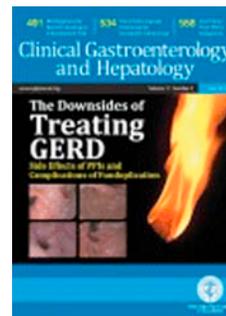


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Resveratrol Does Not Benefit Patients with Non-alcoholic Fatty Liver Disease

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Title

Resveratrol Does Not Benefit Patients with Non-alcoholic Fatty Liver Disease

Short title

Resveratrol in NAFLD

Authors

Veronique S. Chachay^{1,2}, Graeme A. Macdonald^{3,4}, Jennifer H. Martin³, Jonathan P. Whitehead⁵, Trisha M. O'Moore-Sullivan^{3,6}, Paul Lee^{3,6}, Michael Franklin⁷, Kerenaftali Klein⁸, Paul J. Taylor⁷, Maree Ferguson^{2,9}, Jeff S. Coombes⁹, Gethin P. Thomas¹, Gary J. Cowin¹⁰, Carl M.J. Kirkpatrick¹¹, Johannes B. Prins^{1,5} and Ingrid J. Hickman^{1,2,5}

¹The University of Queensland Diamantina Institute, The University of Queensland, Translational Research Institute*; ²^Nutrition and Dietetics*; ³School of Medicine Metro-South**; ⁴^Gastroenterology and Hepatology*; ⁵Mater Medical Research Institute*; ⁶^Endocrinology*; ⁷^Clinical Pharmacology*; ⁸Queensland Clinical Trials & Biostatistics Centre**; ⁹School of Human Movement Studies**; ¹⁰Centre for Advanced Imaging**; ¹¹Centre for Medicine Use and Safety, Monash University, Melbourne, Australia.

^The department of - The Princess Alexandra Hospital. *The University of Queensland.

*Brisbane, Australia. Corresponding author: v.chachay@uq.edu.au

The authors have no conflict of interest to declare.

Corresponding author:

Veronique Chachay
The University of Queensland Diamantina Institute
Translation Research Institute
Level 5, 37 Kent Road
Woolloongabba QLD 4102
Brisbane, Australia
(+) 61 411403936
v.chachay@uq.edu.au

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Authors' contributions

Veronique S. Chachay: study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; statistical analysis; obtained funding.

Graeme A. Macdonald: study concept and design; analysis and interpretation of data; critical revision of the manuscript for important intellectual content; clinical support; study supervision.

Jennifer H. Martin: study concept and design; analysis and interpretation of data; critical revision of the manuscript for important intellectual content; clinical and technical support; study supervision.

Jonathan P. Whitehead: acquisition of data; analysis and interpretation of data; critical revision of the manuscript for important intellectual content; technical and material support.

Trisha M. O'Moore-Sullivan: acquisition of data; critical revision of the manuscript for important intellectual content; clinical support; study supervision.

Paul Lee: important intellectual input; collection of data and clinical support.

Michael Franklin: acquisition of data; critical revision of the manuscript for important intellectual content; technical and material support.

Kerenaftali Klein: statistical analysis advising

Paul J. Taylor: acquisition of data; critical revision of the manuscript for important intellectual content; technical and material support.

Maree Ferguson: study concept and design; critical revision of the manuscript for important intellectual content.

Jeff S. Coombes: analysis and interpretation of data; critical revision of the manuscript for important intellectual content; technical and material support.

Gethin P. Thomas: study concept and design; analysis and interpretation of data; critical revision of the manuscript for important intellectual content; technical and material support.

Gary J. Cowin: study concept and design; acquisition of data; analysis and interpretation of data; critical revision of the manuscript for important intellectual content; technical and material support.

Carl M.J. Kirkpatrick: study concept and design; analysis and interpretation of data; critical revision of the manuscript for important intellectual content; clinical and technical support; study supervision.

Johannes B. Prins: study concept and design; interpretation of data; critical revision of the manuscript for important intellectual content; administrative, technical, and material support.

Ingrid J. Hickman: study concept and design; analysis and interpretation of data; critical revision of the manuscript for important intellectual content; obtained funding; technical and material support; study supervision.

Background & Aims: Non-alcoholic 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 upregulating 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). Metabolic, hepatic, inflammatory, and antioxidant activities were measured using plasma samples; transcription of resveratrol target genes was measured in peripheral blood mononuclear cells (PBMC). Resveratrol pharmacokinetics and safety were assessed.

Results: Eight weeks administration of resveratrol did not reduce insulin resistance, steatosis, or abdominal fat distribution, 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, compared with the placebo group. Resveratrol did not significantly alter transcription of *NQO1*, *PTP1B*, *IL6*, or *HO1* in PBMC. Resveratrol was well tolerated.

Conclusions: Eight weeks administration of resveratrol did not significantly improve any features of NAFLD, compared with placebo, but increased hepatic stress, based on increased 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.

KEY WORDS: NAFLD; obesity-related fatty liver; chronic disease; ALT; AST

BACKGROUND

Non-alcoholic 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 non-alcoholic 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 pharmacological therapies for NAFLD.⁵ Weight loss of 7 to 10% of body weight through lifestyle modification has shown the most success to date.^{6, 7} Weight-loss and permanent maintenance of weight lost remain however unrelenting clinical challenges.⁸

IR appears necessary in the development of NAFLD.⁹ IR can result from adipocytes 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 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 over the last three decades in models of adipogenesis, inflammation, oxidative-stress, insulin resistance, 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 AMP-activated kinase (AMPK), nuclear factor (erythroid-derived)-like 2 (Nrf2), and nicotinamide adenine dinucleotide NAD⁺-dependent deacetylase (SIRT1).²⁰ Pre-clinical 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, insulin sensitivity, and reduced hepatic *de-novo* lipogenesis compared to 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 is 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 over 8 weeks would i) improve hepatic and metabolic dysregulation with insulin resistance as primary outcome, assessed by the euglycemic-hyperinsulinemic clamp; and ii) 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 25kg/m² and waist circumference above 90cm 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 40g, 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 (PI) and providing informed written consent, volunteers were allocated to either resveratrol or placebo by computer-generated randomization. Participants underwent baseline measures. Dosing involved three capsules (500mg each) of resveratrol or placebo before breakfast, and another three of the same capsules before bedtime, equating to 3000mg resveratrol daily in the treatment arm. Resveratrol (from *Polygonum Cuspidatum*) was purchased from Biotivia Bioceuticals, 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 post un-blinding. All co-authors had access to the study data, reviewed and approved the final manuscript.

Safety monitoring and compliance

Subjects were reviewed weekly by the PI 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 3000mg was calculated using the body surface area (BSA) normalization method as previously described.³² The highest dosage used in pre-clinical diet-induced hepatic steatosis (400mg/kg in mice),¹⁹ and the reported highest no observed adverse effect level at the time of protocol development (300mg in rats)³³ were converted to determine a human equivalent dose (HED). Obesity factor calculations were performed, as 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 4000mg resveratrol daily over 2 weeks³⁵ 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 as 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, UT), performed in the fasted and insulin stimulated states as previously described.³⁷

Magnetic resonance imaging and spectroscopy

Magnetic resonance data was acquired on a 3T Siemens TRIO (Erlangen, Germany), as 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 UPLC tandem mass-spectrometry protocols are provided in the Supplementary Material.

Peripheral blood mononuclear cells (PBMC) isolation

PBMC purification was performed at baseline, week 1 and week 8 as previously described.⁴⁰ (Supplementary Material)

Target genes transcription

RNA levels of *PTP-1B*, *HO-1*, *NQO1*, and *IL-6* were measured by quantitative reverse transcriptase polymerase chain reaction in PBMC of subjects receiving resveratrol as previously described.⁴¹ (Supplementary Material)

Statistical analyses

The study was powered to detect 25% change (80% power and $p < 0.05$) in the primary outcome (glucose disposal rate as a measure of insulin resistance). Eight participants were required in each arm. To account for a possible 25% drop-out 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 was analyzed with intention to treat. Where data was missing, the previous time-point value was brought forward. All analyses were performed on Prism GraphPad Version 6.0c and SPSS Version 21. 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 ALT, AST, and mRNA levels of target genes at three time-points, was assessed by repeated-measures analysis of variance (ANOVA). Pharmacokinetics parameters were calculated on Microsoft Excel to obtain plasma concentration maximum (C_{max}), average concentration (C_{av}), 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.

Compliance

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 insulin resistance. Resveratrol treatment did not induce improvement in insulin mediated glucose uptake (Table 2, Figure 2A). Fasting glucose, insulin, non-esterified fatty acids (NEFA) and AT-IR did not change with treatment (Table 2, Figure 2B). Resveratrol led to a statistically significant less effective insulin-mediated suppression of plasma NEFA, however not considered clinically significant (Table 2, Figure 2C). Total and high-molecular weight (HMW) adiponectin did not change, however the HMW to total adiponectin ratio decreased significantly with resveratrol (Table 2, Figures 3A, 3B).

Transcription of *NQO1*, *PTP-1B*, *IL-6* and *HO-1* in PBMC did not change significantly with resveratrol. (Table 2, Figures 3E & 3F)

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 2E and 2F). 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 IL-10 and decrease in IL-6, but no change in TNF α , IL-1 β , IL-8 or C-reactive protein. (Table 2, Figures 3C, 3D)

Resveratrol pharmacokinetics

The C_{max} for parent resveratrol was 65.7 ± 35.9 ng/mL (0.29 μ M) at 60 minutes post-ingestion of 1500mg resveratrol in fasted state. The coefficient of inter-individual variation (CV) was 54%. The C_{av} across the dosing interval was 29.3 ± 11.6 ng/mL (CV of 36%). The AUC_{0-24} was 705 ng/mL x hour (CV 36%). (Figure 4)

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 compared to 20% in the placebo group (data not shown). Previous reports have discussed the potential for drug bioavailability interactions, secondary to 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.

Alanine and aspartate aminotransferases (ALT, AST) 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 (Figures 5A and 5B). Cytokeratin 18 (CK-18) fragments and high-mobility-group box-1 (HMGB-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: IGF-1 and IGFBP-3, F2-isoprostanes, blood pressure, vitamin C (Table 2); none changed with resveratrol.

DISCUSSION

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

Resveratrol did not improve typical characteristics of NAFLD

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 resveratrol on insulin sensitivity has been previously reported in overweight or obese healthy subjects at various resveratrol dosages (daily 75mg to 1500mg) using similar gold-standard protocols to assess functional IR.^{29, 31} In contrast, in populations with type 2 diabetes, 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 (10mg daily²⁶, 250mg⁴⁴, or 1000mg⁴⁵). These studies suggest that the severity of metabolic dysregulation prior to resveratrol treatment, and the combination with pharmacological agents that target AMP-kinase activation, could be of importance for measurable benefits of resveratrol.^{46, 47}

PTP-1B, a negative regulator of insulin signaling, was shown significantly repressed in PBMC of healthy humans receiving 40mg resveratrol daily for 6 weeks,²⁸ and suggested as another mechanism for improved insulin sensitivity by resveratrol.⁴⁸ *PTP-1B* transcription remained however unchanged in the present study, consistent with the no improvement in IR. Total and HMW adiponectin did not change, 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 8mg 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 pharmacological, 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 pathological fat accumulation, characteristic of NAFLD.⁵⁰ The subjects in the present study had up to 54% steatosis measured by MRS. 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 present 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 IL-10 and decrease in IL-6 suggest a modest improvement in the circulating inflammatory profile with resveratrol. However mRNA level of *IL-6* in PBMC 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 pre-clinical evidence of the effect of resveratrol on regulators of inflammation and on the endogenous antioxidant defense is abundant.⁴⁷ The clinical evidence is however mixed, 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}

Safety and plasma resveratrol concentration

A rise in ALT and AST is generally attributed to hepatocyte injury, however plasma CK-18 and HMGB-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 since 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 co-morbidities such as NAFLD. This warrants adequate warning and further research. The 3000mg 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 C_{max} and AUC_{0-24} were however 52% and 41% lower respectively after 1500mg resveratrol (Figure 4), than the C_{max} and AUC_{0-24} after 1000mg 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 employed. Methods measure total resveratrol including metabolites,³⁵ or use enzymatic hydrolysis to reconvert metabolites to parent compound prior to 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 is to our knowledge the first study administering the pharmacological dose of 3000mg 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 pre-clinically (from 1mg to 750mg/kg of body weight, or food consumed by animals) without pharmacokinetics data provided, or described rationale for dose selection. When converted through the BSA 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 a HED for bioactive food-constituents considering allometric scaling.⁵⁹ Dose-calculation limitations further exist for an obese population with concurrent co-morbidities. Standards for BSA, absorption, metabolism and body-composition often rely on assumed data from 60kg 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 is 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 (LBM).⁶⁰ Chronic dosing depends on drug clearance, as the aim is to achieve a steady state concentration related to efficacy.⁵⁴ Therefore animal dosage conversion to HED based on LBM may be more adequate for nutraceuticals targeted for chronic disease therapy.

Furthermore, the distinction between prevention and therapy is rarely addressed in pre-clinical studies, the prevention models being most commonly employed to determine the therapeutic potential of an agent. Chronic disease develops over time however, 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 tissue⁶³ has confirmed early animal evidence, and likely predicts accumulation in other tissues.⁶⁴ Sulfate metabolites were reported undergoing hydrolysis back to parent compound intra-cellularly, thus 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 is not representative of actual exposure, and is 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-1 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 if, and at what dose, this change occurs in humans. Further, it is unclear whether a nutraceutical dose would qualify as a “large” dose physiologically, regardless of size, due to its bolus delivery, compared to 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 clinical established NAFLD. We provide evidence that in obese subjects with chronic fatty liver disease, 8 weeks

supplementation of a high pharmacological dose of resveratrol, calculated from preclinical efficacy doses, and based on BSA i) does not improve insulin sensitivity and hepatic steatosis; ii) appears potentially toxic to hepatocytes; and iii) 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 in order 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 insulin resistance. The MRS 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. As resveratrol metabolites were not measured, actual exposure to resveratrol species cannot be determined.

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TABLES AND FIGURES LEGENDS

Table 1: Baseline characteristics of resveratrol and placebo groups

Table 2: Pre and post intervention results for resveratrol and placebo groups

Figure 1: Consort flow diagram

Figure 2: Effects of resveratrol on NAFLD characteristic features

- 2A: Insulin resistance expressed as M over insulin concentration at 2 hours: $[M/I]*100$. (Medians and interquartile ranges)
- 2B: Adipose Tissue Insulin Resistance Index. (Medians and interquartile ranges)
- 2C: Pre and post intervention plasma non-esterified fatty acids suppression during insulin clamp infusion for resveratrol group (Means and standard deviations)
- 2D: Pre and post intervention plasma non-esterified fatty acids suppression during insulin clamp infusion for placebo group (Means and standard deviations)
- 2E: Pre and post intervention hepatic steatosis measured by MRS for resveratrol and placebo groups. (Medians and interquartile ranges)
- 2F: Pre and post intervention abdominal adipose tissue: total (TAT), visceral (VAT) and subcutaneous (SAT) distribution for resveratrol and placebo groups. (Medians and interquartile ranges)

Figure 3: Effect of resveratrol on other metabolic markers

- 3A: Total adiponectin (Medians and interquartile ranges)
- 3B: SA ratio (Total / high-molecular weight adiponectin). Pre and post intervention in resveratrol group, $p=0.02$ (Medians and interquartile ranges)
- 3C: IL-6 (Medians and interquartile ranges). Pre and post intervention in resveratrol group, $p=0.04$ (Medians and interquartile ranges)
- 3D: TNF α (Medians and interquartile ranges)
- 3E: PTP-1B mRNA levels in PBMC (normalized to actin) of participants in resveratrol group at baseline, week 1 and week 8. (Medians and interquartile ranges)
- 3F: NQO1 mRNA levels in PBMC (normalized to actin) of participants in resveratrol group at baseline, week 1 and week 8. (Medians and interquartile ranges)

Figure 4: Pharmacokinetics of resveratrol up to 6 hours post-ingestion of 1500mg after 8 weeks of 3000mg daily supplementation (Means and SEM)

C_{max} : concentration maximum; C_{min} : concentration minimum; C_{av} : concentration average; AUC: area under the concentration versus time curve

Figure 5: Safety monitoring

Alanine (5A) and Aspartate (5B) aminotransferases weekly fluctuations expressed in percentage change from baseline value (Medians and IQR)

5A: Repeated-measures ANOVA. Resveratrol $p= 0.3$; Placebo $p= 0.6$.

5B: Repeated-measures ANOVA. Resveratrol $p= 0.5$; Placebo $p= 0.8$.

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Parameters	Resveratrol n=10	Placebo n=10	p value
Age (years)	48.8±12.2	47.5±11.2	ns
BMI (kg/m ²)	31.8 (30.2-37.0) [^]	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 (mmHg)	130±12	130±10	ns
Diastolic blood pressure (mmHg)	83±7	82±6	ns
HOMA-IR	2.8 (1.8 - 6.1) [^]	2.5 (1.8 - 3.3)	ns
Glucose disposal rate (mg/kgLBW/min)	4.1±1.7	3.8±0.9	ns
M/ 2hr 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) [^]	10 (8 -14)	ns
Total adiponectin (ug/mL)	3.5±1.1	3.8±2.2	ns
High molecular weight adiponectin (ug/mL)	1.2 (0.9 - 1.8)	0.9 (0.8 - 2.3) [^]	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)	0.02
LDL cholesterol (mmol/L)	3.2±0.7	2.8±0.8	ns
ALT (U/L)	45 (41 - 70)	40 (38 - 87) [^]	ns
AST (U/L)	35±9	36±16	ns
IL-6 (pg/mL)	5.6 (0.6 - 31.6) [^]	3.9 (1.1 - 12.0)	ns
IL-10 (pg/mL)	4.1 (1.3 - 12.0) [^]	4.8 (2.1 - 10.7)	ns
Hepatic triglyceride content (%)	25±17	26±12	ns
Resting energy expenditure (kcal/kg/day)	20 (19 - 20)	19 (18 - 21) [^]	ns
Treatment dosage received per kg body weight (mg)	29±4		

Unpaired t-test: means ± SD for data with normal distribution. Mann-Whitney test: medians and (IQR) for data not normally distributed. [^]= Data normally distributed. Significance set at p <0.05

BMI: body mass index; LBW: lean body weight; HOMA-IR: Homeostatic model of assessment – insulin resistance; M: glucose disposal rate; HDL: high density lipoprotein; LDL: low density lipoprotein; ALT: alanine aminotransferase; AST: aspartate aminotransferase; IL: interleukin

Parameter (units)	Resveratrol n=10			Placebo n =10		
	Pre	Post	p value	Pre	Post	p value
Anthropometrics, blood pressure, resting metabolic rate						
Weight (kg)	105.3 ± 15.7	105.3 ± 15.6	0.9	110.7 ± 32.0	111.5 ± 31.4	0.2
BMI (kg/m ²)	31.8 (30.2-37.0) [^]	31.9 (30.0-37.4) [^]	0.9	31.2 (27.4 - 39.3)	31.1 (28.1 - 40.0) [^]	0.2
Systolic blood pressure (mmHg)	130 ± 12	128 ± 11	0.6	130 ± 10	124 ± 7	0.09
Diastolic blood pressure (mmHg)	83 ± 7	82 ± 8	0.6	82 ± 6	81 ± 5	0.6
Resting energy expenditure (kcal/kg/day)	19.9 (19.4-20.5)	19.9 (18.8-20.4) [^]	0.9	19.4 (17.6 - 20.8) [^]	18.9 (16.7 - 22.3) [^]	0.9
Fasting fat oxidation (mg/min/kg)	0.11 (0.09-0.12)	0.13 (0.11-0.14)	0.3	0.11 (0.09-0.15)	0.11 (0.10-0.13)	0.8
Insulin resistance						
M (mg/kgLBW/min)	3.5 (2.7 - 5.3) [^]	3.2 (2.8 - 4.4)	0.3	3.8 (3.2 - 4.5) [^]	3.8 (3.3 - 4.5) [^]	0.6
M/I (kgLBW/min/mU/L)	4.5 (3.1-7.6)	4.5 (2.9 - 6.4)	0.6	4.4 (4.0 - 5.6)	4.5 (3.9 - 6.9)	0.8
HOMA-IR	2.8 (1.8 - 6.1) [^]	3.6 (1.9 - 5.8) [^]	0.1	2.5 (1.8 - 3.3)	3.1 (2.3 - 3.8) [^]	0.4
Glucose (mmol/L)	5.3 ± 0.5	5.4 ± 0.5	0.7	5.6 ± 0.5	5.6 ± 0.5	0.6
Insulin (mU/L)	12 (8 - 25) [^]	15 (8-26) [^]	0.09	10 (8 - 14) [^]	13 (9 - 16) [^]	0.5
Steady state insulin (mU/L)	79 ± 19	79 ± 23	0.9	83 ± 17	79 ± 22	0.5
Adipose Tissue Insulin Resistance (mmol x pmol/L)	8.6 (3.7-10.8)	7.9 (4.9 - 12.7)	0.06	6.2 (4.6 - 9.0)	5.5 (3.5 - 7.3)	0.1
Non-esterified Fatty Acid (NEFA) (mmol/L)	0.5 ± 0.2	0.6 ± 0.1	0.7	0.7 ± 0.3	0.5 ± 0.2	0.01
2-hour NEFA (mmol/L)	0.07 ± 0.04	0.09 ± 0.05	0.04	0.09 ± 0.08	0.07 ± 0.08	0.3
Total adiponectin (TA) (µg/L)	3.1 (2.7 - 4.2) [^]	3.4 (2.9 - 3.7)	0.4	2.8 (2.2 - 5.2) [^]	3.6 (2.3 - 4.8) [^]	0.8
High molecular weight (HMW) adiponectin (µg/L)	1.2 (0.9 - 1.8)	1.0 (0.7-1.4)	0.3	0.9 (0.8 - 2.3) [^]	1.3 (0.7 - 2.2) [^]	0.9
S _A ratio (HMW / TA)	0.4 ± 0.2	0.3 ± 0.1	0.02	0.4 ± 0.1	0.4 ± 0.1	0.6
Hepatic markers						
Hepatic triglyceride content (%)	25 ± 17	27 ± 18	0.3	26 ± 12	25 ± 12	0.3
Alanine aminotransferase (U/L)	45 (41-7)	63 (57 - 83) [^]	0.02	40 (38 - 87) [^]	48 (39 - 98) [^]	0.1
Aspartate aminotransferase (U/L)	35 ± 9	45 ± 15	0.03	36 ± 16	38 ± 15	0.5
Total bilirubin (µmol/L)	15 (12 - 16)	18 (12 - 22) [^]	0.09	12 (11 - 12) [^]	11 (9 - 15) [^]	0.02
Cytokeratin 18 (U/L)	34.3 ± 26.1	48.4 ± 34.1	0.1	41.5 ± 22.3	56.5 ± 36.1	0.2
High-mobility-group-box-1 (ng/mL)	0.8 (0.4-1.5)	0.8 (0.4 - 2.6)	0.6	0.4 (0.3 - 0.6)	0.4 (0.4 - 0.9)	0.3
Kidney function						
Creatinine (µmol/L)	79 ± 16	80 ± 16	0.7	76 ± 14	79 ± 16	0.2
Potassium (mmol/L)	3.9 ± 0.3	4.0 ± 0.2	0.3	4.0 ± 0.2	4.1 ± 0.3	0.7
Inflammation and oxidative stress						
IL-1 beta (pg/mL)	0.22 ± 0.15	0.20 ± 0.12	0.2	0.34 ± 0.27	0.34 ± 0.25	0.9
IL-6 (pg/mL)	5.6 (0.6 - 31.6) [^]	2.5 (0.5 - 19.0) [^]	0.04	3.9 (1.1-12.0)	4.4 (1.7-12.1)	0.5
IL-8 (pg/mL)	8.7 ± 4.2	10.4 ± 6.4	0.2	7.7 ± 4.7	9.1 ± 7.0	0.2
IL-10 (pg/mL)	4.1 (1.3 - 12.1) [^]	6.2 (3.4 - 11.4) [^]	0.03	4.8 (2.1 - 10.7)	6.2 (3.2-18.8)	0.2
C-reactive protein (mg/L)	1.5 (0.3 - 4.0) [^]	0.9 (0.4 - 3.0)	0.3	0.8 (0.4 - 2.0)	0.8 (0.4 - 2.2) [^]	1.0
TNFα (pg/mL)	9.0 (6.7-14.0)	8.9 (7.0 - 12.5)	0.8	7.4 (5.2-11.5)	8.1 (6.1-10.2)	0.6
F2-Isoprostanes (pg/mL)	247 ± 62	229 ± 49	0.4	248 ± 59	276 ± 99	0.3
Plasma antioxidant activity						
Ferric reducing ability of plasma (µmol/L)	1162 ± 144	1181 ± 174	0.6	1107 ± 179	1027 ± 171	0.02
Superoxide dismutase (U/mL)	7.2 (5.5 - 8.0) [^]	6.4 (5.5 - 7.3) [^]	0.2	6.3 (5.6 - 6.7) [^]	7.1 (6.8 - 8.3)	<0.01
Glutathione peroxidase (U/L)	176 (163 - 179)	175 (163 - 194) [^]	0.5	173 (159 - 204) [^]	165 (155 - 208) [^]	0.3
Total antioxidant capacity (mmol/L)	1.7 ± 0.1	1.7 ± 0.1	0.4	1.7 ± 0.1	1.6 ± 0.1	0.06
Vitamin C (µmol/L)	53 ± 16	56 ± 17	0.5	41 ± 16	54 ± 19	0.05
Lipid profile						
Triglyceride (mmol/L)	1.3 (1.2 - 1.7)	1.7 (1.0 - 1.9)	0.9	1.6 (0.8 - 1.8) [^]	1.3 (1.0 - 2.0) [^]	0.5
Total cholesterol (mmol/L)	5.0 ± 0.7	5.1 ± 1.1	0.4	4.4 ± 1.0	4.3 ± 1.2	0.3
HDL (mmol/L)	1.0 (0.9 - 1.1)	1.0 (0.9 - 1.1)	0.7	0.9 (0.8 - 0.9)	0.9 (0.7 - 1.1)	0.9
LDL (mmol/L)	3.2 ± 0.7	3.5 ± 0.9	0.07	2.8 ± 0.8	2.7 ± 0.9	0.08
Abdominal adipose tissue distribution						
Total adipose tissue (cm ²)	612 ± 171	618 ± 162	0.6	539 ± 166	530 ± 161	0.5
Visceral adipose tissue (cm ²)	262 ± 90	256 ± 70	0.5	214 ± 64	200 ± 55	0.2
Subcutaneous adipose tissue (cm ²)	349 ± 156	354 ± 158	0.7	330 ± 140	337 ± 140	0.4
IGF-1 axis						
Insulin-like growth factor (IGF) 1 (ng/mL)	67 ± 26	61 ± 22	0.2	60 ± 23	69 ± 28	0.07
IGF-binding-protein 3 (ng/mL)	4120 ± 1063	4113 ± 1239	0.9	4852 ± 1215	4826 ± 1418	0.8
Resveratrol group only target genes transcription in peripheral blood mononuclear cells (n=9)						
Genes	Paired test for change in RNA levels at time points			Analysis of variance in multiple time points		
	p value time 0 to week 1	p value week 1 to week 8	p value time 0 to week 8			
PTP-1B [^]	0.2	0.2	0.1	0.08		
IL-6	1	0.3	0.2	0.8		
HO-1	0.3	0.2	1	0.1		
NQO1	0.1	0.02	0.4	0.6		

Data not normally distributed: Wilcoxon test, medians and (IQR) (^ = data normally distributed)

Data normally distributed: Paired t-test, means and ± SD. Significance set at p<0.05. Greenhouse-Geisser correction for assumption of sphericity.

Assessed for eligibility
(n=338)

Enrollment

Excluded (n= 318)

- Not meeting inclusion criteria (n=316)
- ▶ Declined to participate (n=2)
- Other reasons (n=0)

Randomized (n= 20)

Allocation

Allocated to resveratrol (n=10)

- Received allocated intervention (n=10)
- Did not receive allocated intervention (n=0)

Allocated to placebo (n= 10)

- Received allocated intervention (n=10)
- Did not receive allocated intervention (n= 0)

Follow-up

Lost to follow-up (n=0)

- Discontinued intervention (n=0)

Lost to follow-up (n=0)

- Discontinued intervention (n=0)
- Did not complete all post-intervention investigations (n=1)

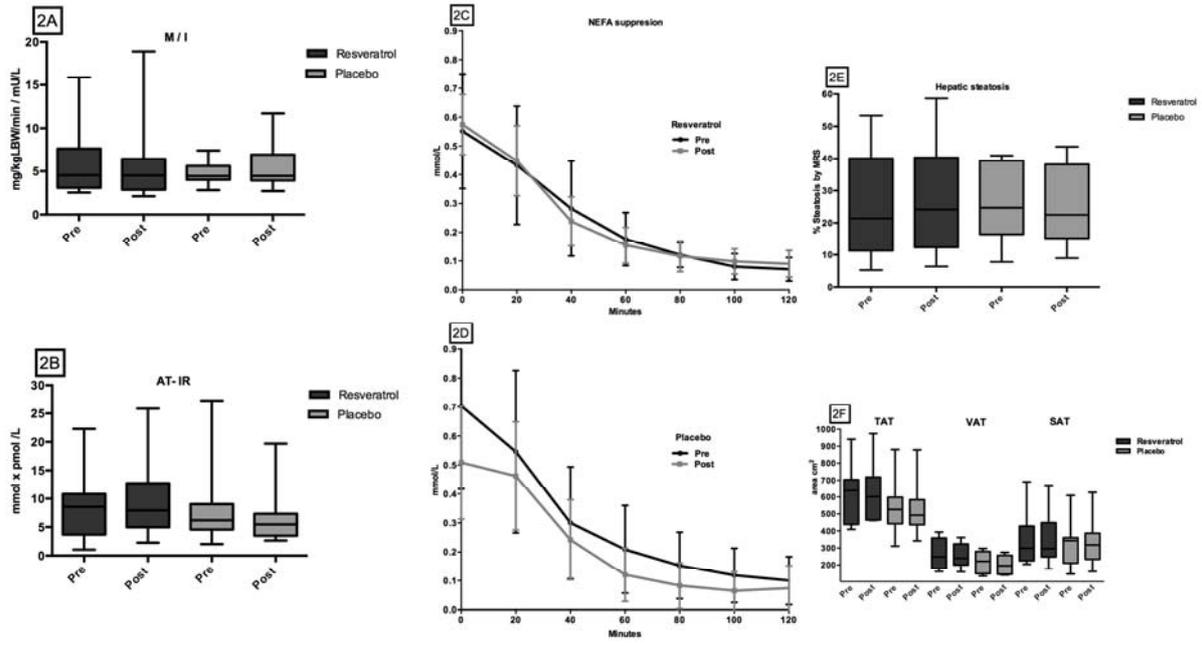
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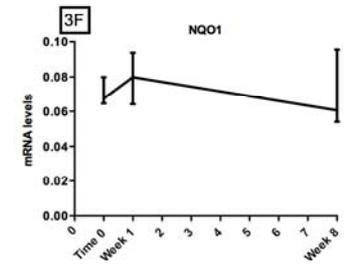
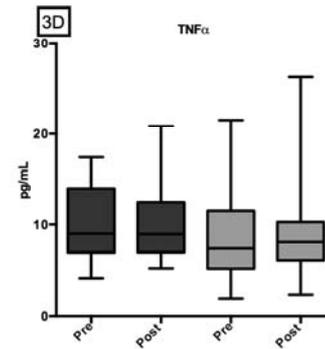
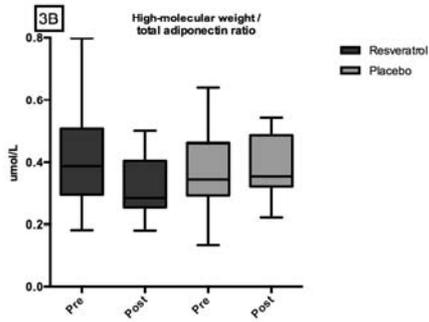
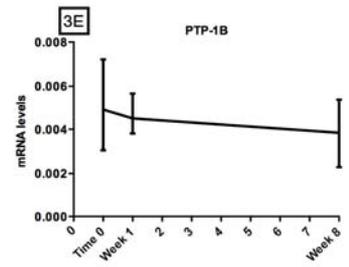
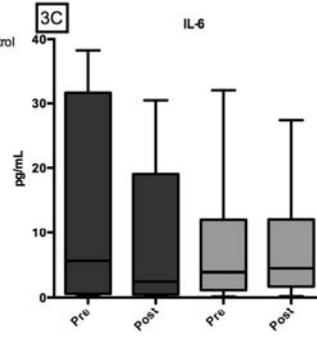
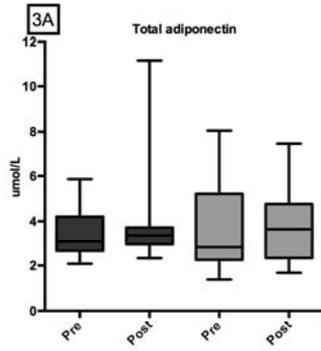
Analysed (n=10)

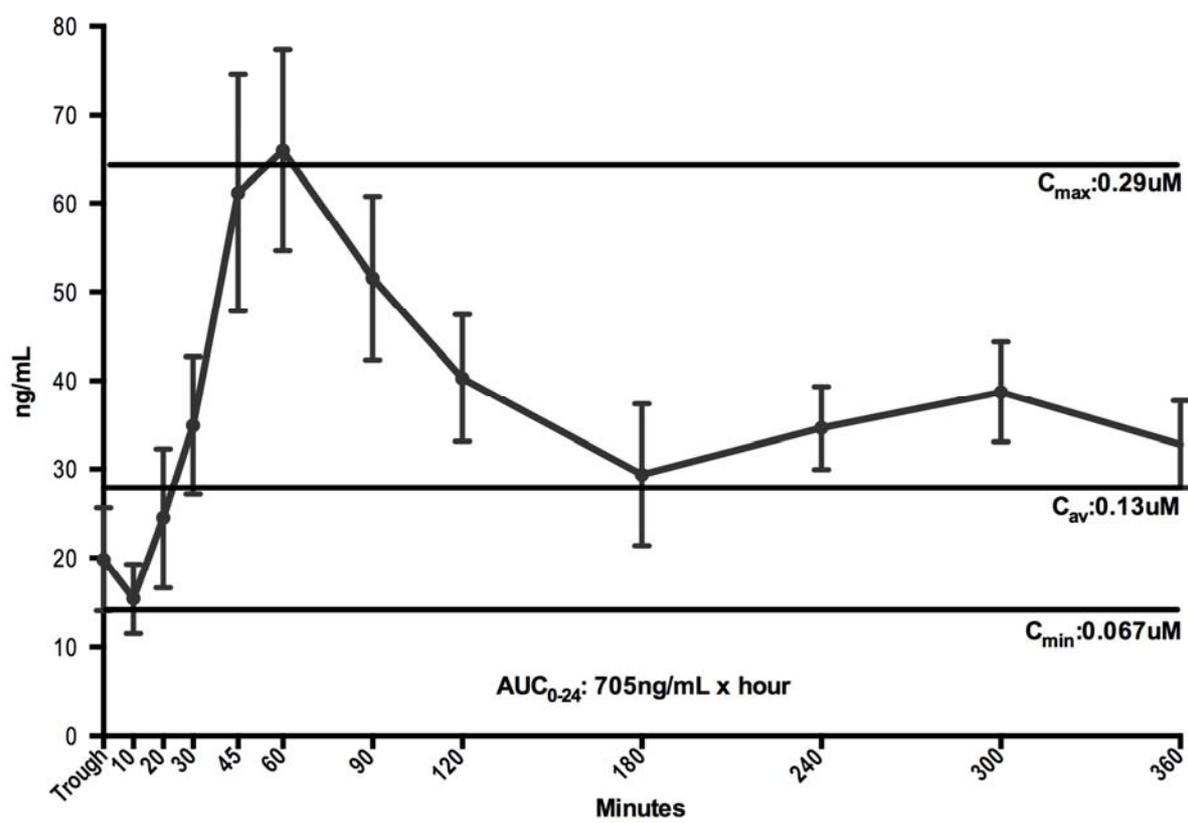
- Excluded from certain analyses (n=0)

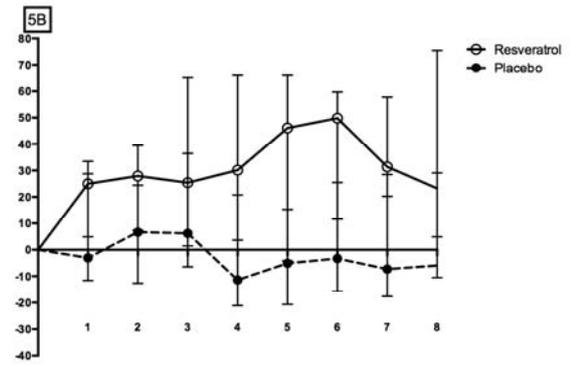
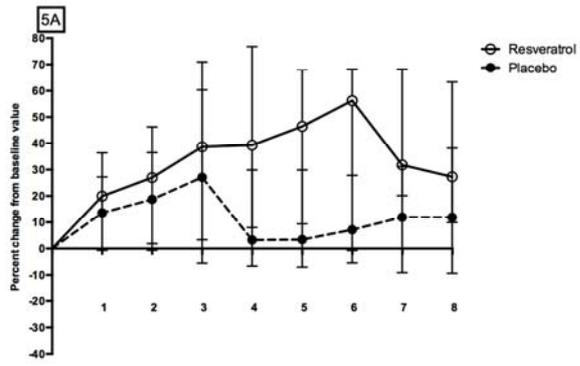
Analysed (n= 10 with exceptions)

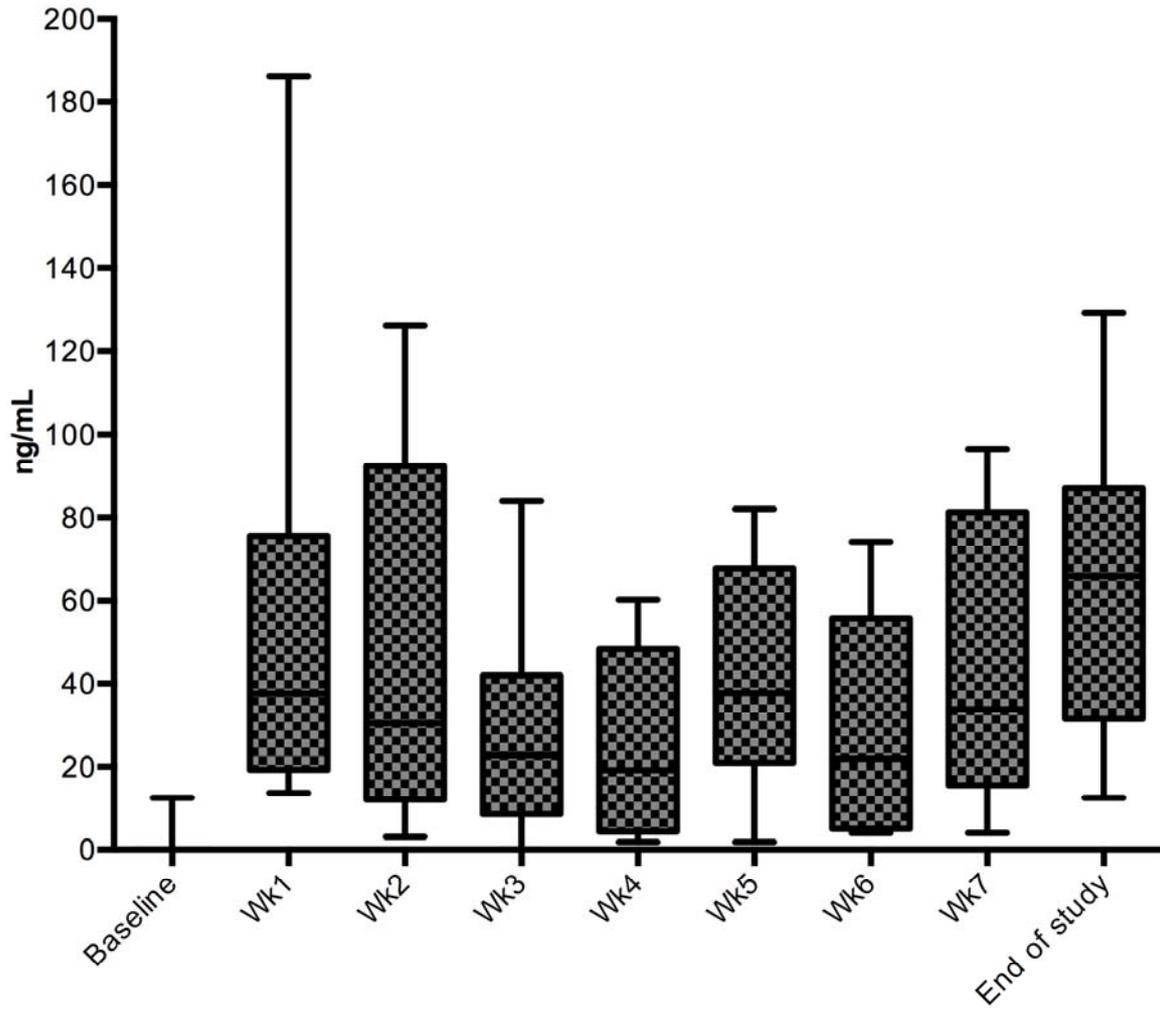
- Excluded from some analyses (n= 3)
- MRI and MRS data missing for 2 patients who could not fit into the MR scanner
- One subject was unable to undergo the end of study euglycemic-hyperinsulinemic clamp due to work commitments. Baseline measures were used in end of study analysis as per intention to treat protocol











Supplementary Material**Sample calculations**

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

Magkos and colleagues (2011)¹ published on the reproducibility of clamp variables in obese people with NAFLD, and also suggested this number of subjects (≤ 8 for paired studies) is required to detect a 25% difference in glucose disposal rate. 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 $p = 0.05$. In addition, as we anticipated a 25% drop out 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 (alanine, aspartate and gamma-glutamyl aminotransferases, 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 minus 80°C to measure resveratrol concentrations post-intervention as described under Pharmacokinetics, 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 prior to sampling, and whether food was consumed since dosing, were recorded.

Participants were provided with 4 weeks supply of capsules, and returned unused capsules prior to 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. Figure 1 shows the concentration of parent resveratrol measured at the weekly reviews.

Figure 1: Resveratrol concentration at weekly reviews in the resveratrol group. (Medians and interquartile ranges)

Euglycemic-hyperinsulinemic clamp (EHC)

Peripheral insulin sensitivity pre and post-intervention was evaluated by the euglycemic-hyperinsulinemic clamp method.² All 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 50mL saline with 1 mL of whole blood, at a rate of 15mL/hr for 2hours. Plasma glucose concentration was monitored every 5 minutes using an automated glucose analyzer (YSI 2300 Stat Plus, YSI Life Sciences, Yellow Springs, OH, interassay coefficient of variation (CV) 2%). A 25% glucose solution was infused at a variable rate, commenced at 2mg/kg body weight /min (DeFronzo et al., 1979), and altered accordingly to maintain fasting blood glucose concentration +/- 10%.

Total glucose disposal rate (M value) was determined as the primary outcome measure. The insulin sensitivity index ($[M/I]*100$) is 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 homeostatic model assessment of insulin resistance (HOMA-IR): fasting glucose (mmol/L) x fasting insulin (mU/L)/22.5 (Matthews et al., 1985); and the adipose tissue insulin resistance index (AT-IR): fasting plasma free fatty acids (mmol/L) x fasting plasma insulin (pmol/L).³

Lean body weight estimation

Lean body weight was estimated for EHC and indirect calorimetry parameter calculations, using the semi-mechanistic model equation where: $LBW(kg)_{Male} = \frac{9270 \times Wt}{6690 + (216 \times BMI)}$, where LBW

is lean body weight in kilogram, Wt is weight in kilogram and BMI is body mass index calculated by weight in kilograms divided by height in meters squared.⁴

Indirect calorimetry

Resting energy expenditure (REE) and respiratory quotient (RQ) were assessed by Indirect calorimetry (TrueOne 2400 Metabolic Measurement System, Parvo Medics, UT), 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 plexiglass hood. Calculations included the average of the last 10 minutes of the test. RQ and REE values were calculated using stoichiometric equations, assuming that the urinary nitrogen excretion rate was negligible.⁵

Magnetic resonance imaging and spectroscopy (MRI and MRS)

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 isocentre for liver MRI and MRS. 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: TR = 5.6, TE = 2.45 and 3.675, Read FOV = 300 mm, phase FOV = 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 min 24 sec. Images were acquired with respiratory gating 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 = 20X20X20mm; 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 H₂O were used to calculate hepatic triglyceride content, where:

$$\text{Hepatic triglyceride content (\%)} = \frac{\text{CH}_2 + \text{CH}_3}{\text{CH}_2 + \text{CH}_3 + \text{H}_2\text{O}} \times 100$$

Following liver MRI and MRS, the patient's bed was moved to position L4 at the gradient isocentre. Dixon images, as described for the liver, were centred on the L4 and acquired.

Four images were acquired and averaged to estimate total, visceral and subcutaneous fat areas. The grow Region Of Interest (ROI) tool in the OsiriX DICOM viewer software package (v.4.1.1 64-bit; Pixmeo Sarl) was used to determine areas of interest.

Plasma biochemistry

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

Liver enzymes, bilirubin creatinine, potassium, iron, transferrin, triglycerides, total cholesterol, HDL and glucose assays were performed on Beckman DxC800 general chemistry analysers (Beckman Coulter, Brea, CA, USA) and VLDL and LDL were calculated using the Friedewald equation (Friedewald 1972). Ferritin assay were performed on Beckman Dxl800 immunoassay analyser (Beckman Coulter, Brea, CA, USA). Serum non-esterified fatty acids (NEFA) concentrations were measured spectrophotometrically (Cobas Mira, Roche Diagnostics, Australia) using a Wako NEFA-HR(2) kit and calibrators (Novachem, Victoria, Australia) with an inter assay CV < 5%.

IGF-1 and IGFBP-3 were assayed by enzyme linked immunoabsorbent assay (R&D Systems, Oxon, UK). The cytokines IL1b, IL6, IL8, IL10, TNF α were assayed using the Merck Millipore high sensitivity Milliplex assay (Merck Millipore Billerica MA USA), with intra and interassay CV < 8%. Leptin was assayed as previously described (Cardinal et al., 2010) using an in-house bead based immunoassay and matched paired antibodies (R&D Systems Minneapolis MN USA), with intra and inter assay CV < 8%. HsC-reactive protein was measured spectrophotometrically (Cobas Mira, Roche Diagnostics, Australia) using a kit and calibrators (Kamiya Biochemical Company, Seattle WA, USA), with intra and inter assay CV < 5%.

Total and high molecular weight adiponectin were assayed with the Adiponectin multimeric ELISA, ALPCO Diagnostics, Salem, NH, USA, as per manufacturer's protocol.

Plasma total antioxidant capacity (TAC) was measured using a method ⁶ adapted for a Cobas Mira (Roche Diagnostics, Australia). Plasma was incubated with met-myoglobin and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS). After incubation, hydrogen peroxide was added and the sample incubated again. Absorbance was measured spectrophotometrically to determine total antioxidant capacity.

Plasma glutathione peroxidase (GPX) was measured using methods⁷ adapted for a Cobas Mira (Roche Diagnostics, Australia). 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 GPX activity.

Ferric reducing ability of plasma (FRAP) was measured using the method as per.⁸ Plasma was incubated with freshly prepared FRAP reagent and absorbance was measured spectrophotometrically (Cobas Mira, Roche Diagnostics, Australia).

Superoxide dismutase (SOD) was measured spectrophotometrically (FLUOstar Optima, BMG Labtech, Germany) using a kit and standards by Cayman Chemical (MI, USA).

F2-Isoprostanes were extracted from plasma using methanolic NaOH. Samples were spiked with 8-iso-PGF_{2α}-d₄ (Cayman Chemicals, USA) as an internal standard and incubated at 42°C for 60 minutes. Samples were then acidified to pH 3 with hydrochloric acid and hexane added before centrifugation. The supernatant was removed and the remaining solution extracted with ethyl acetate and dried under nitrogen. Samples were reconstituted with acetonitrile, transferred into vials with silanized glass inserts and dried. Derivatization used pentafluorobenzylbromide 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 using gas chromatography-mass spectrometry (Varian, Australia) in negative chemical ionization mode.

Plasma cytokeratin 18 fragments were measured by immunoassay with the Peviva M30 Apoptosense ELISA, as per manufacturer's instructions manual (PEVIVA AB, Bromma Sweden)

Plasma high mobility group box-1 was measured by immune assay with the EIAAB assay as per manufacturer's instructions manual (WuhanEIAab Science Co. Ltd. Biopark, Optics Valley, Wuhan China)

Pharmacokinetics

Samples for pharmacokinetics were collected in EDTA 4mL tubes. All samples were analysed for resveratrol concentration post-intervention and data analysis. On the last day of intervention, a trough sample was collected after a 10 hour fast. The morning dose (1500mg resveratrol or placebo) was ingested with 150mL of water, and samples were

collected at 10, 20, 30, 45, 60, 90, 120, 180, 240, 300, 360 minutes. Sampling in the first 120 minutes was performed while fasted. The following 2 hours of sampling were performed in conjunction with the euglycemic-hyperinsulinemic 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 aliquoted for storage at minus 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 ¹³C₆ labelled resveratrol prepared in 0.5M 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 reconstituted in 50 µL of 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 µM, 2.1 x 50 mm) held at 35°C. The mobile phase comprised of 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 using a linear gradient which was started at 10% solvent B and increased to 50% solvent B over 7 minutes. Mass spectrometric detection (Waters Quattro Premier) was by selected reaction monitoring (resveratrol *m/z* 227 → 143; internal standard *m/z* 233 → 149) 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 cells (PBMC) isolation

Peripheral blood mononuclear cells (PBMC) purification was performed from 20mL whole blood collected in heparin tubes at baseline, week 1 and end of study. 10 ml Ficoll-Paque Research Grade (Amersham Pharmacia Biotech, Uppsala Sweden) was carefully added under a layer of 10mL blood, and 20mL saline added. Blood was centrifuged at 1500rpm for 35 minutes at room temperature without braking. The layer above the Fycoll containing the PBMCs was transferred into a new centrifuge tube and the PBMC washed three times in saline. Cells were resuspended, counted, and stored in cryo tubes in 50/50 foetal bovine serum and dimethyl sulfoxide at a concentration of 10x10⁶cells /mL and stored at -80°C.

Target genes transcription by qRT-PCR

Expression levels of *PTB-1B*, *HO-1*, *NQO1*, and *IL-6* were measured by quantitative reverse transcriptase polymerase chain reaction (qPCR) in PBMC of subjects receiving resveratrol, at time 0, week 1 and week 8. These genes were selected to: examine changes in *IL-6* expression by immune cells and correlation with circulating protein; investigate if resveratrol had a molecular effect on the Nrf2 signalling pathway (*HO-1* and *NQO1*); and determine whether resveratrol had an effect on insulin signalling at the receptor site (*PTP-1B*). The time points were chosen to determine if resveratrol had an acute and/or long-term effect.

qPCR was performed as previously described.⁹ Total RNA was obtained using RNeasy kits (Qiagen, Doncaster, Victoria, Australia). The concentration and quality were determined using a spectrophotometer (Nanodrop ND-1000; Biolab, Clayton, Victoria, Australia). Total RNA (500 ng) was reverse transcribed using random hexamers (Promega, Annandale, NSW, Australia) and Superscript III reverse transcriptase as per manufacturer's instructions (Invitrogen). Primers were purchased from Sigma Aldrich (Castle Hill, Victoria, Australia) and sequences were from OriGene (Rockville, MD, USA). qPCR was performed using the SYBR Green detection protocol (SYBR Premix *Ex Taq* Takara Bio, Madison, WI, USA) as per the manufacturer's instructions on a RotorGene 3000 (Corbett Research, Sydney, Australia). Gene expression levels were calculated using the Δ Ct method. Sequences are available on request.

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