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ORIGINAL ARTICLE

Placebo-controlled, randomised clinical trial: high-dose resveratrol treatment for non-alcoholic fatty liver disease

Sara Heebøll^{a,b}, Martin Kreuzfeldt^a, Stephen Hamilton-Dutoit^c, Marianne Kjær Poulsen^d, Hans Stødkilde-Jørgensen^e, Holger Jon Møller^b, Niels Jessen^{f,g}, Kasper Thorsen^g, Ylva Kristina Hellberg^h, Steen Bønløkke Pedersen^e and Henning Grønbaek^a

^aDepartment of Hepatology and Gastroenterology, Aarhus University Hospital and Department of Clinical Medicine, Aarhus University, Aarhus, Denmark; ^bDepartment of Clinical Biochemistry, Aarhus University Hospital and Department of Clinical Medicine, Aarhus University, Aarhus, Denmark; ^cDepartment of Pathology, Aarhus University Hospital and Department of Clinical Medicine, Aarhus University, Aarhus, Denmark; ^dDepartment of Endocrinology and Internal Medicine, Aarhus University Hospital and Department of Clinical Medicine, Aarhus University, Aarhus, Denmark; ^eMR Research Centre, Aarhus University Hospital and Department of Clinical Medicine, Aarhus University, Aarhus, Denmark; ^fResearch Laboratory for Biochemical Pathology, Aarhus University Hospital and Department of Clinical Medicine, Aarhus University, Aarhus, Denmark; ^gDepartment of Molecular Medicine, Aarhus University Hospital and Department of Clinical Medicine, Aarhus University, Aarhus, Denmark; ^hDepartment of Pathology, Hvidovre Hospital, Hvidovre, Denmark

ABSTRACT

Objective “The obesity epidemic” has led to an increase in obesity-related conditions including non-alcoholic fatty liver disease (NAFLD), for which effective treatments are in demand. The polyphenol resveratrol prevents the development of experimental NAFLD through modulation of cellular pathways involved in calorie restriction. We aimed to test the hypothesis that resveratrol alleviates NAFLD in a randomised, clinical trial. **Materials and methods** A total of 28 overweight patients with transaminasemia and histological NAFLD were randomised 1:1 to placebo or resveratrol 1.5 g daily for 6 months. Twenty-six participants completed the trial and underwent repeated clinical investigation, blood work, MR spectroscopy; and 19 participants agreed to a repeat liver biopsy. **Results** Resveratrol treatment was generally not superior to placebo in improving plasma markers of liver injury (primary outcome: alanine transaminase, $p = 0.51$). Resveratrol-treated patients showed a 3.8% decrease in liver lipid content ($p = 0.03$), with no difference between the two treatment arms ($p = 0.38$) and no improvement of histological features. Resveratrol treatment was not associated with improvements in insulin sensitivity or markers of the metabolic syndrome, except for a transient decrease in systolic BP. Microarray analysis and qRT-PCR revealed no major changes in expression profile. Also, we report a serious adverse event in a patient who developed fever and bicytopenia. **Conclusions** In this placebo-controlled, high-dose and long-term study, resveratrol treatment had no consistent therapeutic effect in alleviating clinical or histological NAFLD, though there may be a small ameliorating effect on liver function tests and liver fat accumulation.

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Introduction

The prevalence of non-alcoholic fatty liver disease (NAFLD) is rising, now affecting an estimated one third of population in the Western world and Asia.[1,2] NAFLD is a risk factor for significant liver disease, diabetes, ischaemic heart disease and hepatic cancers.[2,3] Thus, there is an urgent need for effective treatment options. Currently, there is only general consensus as to the benefits of weight loss and life style intervention, though a therapeutic weight loss is neither achievable nor sustainable in many patients.[4,5]

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is a naturally occurring polyphenol, found in minute amounts in

a number of plants.[6] Resveratrol activates AMP-activated protein kinase (AMPK) and silent information regulation 2 homologue 1 (SIRT1), and thereby mimics a condition of caloric restriction as shown in a number of *in vitro* and *in vivo* studies.[7–9] AMPK and SIRT1 pathway activation, anti-inflammatory and anti-oxidative properties provide the rationale for the use of high-dose resveratrol as a treatment for obesity and obesity-related diseases including NAFLD [10]; and the hepatic benefits of resveratrol treatment has been shown in a number of experimental studies.[10]

We have performed a 6-month, high-dose, placebo-controlled, randomised and double-blind clinical trial of

resveratrol treatment in 28 biopsy-verified NAFLD patients. Primarily, we hypothesised that resveratrol would lower plasma transaminases, liver fat content and histological NAFLD activity score (NAS) with superiority to placebo. Second, we speculated that resveratrol treatment would elicit changes in insulin sensitivity and other features of the metabolic syndrome, which are associated with NAFLD severity. Third, we hypothesised that resveratrol treatment would affect cellular pathways in the liver tissue, and that these changes would be measurable in microarray and RT-PCR analyses.

Methods

We conducted a prospective, placebo-controlled, randomised and double-blind clinical trial. Patients were recruited from October 2011 to February 2014. Study procedures were performed at Aarhus University Hospital, Aarhus, Denmark, and participants were screened, seen and allocated by authors SH, MK and HG. The study protocol was approved by the Danish National Committee on Health Research Ethics (No. 20110132) and the Danish Data Protection Agency (No. 1-16-02-471-14). This study was registered in Clinical Trials.gov (NCT01464801).

Study population

All patients, referred to our tertiary hepatology unit with transaminasemia and suspected NAFLD, were screened for the study. Liver steatosis was confirmed by ultrasound. Further, the inclusion criteria included BMI ≥ 25 kg/cm², transaminasemia (alanine transaminase (ALT) $> 70/45$ U/l for men/women) and at least one additional element of the metabolic syndrome, whereas exclusion criteria included diabetes, severe systemic or malignant disease and other causes of liver injury (details in Supplementary Information). All participants gave written informed consent before allocation.

Study procedures

The study design is outlined in Figure 1. Included participants were seen at the baseline visit (V0), at which anthropometric measurements, fasting blood tests, a ¹H-magnetic resonance spectroscopy (MRS) and a liver biopsy were performed. Participants were allocated to either placebo (three times daily) or resveratrol (500 mg three times daily) treatment in a 1:1 ratio. After 2 months, patients were seen at a follow-up visit (V2). Here, general condition, compliance and adverse events were assessed by interview, and anthropometric measurements, questionnaires and blood samples were collected. At 4 months' follow-up, blood samples for

markers of liver injury and drug safety were taken (V4). After 6 months of resveratrol treatment, participants were admitted for end-of-trial tests (V6), including anthropometric measurements, blood tests, questionnaires, a MRS and a liver biopsy.

Biochemistry

Biochemical analyses were performed at the Department of Clinical Biochemistry, Aarhus University Hospital (details in Supplementary Information).

¹H-MR spectroscopy

The intrahepatic lipid (IHL) content was measured by MRS using a Signa Excite 1.5 tesla twin-speed scanner (GE Medical Systems, Waukesha, WI), and the spectra were quantified using the LC model software package version 6.2 (details in Supplementary Information).

Histology

Liver biopsy sections were evaluated in a blinded manner by two experienced pathologists and scored according to the criteria proposed by the NASH-Clinical Research Network.[11] Differentiation between NAFLD and NASH was performed, according to the FLIP algorithm.[12]

Questionnaires

All patients returned a detailed questionnaire on alcohol consumption at V0, V2 and V6. At V2 and V6, patients also committed to a 2-day food registration, a physical activity questionnaire and a structured questionnaire on adverse events (Supplementary Information).

PCR-based methods

Paired baseline and end-of-trial liver biopsies were available for GeneChip analysis in seven resveratrol-treated patients and nine placebo-treated patients. RNA isolation, cDNA synthesis and microarray analysis and quantitative RT-PCR (qRT-PCR) procedures were performed as specified in the Supplementary Information.

Microarray data were deposited in NCBI's Gene Expression Omnibus and are accessible through GEO accession number GSE6842.

Statistics and power calculation

Categorical data are summarised as frequencies (percentages) and continuous variables as means (\pm SD) for parametric data or medians (range) for non-parametric

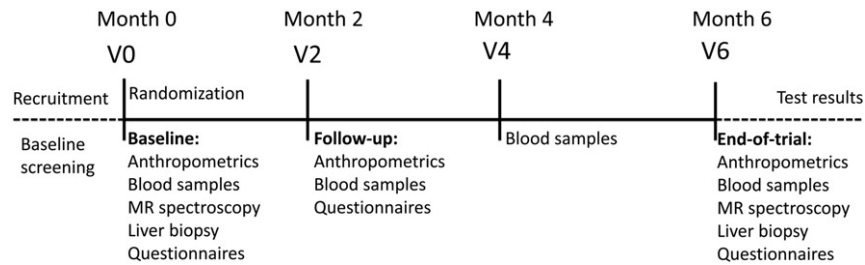


Figure 1. Clinical trial design.

data. Baseline characteristics and differences in treatment effects were compared using the unpaired *t*-test (parametric data), the Wilcoxon rank-sum test (non-parametric data) or the Fishers exact test/Pearson's χ^2 test (categorical data). Changes within each group over the study period were evaluated using the paired *t*-test or the Wilcoxon signed-rank test. The level of statistical significance was set at $p < 0.05$ (two-sided).

Microarray and qRT-PCR data concordance was checked by pairwise correlation and two-way plots. Differential gene expression analysis was performed on transcript values using paired *t*-tests, and using a Benjamini-Hochberg correction of significance level, unless otherwise stated.

Power calculation was based on change in plasma ALT. To detect an ALT difference of 25 U/l ($SD \pm 30$) at a two-sided 0.05 significance level with a power of 0.80, 23 participants had to be included in each group. We therefore planned to include 46 patients, plus two patients to account for attrition. However, patient inclusion was slower than anticipated and prematurely completed after 34 months with a total of 28 patients. This resulted in an actual power for detecting an ALT difference of 0.60, *ceteris paribus*.

Results

Patient inclusion, compliance and study completion

Of the 28 allocated patients, 26 completed the intervention. Of these 26 study participants, seven completed all investigations except the follow-up biopsy (Figure 2). Two resveratrol-treated patients left the study because of adverse events in the first weeks of treatment. Due to ethical concerns, these patients were not included in an intention-to-treat analysis. Patient compliance with study treatment was high (details in Supplementary Information and Table S2).

Baseline characteristics

Baseline characteristics are displayed in Table 1, which generally reveals overall homogeneity comparing the

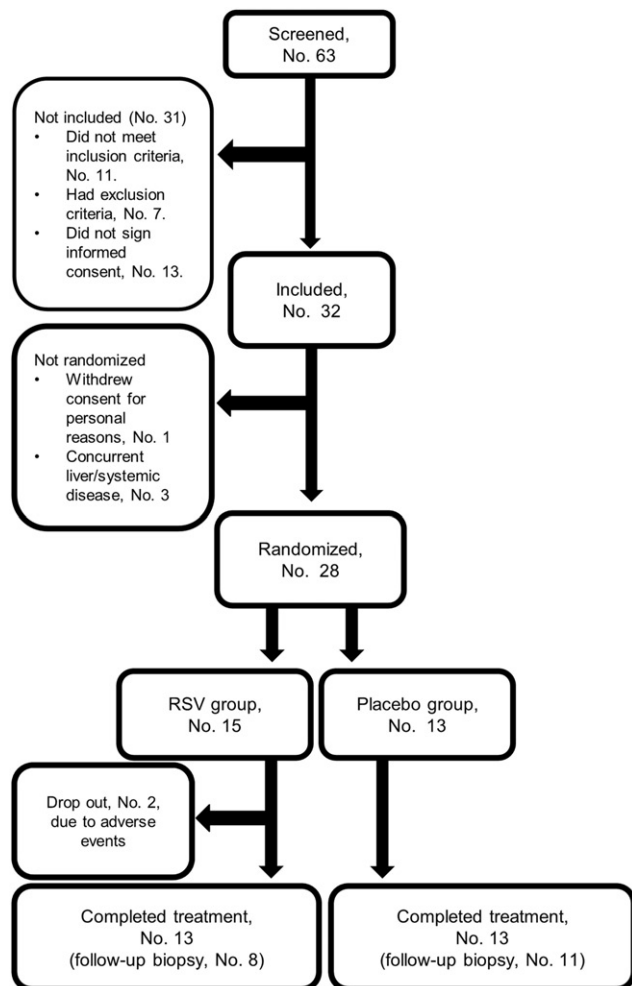


Figure 2. Trial flow diagram.

resveratrol and placebo group. Being part of the inclusion criteria, ALT levels were elevated to median 103 (56–235 U/l) for men and 73 (28–148 U/l) for women. Importantly, patients had liver steatosis as determined by MRS; IHL averaging 29% with a large range (12.6–41.3%). This was also reflected in the histological analysis which showed a median NAS of 4 (1–6), and NASH was present in 12/26 participants (Supplemental Table S4). No patients had fibrosis scores of more than 1a. Moreover, there was no difference in alcohol consumption, caloric intake or exercise expenditure between the study groups

Table 1. Baseline and end-of-trial data for resveratrol and placebo group with within-group and between group comparisons.

	Resveratrol group (no. 13)		Placebo group (no. 13)		Between group difference, <i>p</i> -value	
	Baseline	End-of-trial	<i>p</i> -value ^a	Baseline		End-of-trial
Markers of liver damage – median (range)						
ALT, U/l	104 (56–225)	74 (35–179)	0.049*	86 (28–235)	72 (27–160)	0.514
AST, U/l	50 (25–235)	37 (25–100)	0.045*	45 (25–111)	41 (20–98)	0.176
ALP, U/l	84 (42–146)	86 (48–172)	0.917	82 (48–175)	74 (49–157)	0.603
GGT, U/l	90 (23–214)	68.5 (16–208)	0.075	55 (27–222)	60 (24–448)	0.029*
Bilirubin, µmol/l	9 (5–18)	8 (4–17)	0.525	7 (5–16)	10 (6–12)	0.075
Serum TNFα, µg/l	1.12 (0.83–2.96)	1.11 (0.56–1.95)	0.130	1.09 (0.56–1.95)	1.29 (0.52–2.26)	0.078
CD163, mg/l	2.3 (1.6–6.2)	2.5 (1.9–5.5)	0.677	2.3 (1.0–7.4)	2.7 (1.3–8.5)	0.765
MR spectroscopy – mean (±SD)						
Intrahepatic lipid, %	27.6% (±9.0)	23.8% (±9.7)	0.026*	29.7% (±5.6)	27.7% (±8.8)	0.376
Anthropometric measurements – mean (±SD)						
BMI, kg/m ²	32.1 (±3.1)	31.6 (±3.0)	0.221	32.0 (±5.4)	32.0 (±5.6)	0.844
Weight, kg	97.1 (±11.7)	95.6 (±10.7)	0.254	94.7 (±16.0)	94.7 (±16.1)	0.911
Waist-hip ratio	0.997 (±0.080)	0.999 (±0.070)	0.589	1.002 (±0.074)	1.000 (±0.073)	0.715
Systolic BP, mmHg	142 (±15)	137 (±16)	0.260	136 (±15)	140 (±13)	0.330
Diastolic BP, mmHg	89 (±8)	85 (±10)	0.106	79 (±8)	84 (±9)	0.081
Heart rate, bpm	72 (±9)	69 (±10)	0.467	66 (±10)	67 (±10)	0.864
Insulin resistance and lipid profile						
Glucose, mmol/l ^b	6.1 (±0.5)	6.0 (±0.4)	0.289	5.8 (±0.6)	6.0 (±0.6)	0.250
Insulin, pmol/l ^b	96 (±42)	91 (±38)	0.296	105 (±65)	101 (±70)	0.967
HOMA2-IR ^b	1.85 (±0.81)	1.77 (±0.73)	0.311	2.02 (±1.05)	1.93 (±1.32)	0.935
Mean glucose, mmol/l ^c	6.2 (5.5–7.4)	6.1 (5.5–7.7)	0.746	5.6 (5.3–6.4)	5.9 (5.2–6.6)	0.642
TG, mmol/l ^c	2.0 (1.1–3.7)	2.0 (0.9–3.9)	0.824	1.5 (0.7–3.0)	1.7 (0.7–4.9)	0.035*
HDL, mmol/l ^c	1.0 (0.72–1.9)	1.0 (0.82–1.6)	0.847	1.2 (0.8–1.7)	1.1 (0.83–1.9)	0.725
LDL, mmol/l ^c	3.2 (1.8–6.3)	3.4 (1.8–6.3)	0.922	2.4 (1.7–7.4)	2.8 (1.5–5.6)	0.640

ALP: alkaline phosphatase; BMI: body mass index; BP: blood pressure; bpm: beats per minute; GGT: γ -glutamyl transferase; HDL: high density lipoprotein; HOMA2-IR: insulin resistance as determined by homeostatic model assessment 2; LDL: low density lipoprotein; TG: plasma triglyceride.

Bold font indicates trend difference ($p < 0.1$). **Bold*** indicates statistical difference ($p < 0.05$). ^aIndicates within-group comparison. ^bIndicates value in mean (±SD). ^cIndicates value in median (range).

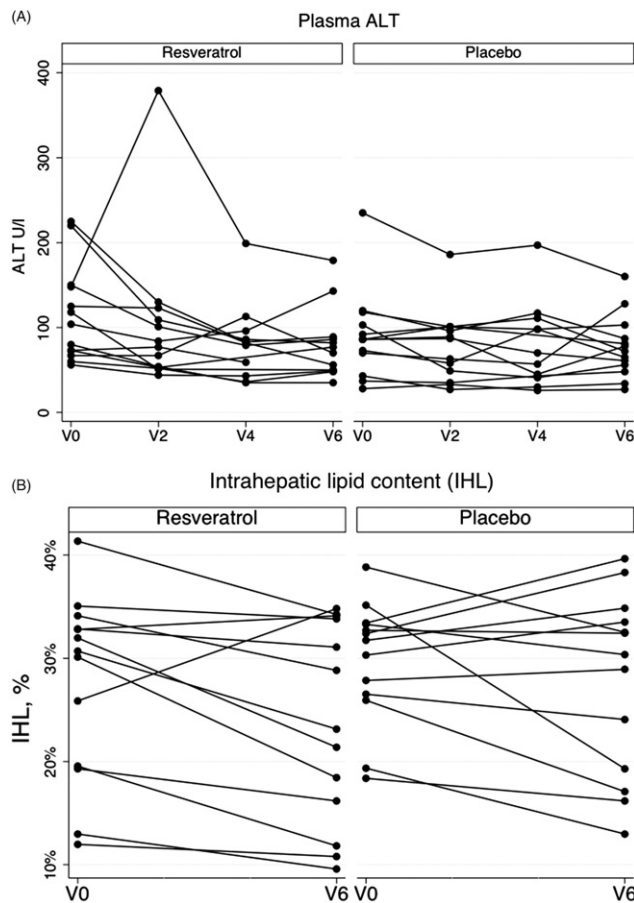


Figure 3. Study endpoints. (A) Change in plasma ALT levels. (B) Change in intrahepatic lipid content (IHL), determined by MR spectroscopy. V0, baseline visit; V2, 2 months' follow-up; V4, 4 months' follow-up; V6, end-of-trial visit.

and the two genders were evenly distributed (Supplemental Table S2).

Markers of liver injury

ALT was significantly reduced in the resveratrol group ($p=0.049$, Table 1 and Figure 3A). The placebo group also showed a reduction in ALT ($p=0.054$), and resveratrol was not superior to placebo ($p=0.51$). Similar patterns were seen for AST and for γ -glutamyl transferase (GGT), whereas alkaline phosphatase (ALP) was elevated in the resveratrol group within the first 2 months of treatment (Supplemental Table S3). Finally, we tested the change in serum CD163 and $\text{TNF}\alpha$, which are markers of histological NAFLD severity and inflammation [13], finding no significant change.

Effect of resveratrol on intrahepatic lipid content and histology

The resveratrol group showed a significant 3.8% ($\pm 5.4\%$) reduction in IHL content ($p=0.026$, Figure 3B and

Table 1). However, the placebo group also had a minor IHL reduction, and resveratrol treatment was not superior to placebo ($p=0.38$). In addition, histological changes did not differ between study groups with regard to steatosis, inflammation, ballooning or fibrosis (data not shown).

Markers of the metabolic syndrome

We found no differences in the change of weight, BMI or waist-hip ratio in the two study groups (Table 1). In plasma/serum, we found no long-term differences in the development of fasting glucose, insulin, HOMA index or lipids (Table 1).

Microarray and PCR data

Global transcriptional changes caused by resveratrol were determined by exploratory microarray analyses. When using a corrected significance level, we identified no change in gene expression in the resveratrol group. When adapting an uncorrected significance level of $p=0.05$ with a fold change in expression in resveratrol samples of ≤ 0.7 or ≥ 1.3 , we identified 18 genes and 16 unnamed transcript clusters that were regulated by resveratrol treatment (Supplemental Table S5). Overall, no genes commonly associated with resveratrol bioactivity or NAFLD pathogenesis were beneficially affected by resveratrol treatment.

Quantitative RT-PCR confirmed no change in gene expression of a number of genes relevant to NAFLD pathogenesis and markers of AMPK or SIRT1 activity (Figure 4 and Supplemental Figure S1).

Tolerability and adverse events

A description of general tolerability is found in the Supplementary Information and Supplemental Tables S6 and S7. Two resveratrol patients left the study due to adverse events: a case of gastrointestinal side-effects and a serious case of febrile leukopenia and thrombocytopenia after 10 days of resveratrol treatment. Fever and lymphopenia recurred upon repeated exposure to resveratrol.

Discussion

In this placebo-controlled trial, a daily dose of resveratrol 1.5 g for 6 months resulted in limited clinical improvements in patients with biopsy-verified NAFLD and NASH. Compared to placebo, resveratrol had no effect on ALT or IHL levels, though we observed minor reductions in both parameters and other biochemical markers of hepatic injury. The ALT reduction

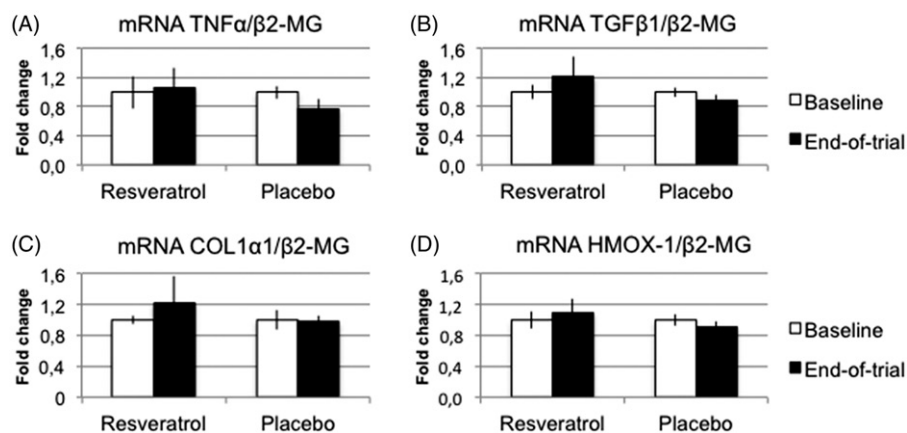


Figure 4. Baseline and end-of-trial quantitative RT-PCR data on liver tissue. (A) TNF α (*TNF*); marker of hepatic inflammation. (B–C) Transforming growth factor β 1 (*TGF β 1*) and collagen 1 α 1 (*COL1A1*); markers of hepatic fibrogenesis. (D) heme oxygenase-1 (*HMOX1*); marker of antioxidant pathway activity, associated with resveratrol bioactivity.

may partly be explained by a regression-toward-the-mean effect, which is supported by a strong correlation between baseline ALT and ALT reduction ($r = -0.73$, $p < 0.01$). Inflammatory markers, serum TNF α and CD163, were unchanged and there was no effect on histological endpoints. Further, resveratrol was not associated with any long-term improvements in insulin sensitivity or other markers of the metabolic syndrome, and there were no major transcriptional changes either as detected by microarray analysis or by targeted qRT-PCR.

The strengths of the present resveratrol trial include the randomised design and the inclusion of biopsy material. This is the first study to report the effects of resveratrol on global and targeted mRNA expression in human hepatic NAFLD tissue.

As evident from baseline clinical and histological characteristics, the participants comprised a wide-spectrum, non-diabetic and non-cirrhotic NAFLD cohort. This makes our findings easily generalisable to the common clinical ambulatory setting, excluding patients with severe NASH fibrosis and incident diabetes.

The major limitation of the study is the small sample size. We did not meet the target number of participants, which limits the use of exploratory subgroup analyses and increases the risk of neglecting a true therapeutic effect of resveratrol (type II error). As we found a minor reduction of ALT, we can speculate that superiority to placebo would appear in a large-scale trial. However, the study holds substantial power to detect a difference in IHL reduction, which did not materialise. Our findings therefore suggest that effect size on NAFLD is less pronounced than expected after numerous reports of beneficial effects in experimental studies.[10] Though this is the longest high-dose resveratrol trial to date, our conclusions are also limited by the relatively short study

duration, which is too short to test resveratrol effects on histological fibrosis.

The use of ALT as a surrogate marker of NAFLD is debatable, though studies have shown that an ALT reduction is associated with a decrease in hepatocyte injury, hepatic inflammation,[14] but not steatosis.[15] There are no other commonly established non-invasive biomarkers for use in clinical NAFLD/NASH trials, and consequently, reduction in ALT was chosen as the primary endpoint for this study. To assist the primary endpoint ALT, we have chosen a number of supportive endpoints, and primary and supportive endpoints were consistent.

The present results confirm our recent experimental findings, where high-dose resveratrol treatment had only limited effect in a rat model with established NASH.[16] Similarly, other recent clinical resveratrol trials have reported ambiguous results. The studies have all been placebo-controlled trials of limited sample size (11–60 participants, total No. 165), short duration (1–3 months), different resveratrol doses (150–3000 mg daily) and endpoints.[13] Steatosis as determined by MRS or ultrasound was reduced by resveratrol in two [9,17] out of six [18–21] of the previous trials. Similarly, aminotransferases and other markers of liver inflammation and injury were reduced in some,[9,17,21] though not in other trials.[18–20] The lack of effect on most features of the metabolic syndrome is also noteworthy and in agreement with most other recent trials.[18,19,22,23]

The challenges of reproducing the beneficial effects of resveratrol in a clinical setting may result from a number of causes, including low resveratrol bioavailability and yet uncertain effects of resveratrol metabolites.[24,25] Although effects on our primary and secondary endpoints were limited, we have indicators of resveratrol bioactivity. The transient decrease in systolic BP in the

resveratrol group marks resveratrol bioactivity (Supplemental Table S3),[26] and similarly, the early increase in plasma ALP level (with no increase in GGT) matches previous reports of a resveratrol-mediated increase in bone-specific ALP.[27] Our results therefore imply that in spite of resveratrol bioactivity, clinical NAFLD was not notably improved.

This study provides the first data on global transcriptional activity in human liver tissue during resveratrol treatment and we did not identify any major modifications. Also, qRT-PCR did not show any of the anticipated beneficial transcriptional changes in resveratrol-treated patients; e.g. a reduction of *TNF* or *COL1A1* expression. In support of these GeneChip findings, Yoshino et al. reported no major resveratrol-mediated effect on expression patterns in muscle and adipose tissue of non-obese women.[20] In contrast, Timmers et al. found an impact on biological pathways linked to mitochondrial function and inflammation in muscle of obese men,[9] which confirmed the results of rodent studies.[28,29] Currently, we have no explanation for the difference in study findings and we are not able to validate our transcriptional findings by protein analysis, which is a limitation of the study. Indeed, our results do not exclude post-transcriptional modulation of protein expression though the lack of clinical effect argues against this.

We confirmed that NAFLD patients tolerated high-dose, long-term resveratrol treatment fairly well,[30] although the occurrence of two severe adverse events highlights the continued need for focussing on the possible harms of high-dose resveratrol treatment.

In this placebo-controlled trial, high-dose resveratrol treatment had only limited therapeutic effect on clinical or histological NAFLD and on the associated metabolic aberrations. No transcriptional changes were observed. Although resveratrol treatment was associated with minor reductions in transaminases and IHL that may prove significant in larger and longer clinical trials, our findings suggest a limited effect-size only.

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Declaration of interest

The authors declare no conflict of interest.

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