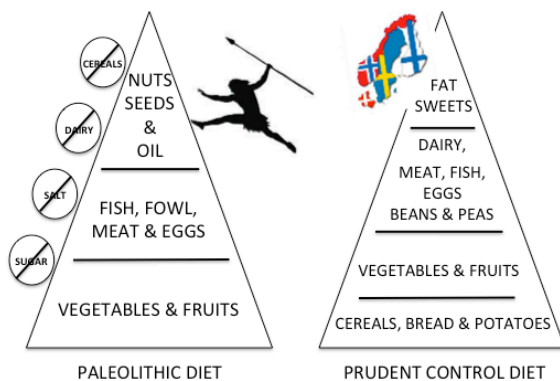


Figure 8.

Food pyramids that indicate the relative proportions of foods included in the two diets studied.

The *ad libitum* CD, a prudent control diet, in accordance the Nordic Nutrition Recommendations, targeted a composition of 15 E% protein, 30 E% fat, and 55 E% carbohydrates. The CD included high-fiber products, meat, fish, vegetables, fruits, and low-fat dairy products (Figure 8).

### ***Dietary assessment***

Dietary intake was assessed with four-day (three weekdays and one weekend day) estimated self-reported food records. The records were collected together with other data, at baseline, at six months, and at 24 months. Subjects were instructed to record all food items and drinks, with the amounts estimated based on household measures or food portion photographs that represented known weights. The food records were then converted to estimate the energy and nutrient intakes based on the Dietist XP 3.0 program and the food composition database maintained by the Swedish National Food Administration (2008).

Each diet group participated in 12 group sessions led by dietitians. The sessions provided information on the diets and instructed participants how to cook, with recipes. During the first six months of the intervention, eight group sessions were held; thereafter, group sessions were held at 9, 12, 18 and 24 months.

### ***Validation of dietary intake***

Energy intake was validated by estimating total energy expenditure based on measures of resting metabolic rate and physical activity. Resting metabolic rate was evaluated with indirect calorimetry, which measured oxygen consumption through breath-by-breath sampling for 30 min. Physical activity was measured with the Actiheart® device (a combined accelerometer and heart rate monitor), which was attached to the chest for seven days.

Protein intake was validated by sampling nitrogen excreted in the urine for three days. Para-aminobenzoic acid was administered in conjunction with the urinary measurements to verify that urine collection was complete. To calculate protein intake, we estimated that the ratio of nitrogen intake to nitrogen excretion in urine was 0.8 and we assumed that the nitrogen content in protein was 16%. We estimated that energy intake would be under-reported by 15%; this was taken into account, when estimating the E% of protein intake (65).

Fatty acid intake was validated by measuring specific fatty acids contained in the plasma CE fraction. All validations were performed concurrent with the recording of dietary intake (67).

### ***Anthropometry***

Body weight was measured to the nearest 0.1 kg on a calibrated digital scale, with the participants wearing light indoor clothing. Body height was measured to the nearest 0.5 cm with a wall-mounted stadiometer, after participants removed their shoes. BMI was calculated as the bodyweight (kg) divided by body height, squared ( $\text{kg}/\text{m}^2$ ).

Waist circumference was measured to the nearest 0.5 cm with a tape measure placed around the middle, between the lowest rib and the iliac crest. The abdominal sagittal diameter was measured with the participant lying in a supine position with legs extended. The sagittal abdominal diameter was measured from the table to the umbilicus, at the umbilical level. Hip circumference was measured around the largest gluteal region. Blood pressure was measured in the supine position.

Whole body dual-energy X-ray absorptiometry simultaneously measured regional fat including android fat and non-fat masses.

### **Blood analyses**

Venous blood samples were taken through a cannula. All samples were drawn under fasting conditions, after 15 min of rest. Plasma levels of glucose, insulin, triglycerides, lipoproteins, and other routine laboratory analyses were measured at the Umeå University Hospital.

#### ***Estimation of insulin resistance***

Insulin resistance was estimated with the homeostasis model assessment (HOMA) formula:  $(G_o \times I_o)/22.5$ , where  $G_o$  is the fasting plasma glucose concentration (mmol/L) and  $I_o$  is the fasting insulin concentration (mIU/L) (68). We also used the triglyceride/HDL ratio as a biomarker of insulin resistance (69).

#### ***Fatty acid content in plasma***

Free fatty acid concentrations were measured with an enzymatic calorimetric method, performed with the NEFA-HR kit.

The analysis of fatty acids in CEs started with the extracting plasma lipids with a mixture of chloroform/methanol. CEs were separated from the other lipids with thin-layer chromatography; they were trans-methylated with methanol and sulfuric acid. Plasma CE compositions were analyzed in duplicate with gas chromatography, coupled with mass spectrometry (70). Individual fatty acids were expressed as the percent of all measured fatty acids. The desaturase activity index for SCD-1 was calculated as the ratio of 16:1/16:0 (71). DNL was estimated with the lipogenic index=16:0/18:2 n-6 (72).

### **Protein analyses**

Serum concentrations of proteins were determined with commercially available immunoassay kits. Concentrations of IL-6, MCP-1, and MIF were analyzed with

a multiplex detection kit. Serum concentrations of CRP were analyzed with an automated, high-sensitivity CRP detection method.

## **Biopsies**

Superficial subcutaneous adipose tissue biopsies were collected with needle aspiration, with the patient under local anesthesia. The biopsies were washed with saline solution, aliquoted into Eppendorf tubes, and immediately snap-frozen in liquid nitrogen. The samples were weighed and stored at -80 °C until analysis.

### ***LPL mass and activity***

The lipoprotein lipase extracted from homogenized subcutaneous fat biopsies was incubated with [<sup>3</sup>H]-oleic acid-labeled triolein. Data are the mean values of three determinations. Enzymatic activity was measured in mU, where 1 mU corresponded to the release of 1 nmol fatty acids per min. LPL mass was determined in triplicate in the same samples, with an enzyme-linked sandwich immunoassay (73).

### ***Real-time polymerase chain reaction (RT-PCR)***

Total RNA was extracted from adipose tissue. Then, cDNA was synthesized with reverse transcription. RT-PCR was performed with pre-designed commercially available gene expression assays. Each gene expression was relatively quantified using  $2^{-\Delta\Delta CT}$ . Expression of each target gene was normalized relative to a control gene, which was selected based on a previous evaluation of endogenous control genes in human adipose tissue (74). The selected control genes were compared with the NormFinder algorithm.

## **Statistical analysis**

A power analysis was performed to estimate an appropriate sample size. This analysis was based on changes in fat mass measured in a pilot study of postmenopausal women on a PD (75). We estimated that 30 participants were required in each group to achieve  $P < 0.05$  with 80% power. Data are expressed as means  $\pm$  SDs. All statistical analyses were performed with IBM SPSS Statistics for Mac, Version 22.0 (IBM Corp., Armonk, NY, USA). In all three substudies included in this thesis, the generalized estimating equation (GEE) was used to explore the effects of time, diet, and the interaction between time and diet. Dependent variables were transformed with natural logarithms, prior to analyses.

In studies I and II, we used a multivariate data analysis strategy to elucidate intervention-related effects on fat metabolism or inflammation profile. We used orthogonal partial least squares analysis effect projections (OPLS-EP) to extract metabolic profiles based on paired analyses of individual effects; i.e., dietary intervention effects. Each subject acted as her own control to minimize confounding factors associated with inter-individual variations.

In study I, univariate linear regression analyses were used to study how the main outcomes related to fat metabolism in adipose tissue were associated with nutrient intake or cardiometabolic risk factors. In study III, correlations between the changes in fatty acids carried in CEs and cardiometabolic risk factors were analyzed with non-parametric Spearman's rank correlation coefficients. The non-parametric Mann-Whitney U test was used to compare groups in clinical measures, nutrient intake, and specific fatty acids carried in CEs.

# Results

The findings from the studies performed in this thesis are presented in each individual article. A summary of the most important findings is presented below.

## Anthropometric measures and blood lipids analyses

After following the diet intervention for 24 months, the 70 healthy postmenopausal obese women showed significant reductions in body weight, with no differences between groups. After six months, abdominal adiposity, measured as android fat (Figure 9A) and the sagittal abdominal diameter (Figure 9B), was significantly lower in the PD group compared to the CD group. In addition, the PD group showed lower triglyceride concentrations at six months (Figure 9C) and lower triglyceride/HDL ratios (Figure 9D) at six and 24 months, compared to the CD group.

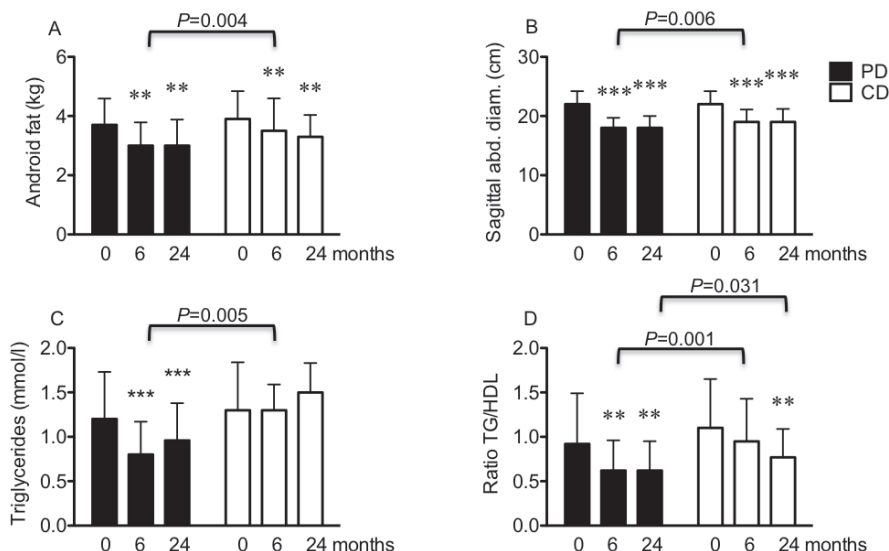


Figure 9.

Changes in body composition and blood lipids during 24 month intervention with a Paleolithic diet or a prudent control diet; (A) android fat weights; (B) sagittal abdominal diameters; (C) plasma triglyceride concentrations; and (D) the ratio of TG/HDL. Data are shown as the mean  $\pm$  SD. Differences compared to baseline were analyzed with GEE: \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Differences between groups are analyzed with Mann-Whitney U test and shown with exact P-values. CD, prudent control diet; GEE, generalized estimating equation; HDL, high-density lipoproteins; PD, Paleolithic diet; Sagittal abd. diam., sagittal abdominal diameter; TG, triglycerides

## Food intake and validation

The diets were given *ad libitum*; in other words, the significant reductions in energy intake observed during the intervention in both diet groups were due to self-restriction. We found no differences in total energy intakes between groups at any time point (Figure 10A). The energy expenditure due to physical activity (PAEE, Figure 10B) was measured with the Actiheart® device. The PAEE was unchanged in both diet groups during the entire intervention, with no difference between groups. We estimated that dietary intake was under-reported by 15% in both groups. This percentage was estimated by comparing the reported energy intake to the total energy expenditure, calculated as the PAEE plus the resting energy expenditure, and taking weight loss into account.

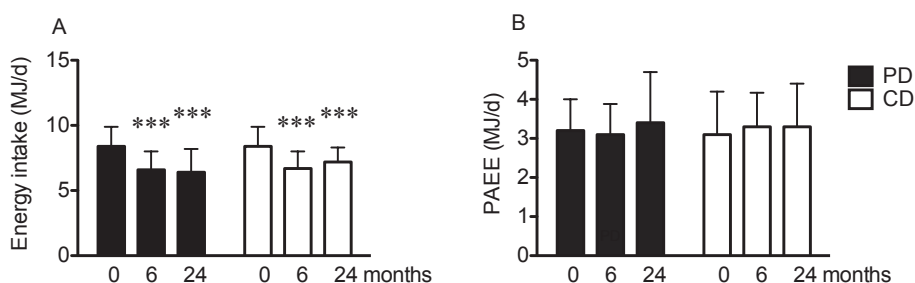


Figure 10.

Changes in energy intake and expenditure. (A) Energy intakes and (B) PAEE were compared between participants on a Paleolithic diet or a prudent control diet for 24 months. Data are the mean  $\pm$  SD. Differences compared to baseline were calculated with a GEE: \*\*\* $P < 0.001$ . CD, prudent control diet; GEE, generalized estimating equation; PAEE, physical activity energy expenditure; PD, Paleolithic diet

The SFA and carbohydrate intakes reported by the PD group were significantly lower than those reported by the CD group at six months ( $P = 0.022$ ,  $P < 0.001$ , respectively) and 24 months ( $P = 0.009$ ,  $P < 0.001$ , respectively; Figure 11). The intakes of proteins, MUFAs, and PUFAs were significantly higher in the PD group compared to the CD group at six and 24 months ( $P < 0.001$ , for all).



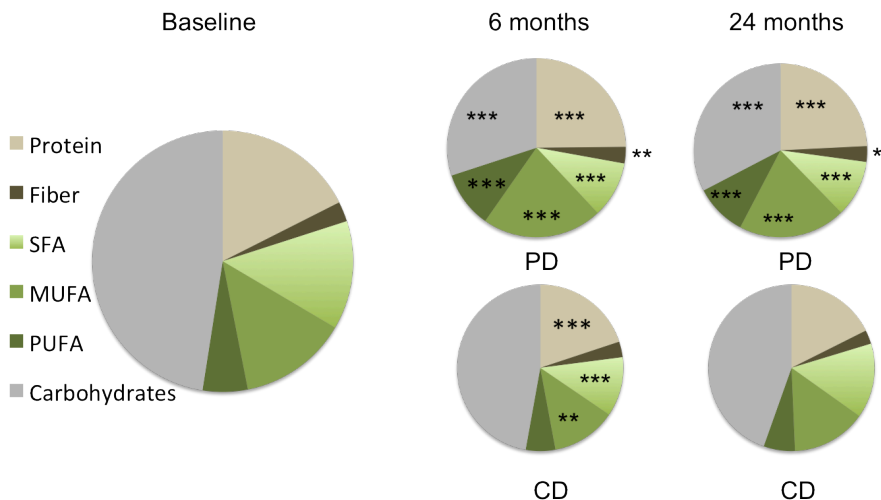


Figure 11.  
Changes in macronutrient intakes for cohorts on a Paleolithic diet or a prudent control diet. Differences compared to baseline were analyzed with a GEE; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . CD, prudent control diet; GEE, generalized estimating equation; MUFA, monounsaturated fatty acids; PD, Paleolithic diet; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids

The changes in reported PUFA intakes correlated with the changes in the PUFAs found in CEs observed between baseline and six months and between six and 24 months. These results confirmed the reported increases in PUFA intake in the PD group (Figure 12). However, the reported intakes of SFA and MUFA were not correlated with the changes in the SFA and MUFA contents found in CEs.

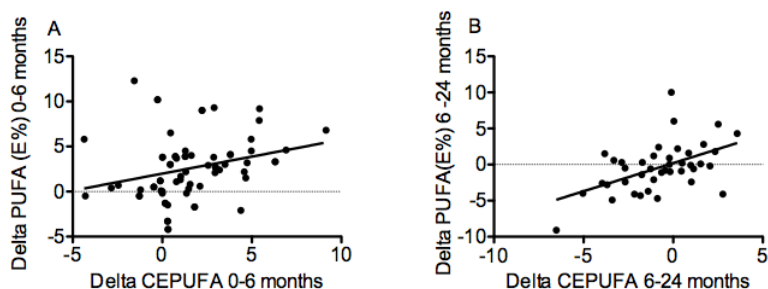
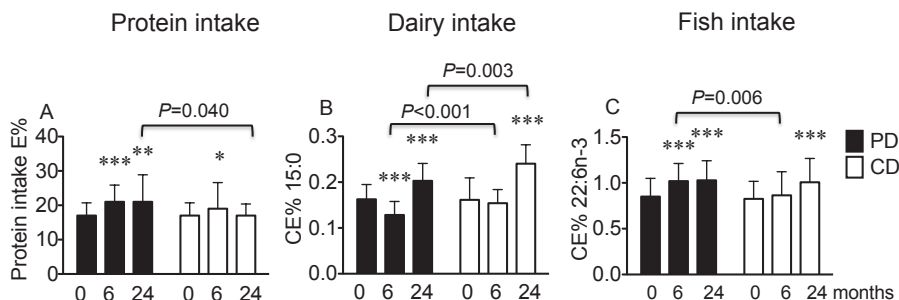


Figure 12.  
Changes (delta values) in reported intakes of PUFAs (%E) and the changes in PUFAs carried in CEs (CEPUFA). Changes were evaluated between (A) baseline and six months and (B) between six and 24 months. Correlations were analyzed with Spearman's coefficients; 0-6 months:  $R = 0.36$ ,  $P = 0.008$ ; 6-24 months:  $R = 0.48$ ,  $P = 0.002$ . CE, cholesterol ester; E%, energy percent; PUFA, polyunsaturated fatty acids

We validated protein intakes (E%) by measuring nitrogen excretion in the urine. The results confirmed the reported increased intakes in the PD group, with a significant difference between groups at 24 months (Figure 13A). The nitrogen excretion levels also confirmed the reported increased protein intake (E%) in the CD group at six months.



**Figure 13.** Validations in reported diet intakes for participants on a Paleolithic or a prudent control diet (A) total protein intakes (E%), based on urinary nitrogen excretion analyses. (B) Dairy intakes, based on the percent of pentadecylic acid (15:0) in CEs; and (C) fish intakes, based on the percent of 22:6 n-3 in CEs. Data are shown as the mean  $\pm$  SD. Differences compared to baseline were calculated with a GEE: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . The differences between groups are analyzed by Mann-Whitney U test and presented as exact P-values. CD, prudent control diet; CE, cholesterol ester; E%, energy percent; GEE, generalized estimating equation; PD, Paleolithic diet

The biomarker for dairy products, pentadecylic acid (15:0), was significantly lower in the PD group than in the CD group during the entire intervention period (Figure 13B). The biomarker for fish-derived fatty acids, 22:6 n-3 (DHA), was significantly higher at six months in the PD group than in the CD group (Figure 13C).

## Study I: Fat metabolism

In the PD group, significant reductions compared to baseline were observed in the expression levels of *LPL*, *ATGL* (data not shown), *CD36*, *DGAT2*, and *FAS* genes (Figure 14A-C) at six months. *DGAT2* and *FAS* expression decreased significantly more in the PD group than in the CD group. In contrast, the expression of the gene that encodes perilipin was unaltered in both diet groups, after six months (data not shown).

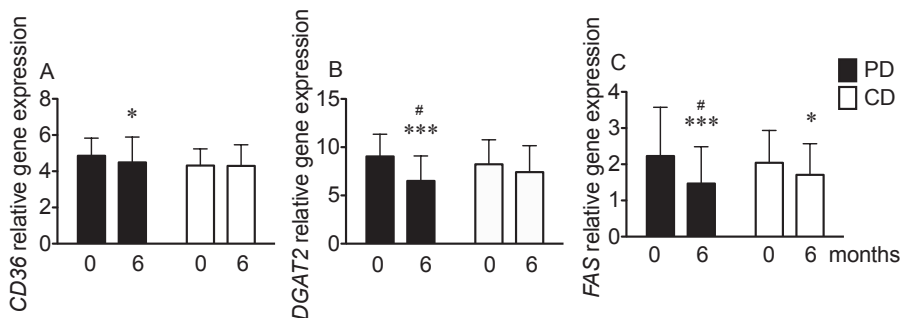


Figure 14.

Changes in relative gene expression levels in adipose tissue. Expression levels of (A) *CD36*; (B) *DGAT2*; and (C) *FAS* were measured in samples from participants on a Paleolithic diet or a prudent control diet. Data are shown as the mean  $\pm$  SD. Differences compared to baseline were calculated with a GEE: \* $P < 0.05$ , \*\*\* $P < 0.001$ . The differences in changes (diet  $\times$  time) between groups were calculated with a GEE: # $P < 0.05$ . CD, prudent control diet; *DGAT2*, diglyceride acyltransferase 2; *FAS*, fatty acid synthase; GEE, generalized estimating equation; PD, Paleolithic diet

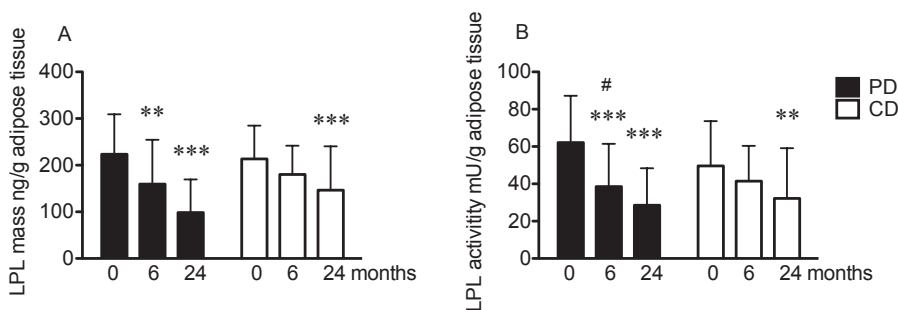


Figure 15.

Changes in LPL mass and activity. (A) LPL mass and (B) LPL activity measured in adipose tissue samples from participants on a Paleolithic diet or a prudent control diet. Data are expressed as the mean  $\pm$  SD. Differences compared to baseline were calculated with a GEE: \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . The differences in changes (diet  $\times$  time) between groups were calculated with a GEE: \* $P < 0.05$ . CD, prudent control diet; GEE, generalized estimating equation; LPL, lipoprotein lipase; PD, Paleolithic diet.

Compared to baseline, LPL mass and activity decreased significantly in the PD group after six and 24 months, and LPL activity in the CD group, after 24 months (Figure 15A, B). After six months, LPL activity changed significantly more in the PD group than in the CD group. Both total and low-density lipoprotein (LDL) cholesterol decreased significantly in both groups at six months. In addition, LDL was decreased in the PD group at 24 months compared to baseline. Circulating triglyceride levels were significantly reduced in the PD group after 6 and 24 months compared to baseline (data not shown).

### ***Linear regression analyses***

We used linear regression analyses to test whether the main outcomes related to adipose tissue fat metabolism (i.e., *CD36*, *DGAT2*, *FAS*, *SCD1* activity index, LPL mass, and LPL activity) were related to the sagittal abdominal diameter, insulin levels, or nutrient intake levels (i.e., carbohydrates and PUFAs).

At 6 months, we found that, in the PD group, the sagittal abdominal diameter was associated with alterations in fat metabolism, including changes in *CD36* ( $R=0.25$ ,  $P<0.05$ ) and *DGAT2* ( $R=0.48$ ,  $P<0.001$ ) expression. We also found a significant association between circulating fasting insulin levels and LPL mass ( $R=0.42$ ,  $P<0.05$ ) in the PD group.

Moreover, in the PD group, we found that the reported carbohydrate intake was associated with *DGAT2* ( $R=0.38$ ,  $P<0.01$ ) and *FAS* ( $R=0.27$ ,  $P<0.05$ ) expression levels, LPL activity ( $R=0.58$ ,  $P<0.001$ ), LPL mass ( $R=0.36$ ,  $P<0.05$ ), and the *SCD1* activity index ( $R=0.51$ ,  $P<0.001$ ). Moreover, the reported PUFA intake was associated with *DGAT2* ( $R=0.42$ ,  $P<0.001$ ) and *FAS* ( $R=0.34$ ,  $P<0.01$ ) expression levels and the *SCD1* activity index ( $R=0.52$ ,  $P<0.001$ ).

## Study II: Inflammation

Circulating CRP levels decreased significantly in the PD group throughout the intervention period (Figure 16A). Concomitantly, IL-6 and TNF- $\alpha$  concentrations decreased, compared to baseline, in both groups after 24 months (Figure 16B, C). At six months, IL-6 and TNF- $\alpha$  levels increased, compared to baseline, in the CD group. Circulating MCP1 and MIF were unchanged compared to baseline in both groups after 24 months (data not shown).

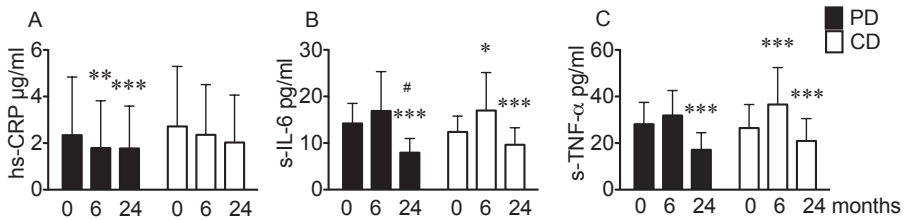


Figure 16.

Changes in circulating inflammatory markers. Changes in plasma levels of (A) hs-CRP; (B) s-IL-6; and (C) s-TNF- $\alpha$  measured in participants on a Paleolithic diet or a prudent control diet. Data are shown as the mean  $\pm$  SD. Differences compared to baseline were calculated with a GEE: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . # $P < 0.05$ . CD, prudent control diet; GEE, generalized estimating equation; hs-CRP, highly-sensitive C-reactive protein; IL-6, interleukin 6; PD, Paleolithic diet; s, serum; TNF- $\alpha$ , tumor necrosis factor- $\alpha$

Adipose expression of *TLR4* decreased significantly at 24 months in both groups (Figure 17A). In the PD group, *IL-6* and *MCP-1* expression levels decreased progressively during the intervention period (Figure 17B, C). In the CD group, *IL-6* expression decreased significantly after 24 months of intervention. In contrast, *MIF* expression increased in both groups after 24 months of intervention (Figure 17D). *TNF- $\alpha$*  gene expression remained unaltered throughout the study in both groups (data not shown).

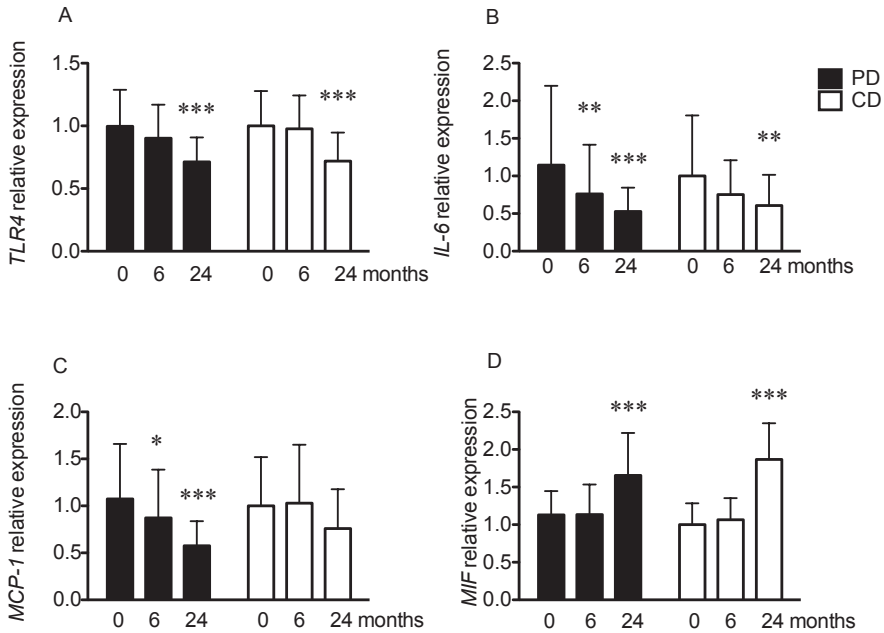
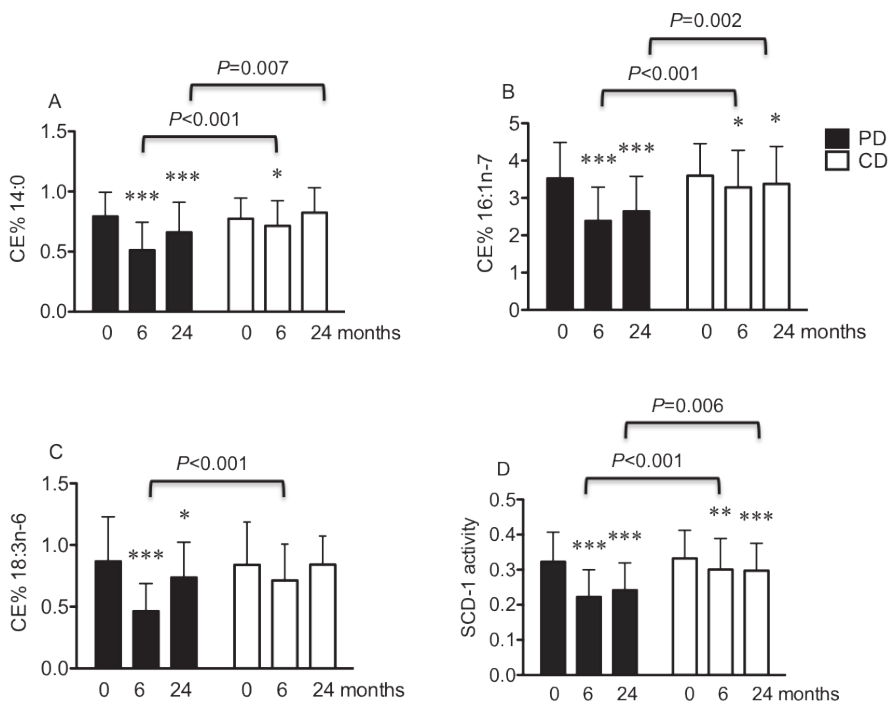


Figure 17. Changes in relative gene expression in adipose tissues. (A) TLR4; (B) IL-6; (C) MCP-1; and (D) MIF expression levels were evaluated in samples from participants on a Paleolithic diet or a prudent control diet. Data are shown as the mean  $\pm$  SD. Differences compared to baseline were calculated with a GEE: \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ . CD, prudent control diet; GEE, generalized estimating equation; IL-6, interleukin 6; MCP-1, monocyte chemoattractant protein 1; MIF, macrophage migration inhibitory factor; PD, Paleolithic diet; TLR4, toll-like receptor 4

### Study III: Circulating fatty acids in cholesterol esters

The PD affected changes in the specific fatty acids found in CEs to a greater extent than the CD. Compared to the CD group, the PD group had significantly lower percentages of myristic acid (14:0), palmitoleic acid (16:1 n-7), and  $\alpha$ -linolenic acid (18:3 n-6) at six months, and lower levels of myristic acid (14:0) and palmitoleic acid (16:1 n-7), at 24 months (Figure 18A-C, next page). The levels of palmitic acid (16:0) and dihomo- $\gamma$ -linoleic acid (20:3 n-6) were significantly lower in the PD group compared to the CD group, after 24 months ( $P=0.035$ ;  $P=0.004$ , respectively; data not shown). The index of SCD-1 activity was significantly lower in the PD group compared to the CD group, at six and 24 months (Figure 18D).



**Figure 18**

Changes in fatty acids carried in CEs. Changes detected in (A) myristic acid (14:0); (B) palmitoleic acid (16:1 n-7); and (C)  $\alpha$ -linolenic acid (18:3 n-6) levels, and in (D) the index of SCD-1 activity measured in participants on a Paleolithic diet or a prudent control diet. Data are shown as the mean  $\pm$  SD. Differences from baseline were calculated with a GEE: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . The differences between groups are analyzed by Mann Whitney U test and shown with exact P-values. CD, prudent control diet; CE, cholesterol ester; GEE, generalized estimating equation; PD, Paleolithic diet; SCD-1, stearyl-CoA desaturase 1

### Correlation analyses

At six months, we found that changes in HOMA were significantly correlated with changes in the concentrations of the fatty acids, 14:0 ( $R=0.44$ ,  $P<0.001$ ), 16:1 n-7 ( $R=0.44$ ,  $P<0.001$ ), and 18:3 n-6 ( $R=0.42$ ,  $P=0.001$ ), and in the SCD-1 activity index ( $R=0.38$ ,  $P<0.001$ ). Furthermore, at six months, we found a significant association between changes in the triglyceride/HDL ratio and changes in the SCD-1 activity index ( $R=0.33$ ,  $P=0.013$ ). Finally, we found that changes in android fat, from baseline to six months, were significantly associated with changes in specific fatty acids: 14:0 ( $R=0.34$ ,  $P=0.011$ ), 16:1 n-7 ( $R=0.44$ ,  $P<0.001$ ), and 18:3 n-6 ( $R=0.51$ ,  $P<0.001$ , respectively).

# Discussion

## Diet intervention

The study cohort of postmenopausal obese women achieved substantial reductions in BMI and abdominal adiposity during the first six months of the interventions. Moreover, they maintained weight loss at the 24 month follow up. The diets were remarkably successful; most previous studies reported weight regain in about half of the participants after one year. Weight was probably regained due to the fact that energy restriction increases hunger levels and provokes energy expenditure adaptations by changing the secretion of hormones from the gut and adipose tissue. These alterations in appetite regulation factors from the gut and adipose tissue, combined with increase in metabolic efficiency, encourage weight regain, and can persist for years after diet-induced weight loss. (44, 76)

There are several possible explanations for the high adherence and the good weight maintenance observed in our study. First, we included postmenopausal women that were highly motivated to lose weight. Second, the intervention included behavioral strategies that, in earlier studies, led to successful weight management (46). These behavioral strategies included diet-specific group sessions that aimed to improve knowledge about nutrition, cooking procedures, and the importance of physical exercise, which increased participants' motivations for achieving better health. Studies have also shown that self-monitoring diet intake and physical activity may improve and maintain behavioral changes associated with weight maintenance (46). The group sessions, food records, and physical activity measurements were most frequently performed the first six months of intervention, but the frequency was substantially reduced thereafter. Nevertheless, weight maintenance continued up to 24 months. Notably, many of the participants were repeat dieters; this behavior is associated with changes in metabolism that increase metabolic efficiency and lower energy demands. Thus, those individuals may have experienced a reduced response to this type of intervention.

The diets were given *ad libitum*. This approach may have reduced the feeling of being restricted to a diet and prevented increases in hunger. These features could have improved adherence, weight loss, and maintenance on a long-term basis (45). Macronutrient composition can also influence dietary adherence. We expected that the increased protein intake in the PD would contribute to increased satiety, due to the modulation of appetitive signaling. Moreover, elevated protein intake might have increased energy expenditure by preserving lean mass and elevating thermogenesis (15). However, an estimation of the



nitrogen excreted in urine revealed that protein intake initially increased in both groups, but at 24 months, the PD group had only a slightly higher protein intake than the CD group. This finding might explain the nearly equal reductions in energy intake observed in both diet groups during the intervention. We had also expected that the PD would have reduced energy intake to a greater extent than the CD, because the PD had higher unsaturated fatty acid (77) and viscous fiber contents (78) and a lower carbohydrate content (79), which may modulate effects on appetite signaling.

The higher PUFA intake and the reported lower carbohydrate intake with the PD compared to the CD can contribute to the more pronounced reduction in abdominal adiposity after six months in the PD compared to the CD group. Indeed, diets with a low glycemic index and high PUFA contents were shown to reduce the levels of many factors involved in fat storage, including fatty acid and triglyceride synthesis, as discussed below.

### ***Diet validation***

As mentioned, the protein intake in the PD group increased (80), but it never reached the goal of 30 E% protein intake. This failure may have been a consequence of the difficulties in changing dietary habits. In particular, a high content of protein at breakfast was difficult, according to the participants. In addition, the increased cost associated with consuming food with high protein contents may have affected the lower intake (81). The CD group initially increased their protein intake, according to both diet records and nitrogen excretions measured in the urine. This behavior may have been due to cross talk between intervention groups, despite separate group sessions, because participants lived in a limited geographical recruitment area.

The PD group reduced their SFA and carbohydrate intakes and increased their MUFA and PUFA intakes. The self-reported food records indicated that the groups showed significant differences in their intakes of all these nutrients during the intervention period. Like the validation of protein intake, the PUFA intake could be validated in an objective way by analyzing the relative proportion of fatty acids in CEs (82). This measurement reflected the fatty acid intake during the few weeks prior to the analysis, including the four days of recording food intake. The reported increase in PUFA intake in the PD group correlated with the proportion of PUFAs in CEs; thus, the increased PUFA intake was validated in the PD group without quantifying the actual PUFA intake.

The fatty acids, 15:0 and 22:6 n-3, served as biomarkers for dairy products (83) and fish (84), respectively. These markers indicated that the PD group had

reduced their intake of dairy products and increased fish intake, at six months, consistent with the guidelines of the diet regime. The 15:0 biomarker was lower in the PD group than in the CD group throughout the intervention period, but it increased in the PD group after six months. This increase indicated that adherence to the diet had weakened, because dairy products were excluded from the PD. The fish intake increased in both groups; at 24 months, there was no difference between groups in fish consumption. This finding probably resulted from the efforts to encourage an increase fish intake in both groups.

The physical activity levels measured with the Actiheart® device remained unchanged during the entire intervention period in both groups, despite information and encouragement to increase the amount of physical activity. The discrepancy between reported energy intakes and total energy expenditures indicated that energy intake was under-reported by 15% in both diet groups. Under-reporting total energy intake is common in dietary trials, and it is more extensive in studies on obesity (85).

## **Fat storage**

Obesity is characterized by excessive lipid accumulation. DNL and triglyceride-rich lipoproteins are important sources for the lipids stored in adipose tissue and in ectopic depots (86). LPL is the gate-keeper enzyme for fat storage, because LPL is the primary enzyme involved in the lipolysis of triglyceride-rich lipoproteins in the circulation (Figure 19) (32). After six months of the intervention, the LPL activity was reduced significantly more in the PD group than in the CD group. This reduced activity was associated with significant reductions in LPL mass, abdominal adiposity, circulating triglycerides, total cholesterol, and LDL cholesterol, compared to baseline. These findings were consistent with findings from an earlier hypocaloric dietary intervention study in postmenopausal women. In that study, a reduction in abdominal fat was followed by reductions in LPL activity, cholesterol levels, and triglyceride levels in the circulation (87). At six months, the difference between diet groups in the reduction of LPL activity might have been a consequence of the lower carbohydrate intake in the PD; indeed, LPL activity in adipose tissue is regulated by glucose and insulin, mainly at the post-translational level (33). This notion fit with our finding that the reported carbohydrate intake was significantly associated with LPL mass and activity and that LPL mass was significantly associated with circulating insulin levels.

The free fatty acid transporter, CD36, is upregulated in obesity and type 2 diabetes (88). The PD group exhibited a significant reduction in *CD36* expression in subcutaneous adipose tissue at six months. A previous study showed that a genetic *CD36* deficiency in humans was associated with a

reduction in total fat mass. That association might at least partly explain the association we observed between reductions in abdominal adiposity and reductions in *CD36* expression in the PD group (34).

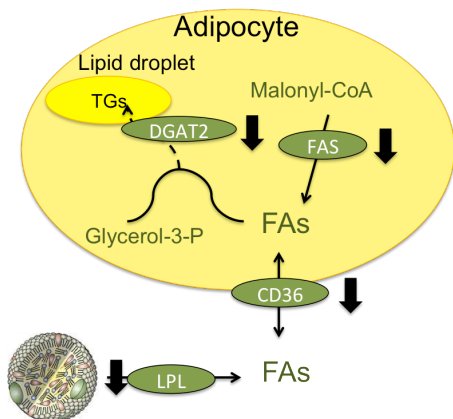


Figure 19.

*The Paleolithic diet caused changes in the expression of key proteins in lipogenesis and lipolysis and in LPL activity. After six months with Paleolithic diet, LPL mass and activity declined, and the expression levels of key enzymes were downregulated (heavy arrows). DGAT2, diglyceride acyltransferase 2; FAs, fatty acids; FAS, fatty acid synthase; Glycerol-3-P, glycerol-3 phosphate; LPL, lipoprotein lipase*

DGAT2 catalyzes the final, and probably rate-limiting, step in triglyceride synthesis (89). At six months, the PD group showed a more pronounced reduction in *DGAT2* expression than the CD group. This indicates that the fat storage rate was lower in the PD group than in the CD group. The expression of *DGAT2* in the PD group was associated with abdominal adiposity and with carbohydrate and PUFA intakes. These findings were consistent with previous studies, which showed that diets caused reduced *DGAT2* expression in adipocytes, due to reductions in adipose mass and nutritional factors, like glucose and PUFAs (90, 91).

Obesity and diabetes are associated with significant elevations in DNL and other avenues of fatty acid synthesis. DNL is catalyzed by the multienzyme FAS, and it is followed by increased triglyceride synthesis (92, 93). When DNL is elevated, fatty acid oxidation is reduced, due to an increase in the concentration of the intermediate metabolite, malonyl-CoA, which also indirectly increases fat storage. We found that FAS expression was reduced more in the PD group than in the CD group at six months. Moreover, in the PD group, FAS expression was associated with carbohydrate and PUFA intakes, consistent with results from previous studies (37, 94).

Hepatic DNL is associated with a high plasma SCD-1 activity index (95). In addition, the SCD-1 activity index in both the hepatic lipid fraction and the CE fraction was shown to correlate with hepatic SCD-1 expression (96). We found that the PD group exhibited a more pronounced reduction in the SCD-1 activity index during the entire intervention, compared to the CD group. This suggests that DNL was reduced in the liver. The reduced SCD-1 activity was associated with the increased PUFA intake in the PD group, consistent with findings from a previous study (97).

In the PD group, a more pronounced reduction in lipogenesis-promoting factors at six months was followed by a reduction in the concentrations of circulating lipids, including triglycerides. During negative energy balance, the fat storage capacity of adipose tissue is reduced, combined with a putative reduction in adipocyte cell size. These features may be metabolically beneficial, because, presumably, the secretion of fatty acids and proinflammatory adipokines would also be reduced. Moreover, the reduced levels of triglycerides in the PD group can reduce ectopic fat storage, and thus, the risk of cardiometabolic diseases. Consistent with this notion, the PD group had lower liver fat contents than the CD group after six months of intervention (98).

During weight loss, fat storage is reduced and lipolysis is reduced in the smaller fat cells, due to the downregulation of *ATGL* expression seen in both diet groups. However, in this state, adipocytes also experience an increased drive to store fat (99). MIF seems to play an important role in fat accumulation (100, 101). The upregulated expression of *MIF* at 24 months may be an adaptive mechanism to compensate for lost triglycerides, by reinstating intracellular fat storage in adipose tissue. Moreover, increased MIF activity could prevent ectopic fat deposition in organs like muscles and liver, during excessive energy intake. In contrast to the increased expression of *MIF* in adipose tissue at 24 months, both groups exhibited reductions in the SCD-1 activity index, LPL mass, and LPL activity at 24 months. These reductions indicated reductions in hepatic DNL and lipogenesis. These contradictory findings may indicate a possible breakpoint between weight maintenance and a more general weight gain at the end of the intervention in both groups.

## **Insulin resistance**

Several important factors have been implicated in the pathogenesis of insulin resistance, including the innate immune pathways and the accumulation of ectopic fat metabolites. Thus, we analyzed proinflammatory factors and circulating fatty acids as risk factors linked to insulin resistance. This investigation aimed to elucidate the effects of the PD and CD on insulin sensitivity in postmenopausal obese women (102).

### Proinflammatory factors

Fat biopsies taken at baseline, six, and 24 months provided the opportunity to investigate longitudinal changes in gene expression in adipose tissue. The expression of inflammation-related genes declined in association with reductions in the sagittal abdominal diameter and android fat mass.

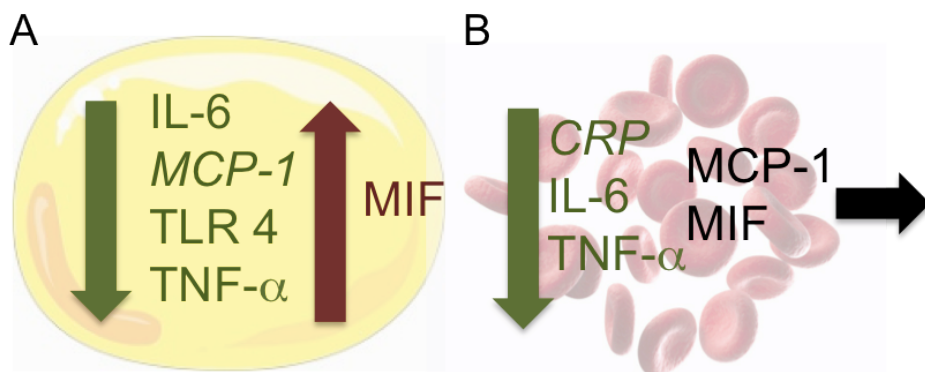


Figure 20.

Changes in inflammatory markers after 24 month diet intervention. Changes were observed in (A) gene expression of inflammatory proteins in adipose tissue, and (B) circulating inflammatory markers. Green = decreased levels compared to baseline. Red = increased levels compared to baseline. Black = unaltered levels compared to baseline. The gene expression of MCP-1 and concentration of CRP in circulation (both italicized) were reduced only in the Paleolithic diet group. CRP, C-reactive protein; IL-6, interleukin 6; MCP-1, monocyte chemoattractant protein 1; MIF, macrophage migration inhibitory factor; TLR-4, toll like receptor 4; TNF- $\alpha$ , tumor necrosis factor alpha

*TLR4* expression is increased in the adipose tissue of obese individuals. This finding supported a previous study that showed that these receptors were involved in low-grade inflammation associated with hypertrophic adipose tissue (103). SFA and lipopolysaccharides bind to TLR4 and stimulate the synthesis of pro-inflammatory adipokines in adipocytes (104). We found that TLR4 mRNA declined significantly in both diet groups between six and 24 months of the intervention (Figure 20). This decline may be associated with long-term, favorable metabolic and inflammatory outcomes. Consistent with the reduced TLR4 mRNA levels, the expression of proinflammatory adipokines, such as *IL-6*, decreased in both groups. Moreover, *MCP-1* expression was reduced in the PD group at 24 months. All three proteins, TLR4, *IL-6*, and *MCP-1*, have been shown to activate kinases that directly blocked insulin signaling and promoted insulin resistance (105).

After 24 months of intervention, both groups exhibited reductions in the circulating levels of *IL-6*, in accordance with the reduced expression of *IL-6* in

adipose tissue. In addition, the circulating levels of TNF- $\alpha$  decreased, despite unchanged expression in adipose tissue. The reduced serum concentrations of IL-6 and TNF- $\alpha$  are consistent with an earlier dietary intervention study that demonstrated reductions in the circulating levels of these cytokines after weight loss, followed by improved insulin sensitivity (106). The discrepancy between the TNF- $\alpha$  expression in adipose tissue and in the circulation might be explained by the fact that TNF- $\alpha$  is mainly secreted by monocytes in the circulation. Therefore, changes in serum levels of TNF- $\alpha$  may be more affected by the secretion from monocytes than from adipose tissue (107).

Circulating CRP levels decreased significantly in the PD group throughout the intervention, but no significant changes in CRP were observed in the CD group. These findings were consistent with a previous study that showed a significant reduction in CRP concentrations associated with weight loss on a low-carbohydrate Mediterranean diet, but weight loss on a low-fat diet resulted in unchanged CRP levels (108).

MCP-1 plays an important role in recruiting monocytes to tissues. MCP-1 has been linked to inflammation, atherosclerosis, and insulin resistance (109). Circulating concentrations of MCP-1 were unchanged in both groups after 24 months of diet intervention, despite a significant reduction in *MCP-1* expression observed in the PD group. The stable serum concentration of MCP-1 was consistent with a previous study that showed unaltered circulating MCP-1 levels during diet-induced weight loss (110).

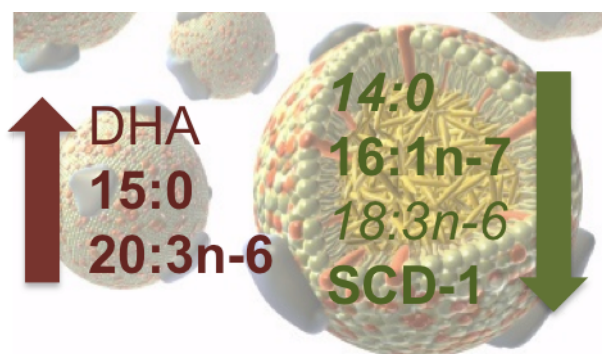
MIF promotes cell recruitment (111), and it is a biomarker for different diseases with inflammatory components, including cardiovascular disease and insulin resistance (111). Consistent with earlier studies, we observed a significant reduction in circulating MIF levels, with a concomitant reduction of body weight, in both diet groups at six months (112). The reduction in circulating MIF at six months reflected the improvements in risk factors of cardiovascular disease, including blood pressure, total cholesterol, and LDL cholesterol (112). After 24 months, circulating MIF concentrations returned to baseline levels in both groups, despite the increased *MIF* expression detected in adipose tissue samples after 24 months. This discrepancy may be explained by the paracrine effect of MIF on adipose tissue, which induces fat storage (113).

In contrast to our hypothesis that the different diets would have different effects on inflammatory markers, we found no major differences in the inflammatory markers between the two diets. This finding suggested that the observed changes were mainly due to weight loss, with two exceptions. First, we did observe a diet-time interaction effect regarding serum IL-6 levels; and second,

we observed significant reductions in circulating CRP levels and *MCP-1* gene expression in the PD group, but not the CD group. Importantly, reduced expression of proinflammatory proteins, including TLR4, IL-6, and MCP-1, may reduce the risk of disturbances in the internal adipocyte insulin signaling pathways. In addition, the reduced levels of proinflammatory cytokines in the circulation may improve insulin sensitivity in muscles and liver (114).

### ***Fatty acids carried in cholesterol esters***

Fatty acids carried in plasma CEs can be used as biomarkers of dietary fat quality. In addition, fatty acid ratios can serve as indicators of endogenous desaturation. The omega-3 PUFA from fish, DHA, has a wide spectrum of biological actions involving membranes, signal transduction, and gene expression. These actions suggest that DHA plays an important role in the prevention of inflammatory diseases, including insulin resistance and cardiovascular diseases (115). The proportion of DHA in CEs increased in both groups after 24 months, but the PD group showed significantly higher DHA levels in CEs at six months. The increased DHA levels at six months in the PD group may have contributed to the significant reductions in inflammatory markers. Indeed, reduced serum CRP levels and reduced *IL-6* and *MCP-1* gene expression in adipose tissue were only observed in the PD group at this time.



*Figure 21.*

*Changes in specific fatty acids in plasma cholesterol esters and stearoyl-CoA desaturase 1 activity after 24 month diet intervention. Green = reduced levels compared to baseline. Red = increased levels compared to baseline. Italicized: only reduced in the Paleolithic diet group. Bold: significantly lower levels in the Paleolithic diet group compared to the prudent control group at 24 months. DHA, docosahexaenoic acid; SCD-1, stearoyl-CoA desaturase 1*

The proportions of fatty acids 14:0, 16:1 n-7, and 18:3 n-6, carried in plasma CEs, were significantly more reduced in the PD group than in the CD group during the intervention period (Figure 21). Similar changes in the 14:0, 16:1 n-7, and 18:3 n-6 proportions of CEs have been associated with reduced abdominal



adiposity and improved insulin sensitivity (97, 116, 117). Consistent with these results, we found that the changes in 14:0, 16:1 n-7, and 18:3 n-6 in plasma CEs were significantly correlated with reductions in android fat and insulin resistance after six months, when the metabolic changes were most pronounced in the PD group. In addition, the PD group had significantly lower levels of 16:0 and 20:3 n-6 in CEs at 24 months compared to the CD group. These fatty acids were linked to increased diabetes risk in the European Prospective Investigation into Cancer and Nutrition study (118, 119).

Estimated desaturase activities (product/precursor ratios) in CEs were closely related to desaturase activities in other body tissues (71). The synthesis of monounsaturated fatty acids is catalyzed by SCD-1, which is regulated by dietary PUFA and SFA intakes (97). The SCD-1 activity index was significantly reduced in the PD group compared to baseline and compared to the CD group. This reduction may have resulted from an increased ratio of PUFA/SFA in the diet (97). A high SCD-1 activity index has been linked to insulin resistance (120); indeed, insulin resistance markers, including HOMA and the triglyceride/HDL ratio, were correlated with the SCD-1 activity index in the PD group after six months.

The postmenopausal obese women included in this study were healthy, with relatively well-preserved insulin sensitivity. Nevertheless, the PD group showed improved insulin sensitivity, as estimated with the HOMA-IR index, at six months, when adherence to the diet was highest. In addition, in the PD group, the ratio of triglycerides/HDL, another predictor of insulin resistance, decreased significantly compared to baseline and compared to the CD group during the entire intervention. The observed improvement in insulin sensitivity in the PD group may be related to fact that the inflammatory profiles were slightly more beneficial in the PD than in the CD group. In addition, the reduction in *MCP-1* expression with a PD may have reduced adipose tissue inflammation by lowering the macrophage infiltration rate and their polarization. Finally, the reduction in CRP levels may have improved systemic inflammation in the PD compared to the CD group. The improved fatty acid profile in the PD group, with reduced levels of specific fatty acids and desaturase activities, compared to the CD group, also might have contributed to the improved insulin sensitivity in the PD group.



## Strengths and limitations

### *Study design*

A randomized controlled trial is often considered the gold standard of clinical trials. Due to the reduction of bias, these studies provide a robust method for determining the specific effects of a novel treatment compared to a conventional treatment, while other variables remain constant (121). However, some key aspects of the trial should be taken into consideration. For example, it might be difficult to maintain blinding of the participants and researchers to avoid contamination between the intervention groups. In the present study, it was not possible to blind either the participants or the nutritionists, but most researchers and nurses were blinded. Despite our efforts, we observed increased protein intakes in the CD group, probably due to some contamination between groups. Adding a free-living observational group to our study would have given us the opportunity to evaluate any changes in diet that occurred in the general population of postmenopausal women. That study design might have highlighted the beneficial behavioral effects of being included in either of the intervention groups. Hence, it should be considered that the women in both dietary groups most likely changed to a healthier diet, when they started the intervention.

Evaluating dietary intake in free-living subjects is always a challenge. Regardless of the methods used, there are problems with both under- and over-estimations of food intake. Selective under-reporting generally occurs for foods that are either known to be unhealthy or are not included in the diet. Over-reporting generally occurs for foods that are known to be healthy or are emphasized in the diet (122). Therefore, it is important to validate food reports with objective measurements. We validated protein intakes by measuring nitrogen excretion in the urine (65). Furthermore, we validated intake of fatty acids and specific food sources by analyzing the fatty acids carried in CEs (67). According to these objective measurements, we estimated that protein intake, which was in focus in the PD, was slightly over-reported, and dairy products, which were excluded from the PD, were under-reported in the PD group.

Another source of error was that participants tend to eat less or healthier during the periods that dietary intakes are assessed (122). Therefore, it is important to evaluate energy intake relative to energy expenditure during the time of the assessments. In this study, we estimated total energy expenditure based on the PAEE and resting metabolic rate, and we took weight loss into account. Our results showed that the total energy intake was under-reported by 15% in both groups. As mentioned above, under-reporting is not unusual in diet studies, and it is particularly common among obese subjects (122).

The diets were *ad libitum*, which is important in a clinical setting. Thus, self-restricted reductions in energy intakes during the interventions were followed by weight loss. The changes in body weight made it difficult to differentiate whether the effects were due to changes in macronutrient composition or due to lower energy intake. The decline in energy intake was not significantly different between the diet groups during the interventions. However, a constant energy intake is preferable for evaluating the impact of a macronutrient change on metabolic factors. Another limitation of this study was that we were not able to validate the self-reported intakes of total fat, carbohydrates, and fiber. Thus, it was difficult to draw any conclusions about the contributions of these nutrients to the observed metabolic changes. In particular, it would have been interesting to confirm carbohydrate intakes, because carbohydrates are associated with many metabolic risk factors for cardiovascular diseases and insulin resistance. Moreover, it would have been very interesting to investigate the stool for fiber content and microbiota composition. That analysis might have provided additional explanations for the observed metabolic outcomes related to inflammatory markers.

The study duration time of 24 months proved to be important, because some metabolic alterations first appeared only after a period of weight maintenance. The relatively low drop-out rate was a strength of the study. However, unfortunately, the CD group had a higher drop-out rate than the PD group. This difference might have influenced our ability to detect differences between the groups. Importantly, the general estimating equation analysis used in our study diminished the effects of the change in variance due to the higher dropout rate.

### ***Subjects***

Postmenopausal women carry high risks of abdominal adiposity, cardiovascular diseases, and type 2 diabetes, probably due to estrogen deficiency (123). The women included in this study were postmenopausal and obese, but otherwise healthy. Excluding pre- and peri-menopausal women from the study reduced the influence of hormonal fluctuations, due to the menstrual cycle.

### ***Measurements and analyses***

One major limitation of our study was that, although the fat biopsies were sufficient for studying gene expression of inflammatory factors, they were insufficient for studying key proteins in fat metabolism at 24 months. We found contradictory results regarding fat storage at 24 months. The *MIF* gene expression increased, which indicated increased fat storage capacity; but the reductions in the SCD-1 activity index, LPL mass, and LPL activity pointed in the opposite direction, i.e., to reduced lipogenesis. Therefore, it would have been very interesting to analyze the expression of genes that encoded other key

proteins involved in lipogenesis and lipolysis at 24 months. It might also have been interesting to analyze fat biopsies more frequently during the intervention. With these additions, we might have been able to study changes in the expression of factors involved in fat storage during weight maintenance.

Another limitation of the study was that we did not measure fat cell size. This would have made it possible to validate changes in lipogenesis-promoting factors, particularly LPL activity and LPL mass, which is expressed in units per g of adipose tissue. Fat cell size is expected to decrease with weight loss, due to the reduced availability of triglycerides. This limitation may have led to an underestimation of the reduction in LPL activity in adipose tissue. Furthermore, it would have been valuable to measure the fatty acids stored in adipocytes to validate long-term fatty acid intakes. Indeed, alterations in fatty acids were a major feature of the metabolic improvement observed in the PD group (66).

The estimation of protein intake based on the 24-h nitrogen excretion in urine depends on the assumption that subjects are in nitrogen balance. Daily variations imply that individuals are unlikely to be in balance for any one day; therefore, measurements of intake and output are required over several days to estimate the protein intake. We used 24-h urine sampling over three days to measure the total volume and nitrogen concentration instead of the ideal eight days of sampling. This shortened period may have been a source of error, but it was considered necessary for practical reasons.

## **Implications for further research**

Our results indicated that dietary fatty acid and carbohydrate contents may be crucial for metabolic improvements associated with cardiometabolic diseases, consistent with findings from earlier studies (124). To elucidate further any beneficial long-term effects of a low-glycemic diet with a high unsaturated fatty acid content on metabolic risk factors, future studies could include a diet with a high saturated fatty acid content and low fiber content, like a low carbohydrate-high fat diet. Further studies should also include obese subjects with type 2 diabetes to broaden the range of putative metabolic improvements. Moreover, a study design that favors weight maintenance could elucidate the specific effects of macronutrient composition. In addition to fat biopsies, muscle biopsies are of interest. A study on concomitant RNA expression in fat and muscle could elucidate changes in key pathways linked to fat metabolism, glucose metabolism, and inflammation during the intervention. Those findings could be analyzed for associations with estimated changes in adipocyte cell size, feces fiber content, and the microbiota composition of feces.

## Summary and conclusions

Both *ad libitum* 24-month diet interventions, the Paleolithic diet (PD) and the prudent control diet (CD), induced substantial weight loss in obese postmenopausal women. Reductions in fat mass were accompanied by long-term attenuations of whole-body and adipose-specific inflammation. The lack of major differences in outcome between groups suggested that the improved inflammatory profile was mainly due to the reduction in abdominal adiposity.

In both groups, we found reductions in the gene expression, mass, and activity of the gatekeeper enzyme, lipoprotein lipase, in subcutaneous adipose tissue. However, the PD group exhibited significantly lower lipoprotein lipase activity at six months compared to the CD group. In addition, compared to the CD, the PD led to more pronounced reductions in the expression of genes that encoded lipogenesis-promoting factors, including CD36, diglyceride acyltransferase 2, and fatty acid synthase as well as the stearoyl-CoA desaturase 1 activity index. This finding suggested that lipogenesis was lower in the PD group than in the CD group.

The intake of polyunsaturated fatty acids, dairy products, and fish was validated by measuring biomarkers. The specific fatty acids found in plasma in the PD group confirmed increases in the intakes of polyunsaturated fatty acids and fish, compared to baseline, and reduced dairy intakes, compared to the CD group. Although the two diet groups showed comparable changes in body weight, energy intake, and physical activity at 24 months, the PD group showed greater reductions in metabolic risk factors, including 16:1 n-7, 20:3 n-6, desaturases and the triglyceride/high density lipoprotein ratio. These results suggested that the PD had improved lipid balance, to a greater extent than the CD.

In conclusion, we showed that long-term weight loss in obese postmenopausal women was associated with beneficial alterations in adipose tissue and circulating inflammatory markers. In addition, compared to a CD, the PD produced larger reductions in lipogenic-promoting factors in adipose tissue after six months. Moreover, the PD group exhibited greater reductions in circulating factors related to insulin resistance, such as fatty acids, desaturases, and the triglyceride/high density lipoprotein ratio, compared to the CD group. Our results suggest that the PD may be beneficial for lowering lipogenesis and improving dyslipidemia.

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