Resveratrol Does Not Benefit Patients With Nonalcoholic Fatty Liver Disease

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BACKGROUND & AIMS: **Nonalcoholic fatty liver disease (NAFLD), characterized by accumulation of hepatic triglycerides (steatosis), is associated with abdominal obesity, insulin resistance, and inflammation. Although weight loss via calorie restriction reduces features of NAFLD, there is no pharmacologic therapy. Resveratrol is a polyphenol that prevents high-energy diet-induced steatosis and insulin resistance in animals by up-regulating pathways that regulate energy metabolism. We performed a placebo-controlled trial to assess the effects of resveratrol in patients with NAFLD.**

METHODS: **Overweight or obese men diagnosed with NAFLD were recruited from hepatology outpatient clinics in Brisbane, Australia from 2011 through 2012. They were randomly assigned to groups given 3000 mg resveratrol (n = 10) or placebo (n = 10) daily for 8 weeks. Outcomes included insulin resistance (assessed by the euglycemic-hyperinsulinemic clamp), hepatic steatosis, and abdominal fat distribution (assessed by magnetic resonance spectroscopy and imaging). Plasma markers of inflammation, as well as metabolic, hepatic, and antioxidant function, were measured; transcription of target genes was measured in peripheral blood mononuclear cells. Resveratrol pharmacokinetics and safety were assessed.**

RESULTS: **Eight-week administration of resveratrol did not reduce insulin resistance, steatosis, or abdominal fat distribution when compared with baseline. No change was observed in plasma lipids or antioxidant activity. Levels of alanine and aspartate aminotransferases increased significantly among patients in the resveratrol group until week 6 when compared with the placebo group. Resveratrol did not significantly alter transcription of *NQO1*, *PTP1B*, *IL6*, or *HO1* in peripheral blood mononuclear cells. Resveratrol was well-tolerated.**

CONCLUSIONS: **Eight weeks administration of resveratrol did not significantly improve any features of NAFLD, compared with placebo, but it increased hepatic stress, based on observed increases in levels of liver enzymes. Further studies are needed to determine whether agents that are purported to mimic calorie restriction, such as resveratrol, are safe and effective for complications of obesity. Clinical trials registration no: ACTRN12612001135808.**

Keywords: NAFLD; Obesity-Related Fatty Liver; Chronic Disease; ALT; AST.

Abbreviations used in this paper: **ALT, alanine aminotransferase; AMPK, adenosine monophosphate–activated kinase; ANOVA, analysis of variance; AST, aspartate aminotransferase; AT-IR, adipose tissue insulin resistance index; CV, coefficient of interindividual variation; EHC, euglycemic-hyperinsulinemic clamp; HED, human equivalent dose; HOMA-IR, homeostatic model assessment of insulin resistance; IGF, insulin-like growth factor; IL, interleukin; IR, insulin resistance; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; NAFLD, nonalcoholic fatty liver disease; NEFA, non-esterified fatty acid; PBMC, peripheral blood mononuclear cell; REE, resting energy expenditure; SIRT1, nicotinamide adenine dinucleotide NAD^+-dependent deacetylase; T2DM, type 2 diabetes.**

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Nonalcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease in the overweight population, generating significant burden on hepatology clinics and liver transplant centers.[1,2] NAFLD spans a spectrum of disease from benign hepatic triglyceride accumulation (steatosis) to nonalcoholic steatohepatitis[3] and appears to be a precursor to type 2 diabetes (T2DM). Abdominal obesity, insulin resistance (IR), and dyslipidemia are correlated with a greater incidence of NAFLD, also independently associated with an increased risk of cardiovascular events and chronic kidney disease.[4] There are currently no accepted pharmacologic therapies for NAFLD.[5] Weight loss of 7%–10% of body weight through lifestyle modification has shown the most success to date.[6,7] Weight loss and permanent maintenance of weight loss remain unrelenting clinical challenges.[8]

IR appears necessary in the development of NAFLD.[9] IR can result from adipocyte altered cytokine secretion impairing insulin signaling, and lipotoxicity from ectopic fat deposition.[10] Nutrient-poor and energy-dense substrate abundance increases mitochondrial and peroxisomal workload, resulting in increased free radicals production, depleted antioxidant defense, and perpetuating IR further via systemic inflammation activation.[11,12]

Bioactive food constituents propose new treatment approaches in the modulation of inflammation and oxidative stress.[13] The clinical investigation of vitamin E and polyunsaturated fatty acids provides insights into how increased exposure to bioactive food constituents may have benefits in NAFLD, even in the absence of weight loss.[14]

Resveratrol is such a compound. The polyphenol is found in grapes, berries, peanuts, and certain therapeutic plants.[15] Dietary consumption is typically in micrograms.[16] Resveratrol's pleiotropic potential has been explored extensively during the last 3 decades in models of adipogenesis, inflammation, oxidative stress, IR, cancer chemoprevention, and age-related degeneration.[17] Resveratrol's potential role in obesity-related complications lies in the purported calorie-restriction mimicking[18,19] via activation of key regulators of metabolic health, namely adenosine monophosphate–activated kinase (AMPK), nuclear factor (erythroid-derived)-like 2, and nicotinamide adenine dinucleotide NAD^+-dependent deacetylase (SIRT1).[20] Preclinical studies have demonstrated a preventive role of resveratrol in diet-induced NAFLD. Animals fed high-energy diets with resveratrol still gained weight, but hepatic steatosis, IR, and systemic inflammation were significantly prevented.[21–24] Resveratrol activated AMPK and SIRT1 in hepatic and muscle tissue, regulated inflammatory cytokines, increased antioxidant capacity and insulin sensitivity, and reduced hepatic de novo lipogenesis when compared with control regimen. Furthermore, the observed effects on gene expression in muscle, hepatic, and adipose tissue postulated a potential for a sustained therapeutic effect in presence of established dysregulation. No dose therapeutic effect relationship in vivo data are currently available, but observation of extensive enteral metabolism suggests that large doses are required to obtain parent resveratrol concentration matching in vitro concentrations likely to demonstrate efficacy.[25] Initial clinical investigations in obesity have provided variable results, likely explained by the heterogeneity in dosages, subjects' baseline health status, and investigation protocols assessing either chronic or acute effects.[26–31] To date, the therapeutic efficacy of resveratrol in established clinical NAFLD has not been investigated.

We hypothesized that in overweight or obese men with NAFLD, 3000 mg daily resveratrol during a period of 8 weeks would (1) improve hepatic and metabolic dysregulation with IR as primary outcome, assessed by the euglycemic-hyperinsulinemic clamp (EHC), and (2) be safe and result in high parent resveratrol concentration.

Methods

Study Design and Participants

This randomized, double-blind, placebo-controlled trial was approved by the Human Research Ethics Committees of the Princess Alexandra Hospital and the University of Queensland, Brisbane, Australia. Twenty men with body mass index above 25 kg/m^2 and waist circumference above 90 cm were recruited prospectively from hepatology outpatient clinics in 2011 and 2012. The primary inclusion criterion was evidence of hepatic steatosis on ultrasound. Exclusion criteria were any known causes of steatosis: viral hepatitis, daily ethanol consumption above 40 g, or use of steatogenic medications. Patients with evidence of cirrhosis, T2DM, history of chronic kidney disease and serious cardiovascular disorders were also excluded. After screening by the primary investigator and providing informed written consent, volunteers were allocated to either resveratrol or placebo by computer-generated randomization. Participants underwent baseline measures. Dosing involved 3 capsules (500 mg each) of resveratrol or placebo before breakfast and another 3 of the same capsules before bedtime, which equated to 3000 mg resveratrol daily in the treatment arm. Resveratrol (from *Polygonum cuspidatum)* was purchased from Biotivia Bioceuticals (New York, NY), and placebo was identically presented microcellulose-filled capsules. Participants were instructed to maintain usual dietary intake and physical activity throughout the study. All baseline measures were repeated after 8 weeks. Participants and investigating staff were blinded to the randomization until completion of results analyses. Pharmacokinetics and target genes transcription were analyzed after unblinding. All co-authors had access to the study data and reviewed and approved the final manuscript.

Safety Monitoring and Compliance

Subjects were reviewed weekly by the principal investigator to assess adverse events. A clinical pharmacologist independent to the study reviewed the weekly liver and kidney function biochemistry. An audit of returned pills and compliance diaries and weekly blood samples for presence (or absence) of resveratrol were performed. (Supplementary Material).

Dose Calculation

The dose of 3000 mg was calculated by using the body surface area normalization method as previously described.[32] The highest dosage used in preclinical diet-induced hepatic steatosis (400 mg/kg in mice)[19] and the reported highest no observed adverse effect level at the time of protocol development (300 mg in rats)[33] were converted to determine a human equivalent dose (HED). Obesity factor calculations were performed per U.S. Food and Drug Administration guidance for industry for estimating the maximum safe starting dose for therapeutics.[34] In light of a recent clinical report on administration of 4000 mg resveratrol daily during a period of 2 weeks[35] with no toxicity observed, it was deemed unnecessary to apply a safety factor to the HED, as commonly applied for first-time administration in humans.[34]

Euglycemic-Hyperinsulinemic Clamp

Insulin sensitivity was assessed by the euglycemic-hyperinsulinemic clamp (EHC)[36] per protocol previously described[37]; the homeostatic model assessment of insulin resistance (HOMA-IR)[38] and the adipose tissue insulin resistance index (AT-IR)[39] were calculated (Supplementary Material).

Indirect Calorimetry

Resting energy expenditure (REE) and fat oxidation were assessed by indirect calorimetry (TrueOne 2400 Metabolic Measurement System; Parvo Medics, Sandy, UT), which was performed in the fasted and insulin stimulated states as previously described.[37]

Magnetic Resonance Imaging and Spectroscopy

Magnetic resonance data were acquired on a 3T Siemens TRIO (Erlangen, Germany) per protocol (Supplementary Material).

Plasma Biochemistry

Weekly liver and kidney function markers and all plasma biochemistry assays are outlined in the Supplementary Material.

Pharmacokinetics

A 6-hour pharmacokinetics study was performed. Sampling and ultra performance liquid chromatography tandem mass-spectrometry protocols are provided in the Supplementary Material.

Peripheral Blood Mononuclear Cell Isolation

Peripheral blood mononuclear cell (PBMC) purification was performed at baseline, week 1, and week 8 as previously described[40] (Supplementary Material).

Target Gene Transcription

RNA levels of *PTP1B*, *HO-1*, *NQO1*, and *IL6* were measured by quantitative reverse transcriptase polymerase chain reaction in PBMCs of subjects receiving resveratrol as previously described[41] (Supplementary Material).

Statistical Analyses

The study was powered to detect 25% change (80% power and $P < .05$) in the primary outcome (glucose disposal rate as a measure of IR). Eight participants were required in each arm. To account for a possible 25% dropout in participation, 10 participants were recruited per arm. Power calculations were based on the reproducibility of the EHC reported in patients with NAFLD.[42] All data were analyzed with intention to treat. Where data were missing, the previous time-point value was brought forward. All analyses were performed on Prism GraphPad Version 6.0c (LaJolla, CA) and SPSS Version 21 (Chicago, IL). Data distribution for all parameters was tested for normality with the D'Agostino and Pearson omnibus normality test. For normally distributed data, values were compared within group with a two-tailed paired *t* test. For data not normally distributed, the Wilcoxon matched-pairs signed rank test was applied. Change in weekly alanine aminotransferase (ALT), aspartate aminotransferase (AST), and mRNA levels of target genes at 3 time points was assessed by repeated-measures analysis of variance (ANOVA). Pharmacokinetics parameters were calculated on Microsoft Excel (Redmond, WA) to obtain plasma concentration maximum, average concentration, and area under the concentration versus time curve (AUC_{0-24}), assuming the concentration at 24-hour from the trough at time 0.

Results

Baseline Characteristics

Table 1 outlines baseline characteristics and indicates overall homogeneity between the resveratrol and placebo groups.

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Table 1. Baseline Characteristics of Resveratrol and Placebo Groups

Parameters	Resveratrol (n = 10)	Placebo (n = 10)	*P* value
Age (*y*)	48.8 ± 12.2	47.5 ± 11.2	NS
Body mass index (kg/m^2)	31.8 (30.2–37.0)[a]	31.2 (27.4–39.3)	NS
Total weight (*kg*)	105.3 ± 15.7	110.7 ± 32.0	NS
LBW (*kg*)	69.7 ± 5.2	71.9 ± 12.0	NS
Systolic blood pressure (*mm Hg*)	130 ± 12	130 ± 10	NS
Diastolic blood pressure (*mm Hg*)	83 ± 7	82 ± 6	NS
HOMA-IR	2.8 (1.8–6.1)[a]	2.5 (1.8–3.3)	NS
Glucose disposal rate (*mg/kgLBW/min*)	4.1 ± 1.7	3.8 ± 0.9	NS
M/2-h insulin (*mg/kgLBW/mU/L*)	4.5 (3.1–7.6)	4.4 (4.0–5.6)	NS
Glucose (*mmol/L*)	5.3 ± 0.5	5.6 ± 0.5	NS
Insulin (*mU/L*)	12 (8–25)[a]	10 (8–14)	NS
Total adiponectin (*μg/mL*)	3.5 ± 1.1	3.8 ± 2.2	NS
High-molecular-weight adiponectin (*μg/mL*)	1.2 (0.9–1.8)	0.9 (0.8–2.3)[a]	NS
Triglycerides (*mmol/L*)	1.3 (1.2–1.7)	1.6 (0.8–1.8)	NS
Total cholesterol (*mmol/L*)	5.0 ± 0.7	4.4 ± 1	NS
HDL cholesterol (*mmol/L*)	1.0 (0.9–1.1)	0.9 (0.8–0.9)	.02
LDL cholesterol (*mmol/L*)	3.2 ± 0.7	2.8 ± 0.8	NS
ALT (*U/L*)	45 (41–70)	40 (38–87)[a]	NS
AST (*U/L*)	35 ± 9	36 ± 16	NS
IL6 (*pg/mL*)	5.6 (0.6–31.6)[a]	3.9 (1.1–12.0)	NS
IL10 (*pg/mL*)	4.1 (1.3–12.0)[a]	4.8 (2.1–10.7)	NS
Hepatic triglyceride content (%)	25 ± 17	26 ± 12	NS
REE (*kcal/kg/day*)	20 (19–20)	19 (18–21)[a]	NS
Treatment dosage received per kg body weight (*mg*)	29 ± 4		

NOTE. Unpaired *t* test: means ± standard deviation for data with normal distribution. Mann–Whitney test: medians (interquartile ranges) for data not normally distributed. BMI, body mass index; HDL, high-density lipoprotein; LBW, lean body weight; LDL, low-density lipoprotein; M, glucose disposal rate.
[a]Data normally distributed. Significance set at $P < .05$.

Compliance

Supplementary Figure 1 presents the study consort flow diagram. All participants completed the 8-week intervention; 85% of participants completed all study investigations. A compliance audit of returned capsules indicated 90% compliance in the resveratrol group and 75% in the placebo group (data not shown). Weekly plasma concentrations confirmed presence of resveratrol in the intervention group (Supplementary Material).

Insulin Resistance and Target Genes Expression

At baseline, all participants had profound IR. Resveratrol treatment did not induce improvement in insulin-mediated glucose uptake (Table 2, Figure 1*A*). Fasting glucose, insulin, non-esterified fatty acids (NEFA), and AT-IR did not change with treatment (Table 2, Figure 1*B*). Resveratrol led to a statistically significant, less effective insulin-mediated suppression of plasma NEFA, which was not considered clinically significant (Table 2, Figure 1*C*). Total and high-molecular-weight adiponectin did not change; however, the high-molecular-weight to total adiponectin ratio decreased significantly with resveratrol (Table 2, Figure 2*A* and *B*).

Transcription of *NQO1*, *PTP1B*, *IL6,* and *HO-1* in PBMCs did not change significantly with resveratrol (Table 3, Figure 2*E* and *F*)

Hepatic Triglyceride Content, Abdominal Adipose Tissue Distribution, and Resting Energy Expenditure

Resveratrol did not induce change in hepatic steatosis, total abdominal fat, or distribution (Table 2, Figure 1*E* and *F*). REE and fasting fat oxidation remained unchanged (Table 2).

Plasma Lipids, Antioxidant, and Inflammation Markers

No change was observed in plasma lipids or antioxidant activity with resveratrol. There was a significant increase in interleukin (IL) 10 and decrease in IL6 but no change in tumor necrosis factor-α, IL1β, IL8, or C-reactive protein (Table 2, Figure 2*C* and *D*).

Resveratrol Pharmacokinetics

The concentration maximum for parent resveratrol was 65.7 ± 35.9 ng/mL (0.29 μmol/L) at 60 minutes after ingestion of 1500 mg resveratrol in fasted state. The coefficient of interindividual variation (CV) was 54%. The average concentration across the dosing interval was 29.3 ± 11.6 ng/mL (CV 36%). The AUC_{0-24} was 705 ng/mL × hour (CV 36%) (Figure 3).

Table 2. Pre-intervention and Post-intervention Results for Resveratrol and Placebo Groups

Parameter (units)	Resveratrol (n = 10)			Placebo (n = 10)		
	Pre-intervention	Post-intervention	*P* value	Pre-intervention	Post-intervention	*P* value
Anthropometrics, blood pressure, resting metabolic rate						
Weight (*kg*)	105.3 ± 15.7	105.3 ± 15.6	.9	110.7 ± 32.0	111.5 ± 31.4	.2
Body mass index (kg/m^2)	31.8 (30.2–37.0)[a]	31.9 (30.0–37.4)[a]	.9	31.2 (27.4–39.3)	31.1 (28.1–40.0)[a]	.2
Systolic blood pressure (*mm Hg*)	130 ± 12	128 ± 11	.6	130 ± 10	124 ± 7	.09
Diastolic blood pressure (*mm Hg*)	83 ± 7	82 ± 8	.6	82 ± 6	81 ± 5	.6
REE (*kcal/kg/day*)	19.9 (19.4–20.5)	19.9 (18.8–20.4)[a]	.9	19.4 (17.6–20.8)[a]	18.9 (16.7–22.3)[a]	.9
Fasting fat oxidation (*mg/min/kg*)	0.11 (0.09–0.12)	0.13 (0.11–0.14)	.3	0.11 (0.09–0.15)	0.11 (0.10–0.13)	.8
IR						
M (*mg/kgLBW/min*)	3.5 (2.7–5.3)[a]	3.2 (2.8–4.4)	.3	3.8 (3.2–4.5)[a]	3.8 (3.3–4.5)[a]	.6
M/I (*kgLBW/min/mU/L*)	4.5 (3.1–7.6)	4.5 (2.9–6.4)	.6	4.4 (4.0–5.6)	4.5 (3.9–6.9)	.8
HOMA-IR	2.8 (1.8–6.1)[a]	3.6 (1.9–5.8)[a]	.1	2.5 (1.8–3.3)	3.1 (2.3–3.8)[a]	.4
Glucose (*mmol/L*)	5.3 ± 0.5	5.4 ± 0.5	.7	5.6 ± 0.5	5.6 ± 0.5	.6
Insulin (*mU/L*)	12 (8–25)[a]	15 (8–26)[a]	.09	10 (8–14)[a]	13 (9–16)[a]	.5
Steady-state insulin (*mU/L*)	79 ± 19	79 ± 23	.9	83 ± 17	79 ± 22	.5
AT-IR (*mmol* × *pmol/L*)	8.6 (3.7–10.8)	7.9 (4.9–12.7)	.06	6.2 (4.6–9.0)	5.5 (3.5–7.3)	.1
NEFA (*mmol/L*)	0.5 ± 0.2	0.6 ± 0.1	.7	0.7 ± 0.3	0.5 ± 0.2	.01
2-hour NEFA (*mmol/L*)	0.07 ± 0.04	0.09 ± 0.05	.04	0.09 ± 0.08	0.07 ± 0.08	.3
Total adiponectin (*μg/L*)	3.1 (2.7–4.2)[a]	3.4 (2.9–3.7)	.4	2.8 (2.2–5.2)[a]	3.6 (2.3–4.8)[a]	.8
High-molecular-weight adiponectin (*μg/L*)	1.2 (0.9–1.8)	1.0 (0.7–1.4)	.3	0.9 (0.8–2.3)[a]	1.3 (0.7–2.2)[a]	.9
S_A ratio (HMW/TA)	0.4 ± 0.2	0.3 ± 0.1	.02	0.4 ± 0.1	0.4 ± 0.1	.6
Hepatic markers						
Hepatic triglyceride content (%)	25 ± 17	27 ± 18	.3	26 ± 12	25 ± 12	.3
ALT (*U/L*)	45 (41–47)	63 (57–83)[a]	.02	40 (38–87)[a]	48 (39–98)[a]	.1
AST (*U/L*)	35 ± 9	45 ± 15	.03	36 ± 16	38 ± 15	.5
Total bilirubin (*μmol/L*)	15 (12–16)	18 (12–22)[a]	.09	12 (11–12)[a]	11 (9–15)[a]	.02
Cytokeratin 18 (*U/L*)	34.3 ± 26.1	48.4 ± 34.1	.1	41.5 ± 22.3	56.5 ± 36.1	.2
High-mobility group box-1 (*ng/mL*)	0.8 (0.4–1.5)	0.8 (0.4–2.6)	.6	0.4 (0.3–0.6)	0.4 (0.4–0.9)	.3
Kidney function						
Creatinine (*μmol/L*)	79 ± 16	80 ± 16	.7	76 ± 14	79 ± 16	.2
Potassium (*mmol/L*)	3.9 ± 0.3	4.0 ± 0.2	.3	4.0 ± 0.2	4.1 ± 0.3	.7
Inflammation and oxidative stress						
IL1-beta (*pg/mL*)	0.22 ± 0.15	0.20 ± 0.12	.2	0.34 ± 0.27	0.34 ± 0.25	.9
IL6 (*pg/mL*)	5.6 (0.6–31.6)[a]	2.5 (0.5–19.0)[a]	.04	3.9 (1.1–12.0)	4.4 (1.7–12.1)	.5
IL8 (*pg/mL*)	8.7 ± 4.2	10.4 ± 6.4	.2	7.7 ± 4.7	9.1 ± 7.0	.2
IL10 (*pg/mL*)	4.1 (1.3–12.1)[a]	6.2 (3.4–11.4)[a]	.03	4.8 (2.1–10.7)	6.2 (3.2–18.8)	.2
C-reactive protein (*mg/L*)	1.5 (0.3–4.0)[a]	0.9 (0.4–3.0)	.3	0.8 (0.4–2.0)	0.8 (0.4–2.2)[a]	1.0
Tumor necrosis factor-α (*pg/mL*)	9.0 (6.7–14.0)	8.9 (7.0–12.5)	.8	7.4 (5.2–11.5)	8.1 (6.1–10.2)	.6
F2-isoprostanes (*pg/mL*)	247 ± 62	229 ± 49	.4	248 ± 59	276 ± 99	.3
Plasma antioxidant activity						
Ferric reducing ability of plasma (*μmol/L*)	1162 ± 144	1181 ± 174	.6	1107 ± 179	1027 ± 171	.02
Superoxide dismustase (*U/mL*)	7.2 (5.5–8.0)[a]	6.4 (5.5–7.3)[a]	.2	6.3 (5.6–6.7)[a]	7.1 (6.8–8.3)	<.01
Glutathione peroxidase (*U/L*)	176 (163–179)	175 (163–194)[a]	.5	173 (159–204)[a]	165 (155–208)[a]	.3
Total antioxidant capacity (*mmol/L*)	1.7 ± 0.1	1.7 ± 0.1	.4	1.7 ± 0.1	1.6 ± 0.1	.06
Vitamin C (*μmol/L*)	53 ± 16	56 ± 17	.5	41 ± 16	54 ± 19	.05
Lipid profile						
Triglyceride (*mmol/L*)	1.3 (1.2–1.7)	1.7 (1.0–1.9)	.9	1.6 (0.8–1.8)[a]	1.3 (1.0–2.0)[a]	.5
Total cholesterol (*mmol/L*)	5.0 ± 0.7	5.1 ± 1.1	.4	4.4 ± 1.0	4.3 ± 1.2	.3
High-density lipoprotein (*mmol/L*)	1.0 (0.9–1.1)	1.0 (0.9–1.1)	.7	0.9 (0.8–0.9)	0.9 (0.7–1.1)	.9
Low-density lipoprotein (*mmol/L*)	3.2 ± 0.7	3.5 ± 0.9	.07	2.8 ± 0.8	2.7 ± 0.9	.08
Abdominal adipose tissue distribution						
Total adipose tissue (cm^2)	612 ± 171	618 ± 162	.6	539 ± 166	530 ± 161	.5
Visceral adipose tissue (cm^2)	262 ± 90	256 ± 70	.5	214 ± 64	200 ± 55	.2
Subcutaneous adipose tissue (cm^2)	349 ± 156	354 ± 158	.7	330 ± 140	337 ± 140	.4
IGF-I axis						
IGF-I (*ng/mL*)	67 ± 26	61 ± 22	.2	60 ± 23	69 ± 28	.07
IGF-binding protein 3 (*ng/mL*)	4120 ± 1063	4113 ± 1239	.9	4852 ± 1215	4826 ± 1418	.8

NOTE. Data not normally distributed: Wilcoxon test, medians (interquartile ranges). Data normally distributed: paired *t* test, means ± standard deviations. Significance set at $P < .05$.

HMW, high-molecular-weight; TA, total adiponectin.

[a]Data normally distributed.

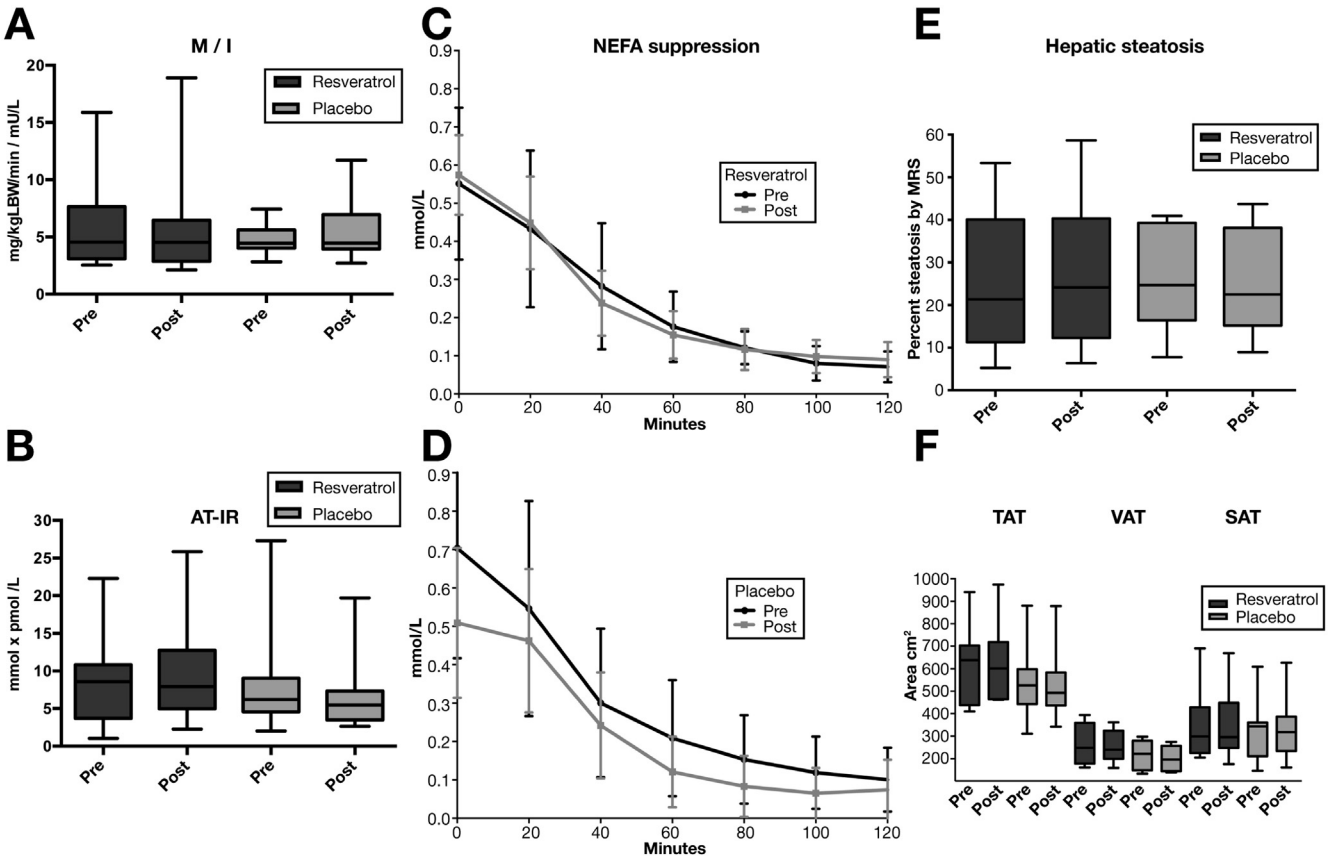

Figure 1. Effects of resveratrol on NAFLD characteristic features. (*A*) IR expressed as M over insulin concentration at 2 hours: [M/I]*100 (medians and interquartile ranges). (*B*) AT-IR (medians and interquartile ranges). (*C*) Pre- and post-intervention plasma NEFA suppression during insulin clamp infusion for resveratrol group (means and standard deviations). (*D*) Pre- and post-intervention plasma NEFA suppression during insulin clamp infusion for placebo group (means and standard deviations). (*E*) Pre- and post-intervention hepatic steatosis measured by magnetic resonance spectroscopy for resveratrol and placebo groups (medians and interquartile ranges). (*F*) Pre- and post-intervention abdominal adipose tissue: total (TAT), visceral (VAT), and subcutaneous (SAT) distribution for resveratrol and placebo groups (medians and interquartile ranges). LBW, lean body weight.

Tolerability and Safety

Resveratrol was well-tolerated. The most frequent adverse event was mild gastrointestinal symptoms described as increased frequency of bowel motions and loose stools. This was reported by 80% of subjects in the resveratrol group compared with 20% in the placebo group (data not shown). Previous reports have discussed the potential for drug bioavailability interactions as a result of inhibition of the phase I drug metabolism enzyme CYP3A4 by resveratrol.[43] No symptom attributed to drug metabolism was observed, and usual medication prescriptions remained unchanged.

ALT and AST levels increased significantly to week 6 (56% and 50% median increase, respectively) in the resveratrol group (Table 2, paired *t* test). Statistical significance was not sustained when overall change across the study was assessed by ANOVA (Figure 4*A* and *B*). Cytokeratin 18 fragments and high-mobility group box-1 levels did not change with treatment (Table 2). Serum creatinine and potassium concentration did not change (Table 2).

Other Parameters

Other variables measured included insulin-like growth factor (IGF)-I and IGF-binding protein 3, F2-isoprostanes, blood pressure, and vitamin C (Table 2); none changed with resveratrol.

Discussion

Daily 3000-mg resveratrol treatment did not induce therapeutic benefits in men with established NAFLD.

Resveratrol Did Not Improve Typical Characteristics of Nonalcoholic Fatty Liver Disease

Insulin resistance. Fasting glucose, insulin, and the M value did not change. The M/I index, or the quantity of glucose disposed per unit of insulin concentration during the EHC (clinically most representative of the degree of IR), did not change (Table 2). The lack of effect of

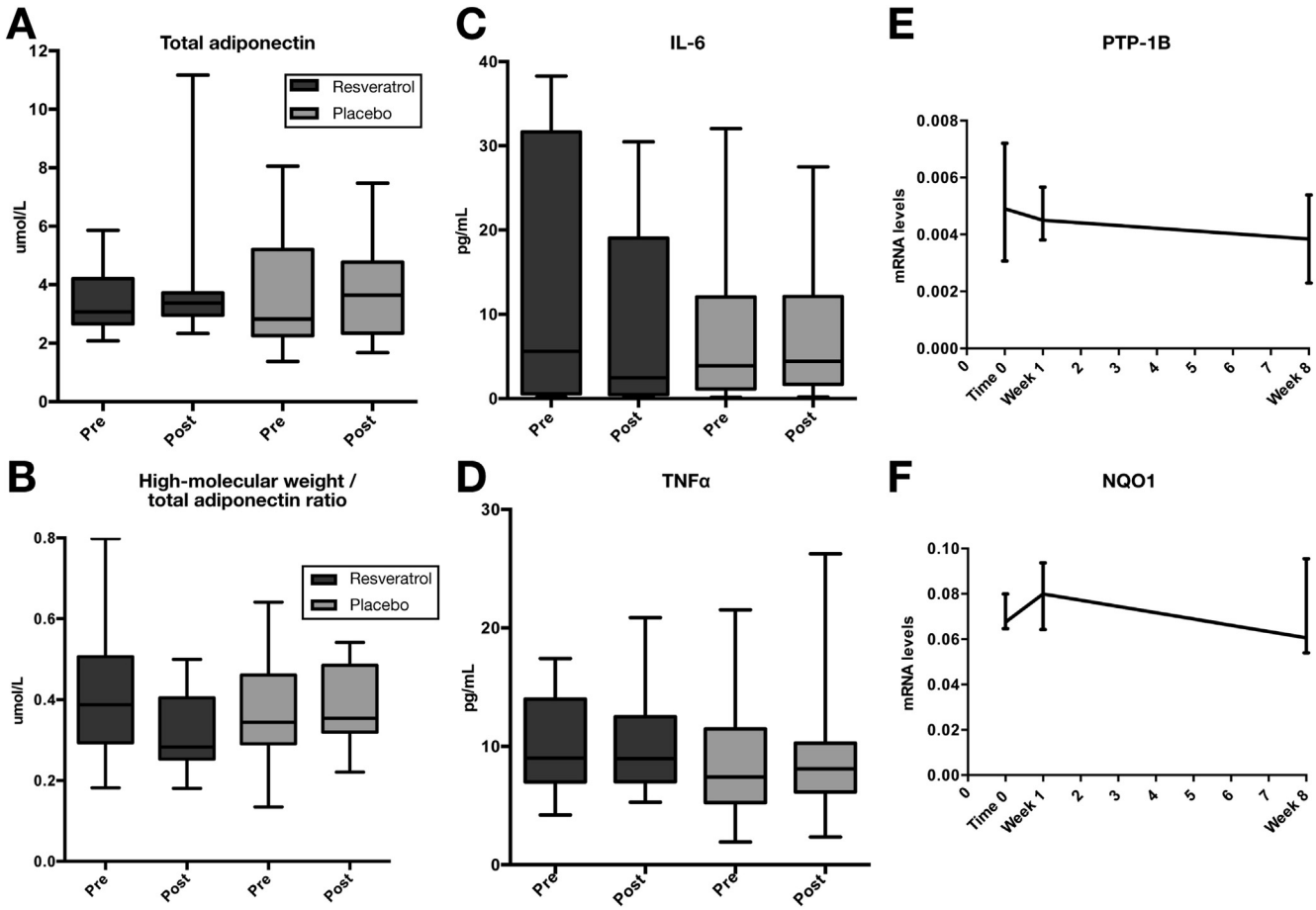

Figure 2. Effect of resveratrol on other metabolic markers. (*A*) Total adiponectin (medians and interquartile ranges). (*B*) SA ratio (high molecular weight/total adiponectin). Pre- and post-intervention in resveratrol group, $P = .02$ (medians and interquartile ranges). (*C*) IL6 (medians and interquartile ranges). Pre- and post-intervention in resveratrol group, $P = .04$ (medians and interquartile ranges). (*D*) Tumor necrosis factor (TNF)-α (medians and interquartile ranges). (*E*) *PTP1B* mRNA levels in PBMCs (normalized to actin) of participants in resveratrol group at baseline, week 1, and week 8 (medians and interquartile ranges). (*F*) *NQO1* mRNA levels in PBMCs (normalized to actin) of participants in resveratrol group at baseline, week 1, and week 8 (medians and interquartile ranges).

resveratrol on insulin sensitivity has been previously reported in overweight or obese healthy subjects at various resveratrol dosages (daily 75–1500 mg) by using similar gold-standard protocols to assess functional IR.[29,31] In contrast, in populations with T2DM, the adjunction of resveratrol to standard care showed significant improvement in mechanisms of glucose control and reduced oxidative stress across a range of daily doses (10 mg daily,[26] 250 mg,[44] or 1000 mg[45]). These studies suggest that the severity of metabolic dysregulation before resveratrol treatment and the combination with pharmacologic agents that target AMPK activation could be of importance for measurable benefits of resveratrol.[46,47]

PTP1B, a negative regulator of insulin signaling, was shown to be significantly repressed in PBMCs of healthy humans receiving 40 mg resveratrol daily for 6 weeks[28] and suggested as another mechanism for improved insulin sensitivity by resveratrol.[48] *PTP1B* transcription remained unchanged in the present study, which was consistent with the no improvement in IR. Total and high-molecular-weight adiponectin did not change, which was also consistent with no effect on IR. This contradicts a recent clinical report of increased circulating adiponectin by 9.6% after 1 year of daily 8-mg resveratrol in combination with grape extract in a population with cardiovascular disease.[49] These overall discrepant results indicate that dose-finding studies including a dose spectrum from dietary to pharmacologic are required.[47]

Hepatic and abdominal fat distribution. Hepatic steatosis, abdominal adipose tissue distribution, and weight remained unchanged, implying that resveratrol did not promote fat oxidation. Indirect calorimetry results for REE and fat oxidation confirmed this. Significant reduction in hepatic fat with resveratrol was previously reported in obese healthy subjects,[30] but this was observed within the clinically normal range of less than 5% steatosis, which may reflect day-to-day variability and not predict improvement in pathologic fat accumulation, characteristic of NAFLD.[50] The subjects in the present study had up to 54% steatosis measured by magnetic

Table 3. Resveratrol Group Only: Target Genes Transcription in PBMCs (n = 9)

Genes	Paired test for change in RNA levels at time points: *P* value, time 0 to week 1	*P* value, week 1 to week 8	*P* value, time 0 to week 8	ANOVA in multiple time points
PTP1B[a]	.2	.2	.1	.08
IL6	1	.3	.2	.8
HO-1	.3	.2	1	.1
NQO1	.1	.02	.4	.6[b]

NOTE. Data not normally distributed: Wilcoxon test, medians (interquartile ranges). Data normally distributed: paired *t* test, means ± standard deviations. Significance set at $P < .05$.
[a]Data normally distributed.
[b]Greenhouse–Geisser correction for assumption of sphericity.

resonance spectroscopy. The mechanism of resveratrol on lipid metabolism has been proposed via activation of AMPK, SIRT1, and the resulting increased fat oxidation.[51] The lipolytic activity of resveratrol has also been reported in human adipocytes via regulation of adipose triglyceride lipase.[52] Increased lipolytic activity presents as an advantage, whereby more fatty acids become available substrate for muscles. However, in the context of reduced hepatic and whole-body fat oxidation ability as has been recently reported in NAFLD patients,[37] this may not translate into measurable benefits. The preventive effect of resveratrol in animals on diet-induced obesity and related hepatic steatosis appears not efficacious in the context of established steatosis, where multiple pathways are dysregulated for some time.

Inflammation and antioxidant activity. The observed increase in IL10 and decrease in IL6 suggest a modest improvement in the circulating inflammatory profile with resveratrol. However, mRNA level of *IL6* in PBMCs did not change. *NQO1* mRNA levels appeared to decrease significantly between week 1 and week 8, suggesting different acute and long-term effects. However, the repeated-measures ANOVA indicated no significant change across time points. The preclinical evidence of the effect of resveratrol on regulators of inflammation and on the endogenous antioxidant defense is abundant.[47] The clinical evidence is mixed, which is likely explained by the baseline health status of subjects, dosage variety, and fasting or post meal-challenge sampling protocols, which assess different outcomes.[28–31,53]

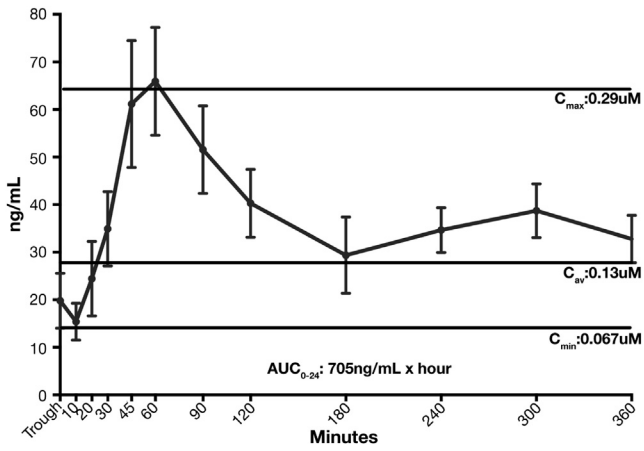

Figure 3. Pharmacokinetics of resveratrol up to 6 hours after ingestion of 1500 mg after 8 weeks of 3000 mg daily supplementation (means and standard error of the mean). C_{av}, concentration average; C_{max}, concentration maximum; C_{min}, concentration minimum.

Safety and plasma resveratrol concentration. A rise in ALT and AST is generally attributed to hepatocyte injury; however, plasma cytokeratin 18 and high-mobility group box-1 concentrations did not increase significantly (Table 2), suggesting there was little hepatocyte necrosis. The repeated-measures ANOVA failed to find statistical significance across the study, and ALT and AST concentrations appeared to fall after week 6. The significant rise up to week 6 is nevertheless concerning, especially because the long-term effects of resveratrol supplementation were not investigated in this study. Moreover, resveratrol is advertised as a calorie-restriction mimic targeting populations likely to present with obesity-related comorbidities such as NAFLD. This warrants adequate warning and further research. The 3000-mg daily dose was chosen after consideration of the lipophilic characteristics of resveratrol and assumed altered pharmacokinetics in obesity with increased fat mass.[54,55] The split-dosing regimen was aimed at increasing exposure to circulating parent compound, despite known extensive metabolism. The mean concentration maximum and AUC_{0-24} were 52% and 41% lower, respectively, after 1500 mg resveratrol (Figure 3) than the concentration maximum and AUC_{0-24} after 1000 mg in healthy subjects previously reported.[56] It is nonetheless difficult to compare the concentration of parent resveratrol in the present study with other clinical data because of variable analytical methods used. Methods measure total resveratrol including metabolites[35] or use enzymatic hydrolysis to reconvert metabolites to parent compound before analysis.[30,31] From our data, it could be speculated that resveratrol kinetics may be altered in obesity with chronic liver disease, resulting in reduced parent concentration. It could also be speculated that the chronic high dose may have resulted in saturation at absorption sites as seen with high-dose lipophilic nutrients when taken out of the food matrix[57] or with micronutrient absorption interactions.[58]

What Defines a Therapeutic Dose?

This study administered the pharmacologic dose of 3000 mg daily for 8 weeks to an obese cohort with chronic liver disease. It is unclear what animal dose should be selected as benchmark for therapeutic HED calculations. Broad dosages have been used preclinically

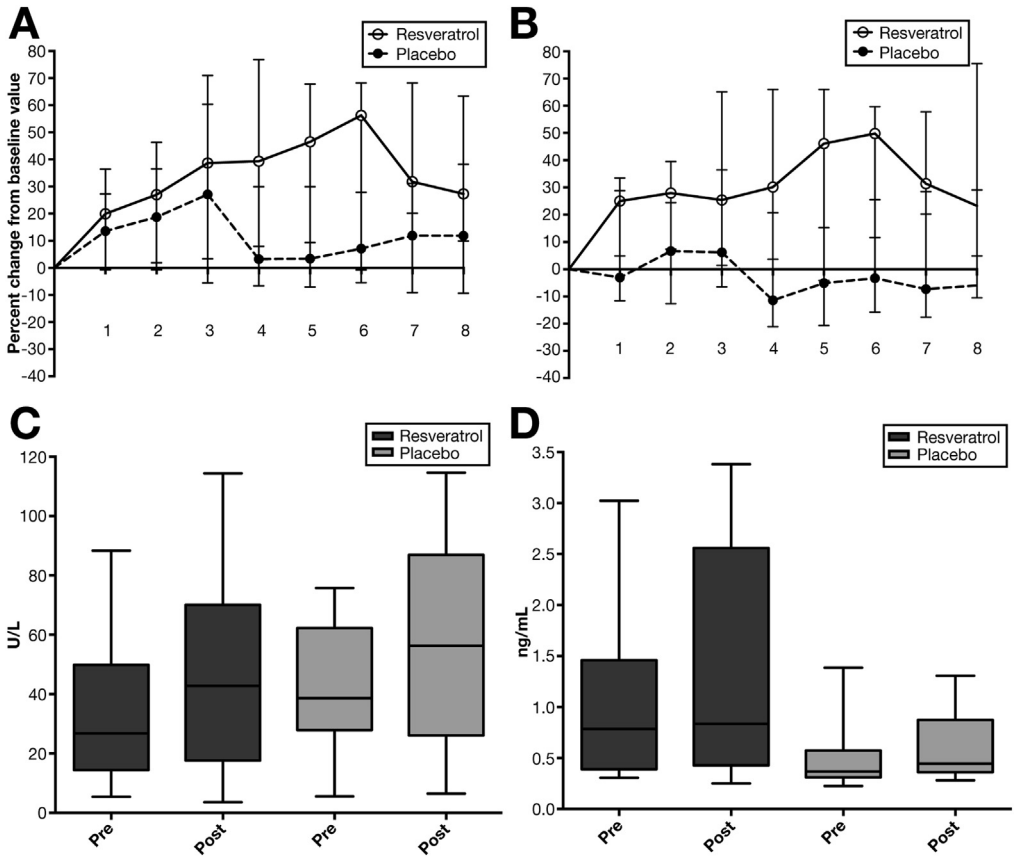

Figure 4. Safety monitoring. ALT (*A*) and AST (*B*) weekly fluctuations expressed in percentage change from baseline value (medians and interquartile ranges). (*A*) Repeated-measures ANOVA. Resveratrol, $P = .3$; placebo, $P = .6$. (*B*) Repeated-measures ANOVA. Resveratrol, $P = .5$; placebo, $P = .8$. (*C*) Plasma cytokeratin-18 fragments for resveratrol and placebo (medians and interquartile ranges). Pre- and post-intervention paired *t* test: resveratrol, $P = .1$; placebo, $P = .2$. (*D*) Plasma high-mobility group box 1 for resveratrol and placebo (medians and interquartile ranges). Pre- and post-intervention paired *t* test: resveratrol, $P = .6$; placebo, $P = .3$.

(from 1 mg to 750 mg/kg of body weight, or food consumed by animals) without pharmacokinetics data provided or described rationale for dose selection. When converted through the body surface area normalization method,[32] the dose received by the participants in the present study equated approximately to that received by mice in the investigation of resveratrol in the prevention of diet-induced obesity complications.[19] There is no agreed consensus on the best method to calculate an HED for bioactive food constituents considering allometric scaling.[59] Dose-calculation limitations further exist for an obese population with concurrent comorbidities. Standards for body surface area, absorption, metabolism, and body composition often rely on assumed data from 60-kg adults[34] and do not take into consideration obesity-related changes leading to altered drug distribution and clearance.[60] Pharmacokinetic monitoring and chemotherapy dosing adjusted to weight are well-documented.[61] Body composition studies in the obese suggest that drug clearance is not proportional to total body weight but correlates more accurately with lean body mass.[60] Chronic dosing depends on drug clearance, because the aim is to achieve a steady-state concentration related to efficacy.[54] Therefore, animal dosage conversion to HED that is based on lean body mass may be more adequate for nutraceuticals targeted for chronic disease therapy.

Furthermore, the distinction between prevention and therapy is rarely addressed in preclinical studies; the prevention models are most commonly used to determine the therapeutic potential of an agent. Chronic disease develops over time, and treatment may consequently involve vastly different pathways than those for prevention.

The Poor Bioavailability of Resveratrol Is Perhaps a Protective Mechanism

The extensive conjugation of resveratrol may indicate a protective mechanism against high concentration of parent compound from bolus nutraceutical doses.[62] Clinical evidence of resveratrol accumulation in colorectal tissue[63] has confirmed early animal evidence and likely predicts accumulation in other tissues.[64] Sulfate metabolites were reported undergoing hydrolysis back to parent compound intracellularly, which is suggested to constitute a "sustained release" pool of parent compound.[65,66] Resveratrol conjugates have also been reported to hold bioactivity potential.[67,68] Therefore, circulating concentrations of parent resveratrol at a given time point are not representative of actual exposure and are potentially an incorrect target for expected efficacy.

The lack of therapeutic effect in the present study questions the dose adequacy and the efficacy of resveratrol in humans with hepatic steatosis. A lack of effect at larger doses was previously reported when markers of

efficacy on the IGF-I axis did not increase proportionally with dose in healthy volunteers.[56] Resveratrol has been shown to have hormetic effects in cell and animal models for a range of disease states, with protective effects at low dose but adverse effects at higher doses.[62,69] However, it is not clear whether, and at what dose, this change occurs in humans. Furthermore, it is unclear whether a nutraceutical dose would qualify as a "large" dose physiologically, regardless of size, because of its bolus delivery, compared with a dietary source.[47] Dose-comparison studies are needed to determine differential dose-dependent effects.

Conclusion

The present study demonstrates that the preventive role of resveratrol observed in diet-induced preclinical models of NAFLD does not translate into a therapeutic role in clinically established NAFLD. We provide evidence that in obese subjects with chronic fatty liver disease, 8-week supplementation of a high pharmacologic dose of resveratrol, calculated from preclinical efficacy doses and based on body surface area, (1) does not improve insulin sensitivity and hepatic steatosis, (2) appears potentially toxic to hepatocytes, and (3) does not result in high concentration of parent resveratrol. Clinical dose-finding studies with rationalized dose selection, pharmacokinetics, and dynamics data are paramount to elucidate the dose-response relationship. The purported calorie-restriction mimicking of resveratrol may require investigation in combination with dietary prescription, standard care, and lifestyle modifications to target adequately the complexity of dysregulation in obesity-related chronic disease. Caution is warranted for use in obesity with chronic liver disease until further research determines safety.

Limitations

This study was powered to detect 25% change in glucose disposal rate during the EHC. There is potential for type 2 errors in all other variables. The EHC protocol did not include glucose tracers and thus did not allow for distinction between specific sites of IR. The magnetic resonance spectroscopy method was unable to determine severity of liver disease or changes to hepatic fibrosis and inflammation. The study involved exclusively male participants. The pharmacokinetics protocol was included to monitor presence of parent resveratrol during the study and dosage kinetics. Because resveratrol metabolites were not measured, actual exposure to resveratrol species cannot be determined.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Clinical Gastroenterology and Hepatology* at www.cghjournal.org, and at http://dx.doi.org/10.1016/j.cgh.2014.02.024.

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Conflicts of interest
The authors disclose no conflicts.

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Supplementary Material

Sample Calculations

The study was powered for the primary outcome measure of glucose disposal rate (as a measure of peripheral insulin sensitivity) by using the EHC technique. It was deemed by the clinical investigators that a 25% difference in peripheral IR (similar to that seen with moderate lifestyle intervention) would be a clinically significant treatment effect for a nutraceutical therapy.

Magkos et al[1] published on the reproducibility of clamp variables in obese people with NAFLD and also suggested this number of subjects ($\leq$8 for paired studies) is required to detect a 25% difference in glucose disposal rate. By using the mean and standard deviation of glucose disposal rate of each group in this study to detect a 25% difference in glucose disposal rate, we calculated that a sample size of 5–8 subjects in each arm was required for 80% power at $P = .05$. In addition, because we anticipated a 25% dropout rate (which did not eventuate), a final sample size of 10 patients per group was planned for recruitment in this study.

Safety and Compliance Monitoring

Subjects were provided with a capsule intake diary chart to be completed daily to record intake and symptoms. Subjects were reviewed weekly by the principal investigator to assess compliance and any adverse events.

Weekly blood samples were taken to monitor liver (ALT, AST, and gamma-glutamyl aminotransferase, alkaline phosphate, and bilirubin), and kidney function (creatinine and potassium). The results were reviewed by an independent clinical pharmacologist. Additional plasma was collected weekly and stored at −80°C to measure resveratrol concentrations post-intervention and demonstrate presence of resveratrol in the plasma weekly throughout the intervention. These samples were taken at random times on the scheduled visit day to facilitate participants' timetable and attendance to review appointment. The time of last dosing before sampling and whether food was consumed since dosing were recorded.

Participants were provided with 4-week supply of capsules and returned unused capsules before receiving the second 4-week supply. An audit of returned capsules was performed. Participants were provided with a compliance diary to be filled daily and were asked verbally about compliance to prescribed capsule intake at the weekly reviews. Supplementary Figure 2 shows the concentration of parent resveratrol measured at the weekly reviews.

Euglycemic-Hyperinsulinemic Clamp

Peripheral insulin sensitivity pre- and post-intervention was evaluated by the EHC clamp method.[2] All evaluations were performed after an overnight fast (10–12 hours), with no vigorous exercise in previous 24 hours. Insulin (Humulin R; Eli Lilly, Indianapolis, IN) was given as a primed continuous infusion (1 mU/kg body weight/min) in 50 mL saline with 1 mL whole blood at a rate of 15 mL/h for 2 hours. Plasma glucose concentration was monitored every 5 minutes by using an automated glucose analyzer (YSI 2300 Stat Plus; YSI Life Sciences, Yellow Springs, OH; interassay coefficient of variation 2%). A 25% glucose solution was infused at a variable rate, commenced at 2 mg/kg body weight/min,[2] and altered accordingly to maintain fasting blood glucose concentration $\pm$ 10%.

Total glucose disposal rate (M value) was determined as the primary outcome measure. The insulin sensitivity index ([M/I]*100), a measure of the quantity of glucose disposal per unit of insulin concentration, was calculated.[2] Additional indices of insulin resistance were calculated including the HOMA-IR: fasting glucose (mmol/L) $\times$ fasting insulin (mU/L)/22.5[3] and the AT-IR: fasting plasma free fatty acids (mmol/L) $\times$ fasting plasma insulin (pmol/L).[4]

Lean Body Weight Estimation

Lean body weight was estimated for EHC and indirect calorimetry parameter calculations by using the semi-mechanistic model equation $LBW(kg)_{Male} = \frac{9270\ x\ Wt}{6690+(216xBMI)}$, where LBW is lean body weight in kilograms, Wt is weight in kilograms, and BMI is body mass index calculated by weight in kilograms divided by height in meters squared.[5]

Indirect Calorimetry

REE and respiratory quotient were assessed by indirect calorimetry (TrueOne 2400 Metabolic Measurement System; Parvo Medics), which was performed in the fasted and insulin stimulated states for 30 minutes each, with subjects lying supine on a bed, awake, and breathing under a ventilated Plexiglas hood. Calculations included the average of the last 10 minutes of the test. Respiratory quotient and REE values were calculated by using stoichiometric equations, assuming that the urinary nitrogen excretion rate was negligible.[6]

Magnetic Resonance Imaging and Spectroscopy

All magnetic resonance data were acquired on a 3T Siemens TRIO (Erlangen, Germany). Subjects were positioned supine head first, with body matrix and large flex coils positioned to cover the region from the liver to L5 with the liver at the gradient isocenter for liver magnetic resonance imaging and magnetic resonance spectroscopy. Standard Localiser and breath-hold TRUE FISP images with full coverage of the liver were acquired for

planning of Dixon images and spectroscopy. Dixon vibe images with automatic generation of water and fat images with the following parameters: repetition time (TR) = 5.6, echo time (TE) = 2.45 and 3.675, read field of view = 300 mm, phase field of view = 75%, base resolution = 320, phase resolution = 100%, slice thickness = 4 mm, number of slices = 48, slice gap = 0 mm, number of averages = 4, acquisition time = 1 minute 24 seconds. Images were acquired with respiratory gating by using the Siemens pressure pad. Spectroscopy was acquired with the spin echo SVS_SE_30 sequence with the following parameters: TR = 2000, TE = 30, voxel size = 20 × 20 × 20 mm; voxel positioned in the posterior region of the left liver lobe, avoiding major vessels and at least 20 mm from edge of the liver; 4 averages; no water suppression; the spectrum acquired with a single breath-hold. Spectroscopy signals values for CH3, CH2, and H_2O were used to calculate hepatic triglyceride content:

$$Hepatic\ triglyceride\ content(\%) = \frac{CH2 + CH3}{CH2 + CH3 + \mathrm{H2O}} \times 100.$$

After liver magnetic resonance imaging and magnetic resonance spectroscopy, the patient's bed was moved to position L4 at the gradient isocenter. Dixon images, as described for the liver, were centered on the L4 and acquired. Four images were acquired and averaged to estimate total, visceral, and subcutaneous fat areas. The Grow Region of Interest tool in the OsiriX DICOM viewer software package (v.4.1.1 64-bit; Pixmeo Sarl, Bernex, Switzerland) was used to determine areas of interest.

Plasma Biochemistry

Insulin was assayed by using an immunoenzymatic assay with chemiluminescence detection (Unicel DxI 800 Immunoassay System; Beckman Coulter, Brea, CA).

Liver enzymes, bilirubin, creatinine, potassium, iron, transferrin, triglycerides, total cholesterol, high-density lipoprotein, and glucose assays were performed on Beckman DxC800 general chemistry analyzers (Beckman Coulter), and very low density lipoprotein and low-density lipoprotein were calculated by using the Friedewald equation. Ferritin assays were performed on Beckman DxI800 immunoassay analyzer (Beckman Coulter). Serum NEFA concentrations were measured spectrophotometrically (Cobas Mira; Roche Diagnostics, Sydney, Australia) by using a Wako NEFA-HR(2) kit and calibrators (Novachem, Victoria, Australia) with an interassay CV <5%.

IGF-I and IGF-binding protein 3 were assayed by enzyme-linked immunosorbent assay (R&D Systems, Oxon, UK). The cytokines IL1b, IL6, IL8, IL10, and tumor necrosis factor-α were assayed by using the Merck Millipore high sensitivity Milliplex assay (Merck Millipore, Billerica, MA), with intra-assay and interassay CV <8%. Leptin was assayed as previously described[7] by using an in-house bead-based immunoassay and matched paired antibodies (R&D Systems), with intra-assay and interassay CV <8%. HsC-reactive protein was measured spectrophotometrically (Cobas Mira; Roche Diagnostics) by using a kit and calibrators (Kamiya Biochemical Company, Seattle, WA), with intra-assay and interassay CV <5%.

Total and high-molecular-weight adiponectin were assayed with the Adiponectin multimeric enzyme-linked immunosorbent assay (ALPCO Diagnostics, Salem, NH) per manufacturer's protocol.

Plasma total antioxidant capacity was measured by using a method[8] adapted for a Cobas Mira. Plasma was incubated with met-myoglobin and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid. After incubation, hydrogen peroxide was added, and the sample was incubated again. Absorbance was measured spectrophotometrically to determine total antioxidant capacity.

Plasma glutathione peroxidase was measured by using methods[9] adapted for a Cobas Mira. Briefly, plasma was incubated with Tris-hydrochloric acid buffer, nicotinamide adenine dinucleotide phosphate, glutathione, and glutathione reductase. T-butyl hydroperoxide was added. Absorbance was measured spectrophotometrically to determine glutathione peroxidase activity.

Ferric reducing ability of plasma was measured by using the method of Benzie and Strain.[10] Plasma was incubated with freshly prepared ferric reducing ability of plasma reagent, and absorbance was measured spectrophotometrically (Cobas Mira).

Superoxide dismutase was measured spectrophotometrically (FLUOstar Optima; BMG Labtech, Offenburg, Germany) by using a kit and standards by Cayman Chemical (Ann Arbor, MI).

F2-isoprostanes were extracted from plasma by using methanolic NaOH. Samples were spiked with 8-iso-$PGF_{2\alpha}$-d_4 (Cayman Chemicals) as an internal standard and incubated at 42°C for 60 minutes. Samples were then acidified to pH 3 with hydrochloric acid, and hexane was added before centrifugation. The supernatant was removed, and the remaining solution was extracted with ethyl acetate and dried under nitrogen. Samples were reconstituted with acetonitrile, transferred into vials with silanized glass inserts, and dried. Derivatization used pentafluorobenzyl bromide and diisopropylethylamine and incubation at room temperature. Samples were then dried under nitrogen before pyridine, bis(trimethylsilyl) trifluoroacetamide, and trimethylchlorosilane were added and incubated at 45°. Finally, hexane was added, and analysis was done by using gas chromatography-mass spectrometry (Varian Medical Systems, Sydney, Australia) in negative chemical ionization mode.

Plasma cytokeratin 18 fragments were measured by immunoassay with the Peviva M30 Apoptosense enzyme-linked immunosorbent assay per manufacturer's instruction manual (PEVIVA AB, Bromma, Sweden).

Plasma high-mobility group box-1 was measured by immune assay with the EIAAB assay per manufacturer's

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instruction manual (WuhanEIAab Science Co Ltd Biopark, Optics Valley, Wuhan, China).

Pharmacokinetics

Samples for pharmacokinetics were collected in ethylenediamine tetraacetic acid 4-mL tubes. All samples were analyzed for resveratrol concentration after intervention and data analysis. On the last day of intervention, a trough sample was collected after a 10-hour fast. The morning dose (1500 mg resveratrol or placebo) was ingested with 150 mL water, and samples were collected at 10, 20, 30, 45, 60, 90, 120, 180, 240, 300, and 360 minutes. Sampling in the first 120 minutes was performed while fasted. The following 2 hours of sampling were performed in conjunction with the EHC clamp, and the remaining hour included a light hospital meal. All samples were spun immediately after collection at 2500 rpm for 10 minutes, and plasma was aliquoted for storage at –80°C. Caution was taken to handle samples with minimal light exposure. Concentration of parent resveratrol without enzymatic hydrolysis was measured including albumin bound and unbound fractions. Plasma samples (100 μL) were thawed; to each was added 50 μL of an internal standard solution containing 2 μg/mL $^{13}C_6$ labeled resveratrol prepared in 0.5 mol/L pH 7 phosphate buffer. The samples were then extracted with ethyl acetate (1 mL) by vortex mixing for 2 minutes. The solvent layer was removed and evaporated to dryness under nitrogen, and the residue was reconstituted in 50 μL 10% methanol. Chromatography of the extract was performed by injection of a 10-μL aliquot onto a Waters Acquity BEH C18 UPLC column (1.7 μmol/L, 2.1 × 50 mm) held at 35°C. The mobile phase comprised 2 mmol/L ammonium acetate and 1 mL/L formic acid in water (solvent A) and 2 mmol/L ammonium acetate and 1 mL/L formic acid in methanol (solvent B). The analytes were eluted by using a linear gradient that was started at 10% solvent B and increased to 50% solvent B over 7 minutes. Mass spectrometric detection (Waters Quattro Premier; Waters Corporation, Milford, MA) was by selected reaction monitoring (resveratrol *m/z* 227 →143; internal standard *m/z* 233 → 149) by using negative electrospray ionization conditions. Both resveratrol and the internal standard eluted at approximately 5.2 minutes. Linearity of the method was shown to be suitable over the range 5–500 ng/mL.

Peripheral Blood Mononuclear Cell Isolation

PBMC purification was performed from 20 mL whole blood collected in heparin tubes at baseline, week 1, and end of study. Ten milliliters Ficoll-Paque Research Grade (Amersham Pharmacia Biotech, Uppsala Sweden) was carefully added under a layer of 10 mL blood, and 20 mL saline was added. Blood was centrifuged at 1500 rpm for 35 minutes at room temperature without braking. The layer above the Ficoll containing the PBMCs was transferred into a new centrifuge tube, and the PBMCs were washed 3 times in saline. Cells were resuspended, counted, and stored in cryo tubes in 50/50 fetal bovine serum and dimethyl sulfoxide at a concentration of 10×10^6cells/mL and stored at –80°C.

Target Genes Transcription by Quantitative Reverse Transcriptase Polymerase Chain Reaction

Expression levels of *PTB1B*, *HO-1*, *NQO1*, and *IL6* were measured by quantitative reverse transcriptase polymerase chain reaction in PBMCs of subjects receiving resveratrol at time 0, week 1, and week 8. These genes were selected to examine changes in *IL6* expression by immune cells and correlation with circulating protein, investigate whether resveratrol had a molecular effect on the Nrf2 signaling pathway (*HO-1* and *NQO1*), and determine whether resveratrol had an effect on insulin signaling at the receptor site (*PTP1B*). The time points were chosen to determine whether resveratrol had an acute and/or long-term effect.

Quantitative reverse transcriptase polymerase chain reaction was performed as previously described.[11] Total RNA was obtained by using RNeasy kits (Qiagen, Doncaster, Victoria, Australia). The concentration and quality were determined by using a spectrophotometer (Nanodrop ND-1000; Biolab, Clayton, Victoria, Australia). Total RNA (500 ng) was reverse transcribed by using random hexamers (Promega, Annandale, NSW, Australia) and Superscript III reverse transcriptase per manufacturer's instructions (Invitrogen, Carlsbad, CA). Primers were purchased from Sigma Aldrich (Castle Hill, Victoria, Australia), and sequences were from OriGene (Rockville, MD). Quantitative reverse transcriptase polymerase chain reaction was performed by using the SYBR Green detection protocol (SYBR Premix *Ex Taq*; Takara Bio, Madison, WI) per the manufacturer's instructions on a RotorGene 3000 (Corbett Research, Sydney, Australia). Gene expression levels were calculated by using the ΔCt method. Sequences are available on request.

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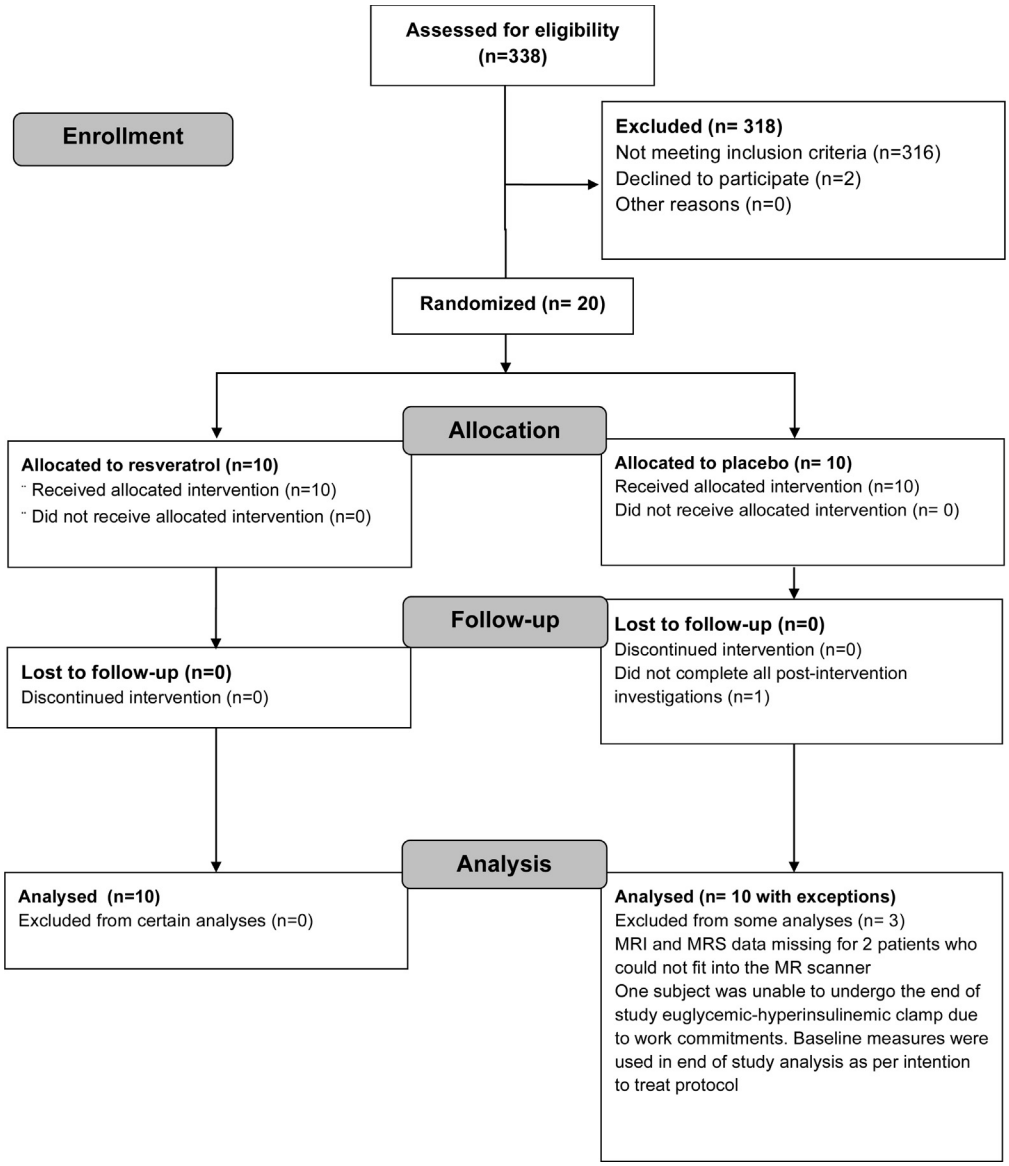

Supplementary Figure 1. Consort flow diagram.

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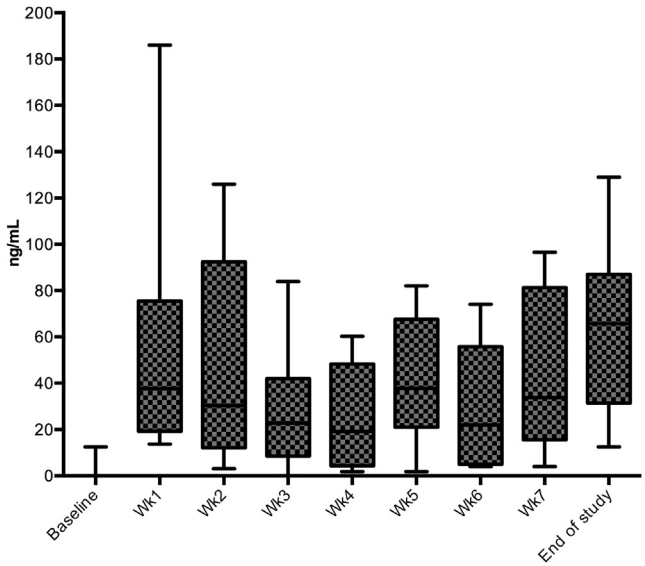

Supplementary Figure 2. Resveratrol concentration at weekly reviews in the resveratrol group (medians and interquartile ranges).