Resveratrol

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Resveratrol

a booster of mitochondria

Marlies de Ligt

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RESVERATROL

A BOOSTER OF MITOCHONDRIA

DISSERTATION

to obtain the degree of doctor at the Maastricht University,
on the authority of the Rector Magnificus,
Prof. dr. Rianne M. Letschert,
in accordance with the decision of the Board of Deans,
to be defended in public on
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by

Marlies de Ligt

Born in Boskoop, The Netherlands, on February 22nd, 1989

Promotors:

Prof. dr. P. Schrauwen

Co-promotors:

Dr. V.B. Schrauwen-Hinderling

Dr. E. Phielix

Assessment committee:

Prof. dr. J. Plat (chairman)

Prof. dr. R. Witkamp (Wageningen University)

Dr. R. Houtkooper (AMC Amsterdam)

Prof. dr. N. Schaper

Prof. dr. A. Schols

*to my parents
for their infinite support*

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CHAPTER 1

General Introduction

GENERAL INTRODUCTION

Over the past decades, the average life expectancy of humans has been gradually increasing. Better nutrition and health care are fundamental factors for this increase in longevity [1]. Although life expectancy is increasing, the prevalence of non-communicable diseases is also rapidly increasing. For example, obesity is becoming a growing health problem all over the world [2]. In turn, this rise in obesity rates is accompanied by an increase in chronic metabolic diseases, such as Type 2 Diabetes Mellitus (T2D) [3]. Therefore, research focus is shifting towards the development of novel therapies that maintain metabolic health even in the presence of obesity.

Type 2 Diabetes Mellitus and Insulin Resistance

Obesity has reached epidemic proportions over the past few decades, and by now affects all ages and socioeconomic groups among the world [4]. The underlying cause is an overall change in lifestyle, leading to a misbalance between energy intake and energy utilization. Obesity is associated with several comorbid conditions, such as non-alcoholic fatty liver disease, hypertriglyceridemia, hypercholesterolemia, hypertension and T2D [5]. Thus, obesity has a great impact on morbidity and mortality rates. A major characteristic of obesity is excessive fat accumulation in adipose tissue, but a fraction of the fat is also stored in non-adipose tissue, the so-called ectopic fat depots [6]. Accumulation of fat in ectopic depots, such as the liver and skeletal muscle, is associated with insulin resistance and decreased muscle mitochondrial fatty acid oxidative capacity [7, 8].

Insulin resistance is the key factor in the development of T2D [9]. Insulin resistance, and accompanying disruptions in glucose homeostasis, can over time lead to damage and dysfunction of several organs such as the eyes, kidneys, nerves, blood vessels and heart [10]. Thus, T2D is a serious chronic disease that arises when several tissues, primarily the liver, skeletal muscle and adipocytes, do not respond properly anymore to the insulin the body produces. Normally, insulin promotes the uptake of glucose in the peripheral tissues and suppresses hepatic glucose production and hepatic glycogenolysis upon feeding [11]. Thus, with decreasing insulin sensitivity insulin production by the pancreatic β -cells increases as a compensatory mechanism to maintain glucose levels. When the insulin production becomes insufficient to compensate for the increased requirements, plasma glucose levels will gradually rise. Ultimately, this can lead to β -cell failure and thereby reduced insulin production and a further rise of plasma glucose levels. Although β -cell failure is crucial for the development of T2D, skeletal muscle insulin resistance is also considered to be a primary defect that is evident in early stages of the development towards T2D [12, 13]. Besides the skeletal muscle, also the liver plays a pivotal role in the pathogenesis of T2D. In the case of hepatic insulin resistance, glucose release by the liver is not suppressed sufficient by insulin, further exacerbating peripheral insulin resistance [14]. Beside decreased insulin sensitivity, decreased metabolic flexibility is also regarded as an early hallmark of diabetes.

Metabolic flexibility is defined as the capacity to switch from predominately fat oxidation in the fasted state, to carbohydrate oxidation upon feeding [15]. Reduced metabolic flexibility results in delayed postprandial glucose clearance and thereby contributes to disturbances in glucose homeostasis.

Mitochondrial Function

The most important roles of mitochondria are production of energy in the form of adenosine triphosphate (ATP), and regulation of cellular metabolism. Considering the pivotal role of mitochondria in cellular energy metabolism, it is not surprising that impairments in mitochondrial function are associated with insulin resistance and metabolic diseases such as T2D [16]. For example, patients with T2D and people at risk of developing T2D are characterised by a decreased mitochondrial fatty acid oxidative capacity [17], show mitochondria smaller in size and number [18, 19], and show decreased levels of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) gene expression [20] in skeletal muscle. PGC-1α is a key regulator of mitochondrial metabolism and biogenesis and expression is linked to insulin resistance [20]. Lifestyle modifications, including calorie restriction and exercise training, are the most effective strategies for targeting mitochondrial function and decrease the risk for developing T2D. For example, six months of caloric restriction in young overweight men led to an increase in muscle mitochondrial biogenesis accompanied by an increase in insulin sensitivity, and expression of silent information regulator 2 (SIRT1) which is a regulator of PGC-1α activity [21, 22]. This indicates interventions that target mitochondrial function could be effective strategies to improve insulin sensitivity and thereby prevent and treat T2D. Unfortunately, adherence to lifestyle interventions programs has proven disappointing on the long term, and for the major part of the population such rigorous lifestyle changes are not realisable. Therefore, alternative treatments which can improve mitochondrial function, and thereby metabolic health, are warranted. A promising candidate with respect to this is resveratrol.

Resveratrol

Resveratrol (*trans*-, 3,5,4'-trihydroxystilbene), is a natural occurring polyphenol present in among others red wine. Interest in resveratrol increased tremendously after it was identified as a small molecule activator of SIRT1 [23]. SIRT1 is a molecule linked to energy metabolism and plays an important role in regulating mitochondrial biogenesis [24]. In addition, further research has shown that besides SIRT1, AMP-activated protein kinase (AMPK) is also a key target of resveratrol [25, 26]. Exercise and calorie restriction are also known activators of both SIRT1 and AMPK [24]. AMPK and SIRT1 have been described to directly affect PGC-1α activity through phosphorylation and deacetylation, respectively [24]. Implying, through stimulation of the AMPK-SIRT1-PGC-1α pathway, resveratrol can have stimulatory effects on mitochondrial biogenesis and fatty acid oxidation [27] (Figure 1).

Figure 1. Proposed working mechanism of resveratrol.

Two interrelated mechanisms of action: 1) Resveratrol directly activates SIRT1, which leads to deacetylation of PGC-1α and LKB1, and activation of AMPK. 2) Resveratrol directly or indirectly activates AMPK, leading to activation of PGC-1α by phosphorylation. In both proposed mechanisms, the subsequent increase in NAD⁺ levels likely contribute to a positive feedback cycle.

The potential of resveratrol as metabolic health enhancer, has been extensively studied in cell and animal studies [28]. Overall, the results of pre-clinical studies have been promising and these findings have created high hopes for the therapeutic value of resveratrol in prevention and treatment of metabolic diseases [25, 26, 29-34]. However, results from human clinical trials are contradictory, potentially due to differences in study set-up with varying doses of resveratrol and treatment duration [27]. This indicates studies need to be performed unravelling which dose is most effective, what treatment duration should be applied, and clinical trials with positive outcomes should be repeated to confirm reproducibility.

Carnitine

Carnitine is essential for maintaining normal mitochondrial function. Carnitines are trimethylated amino acids that can be synthesised by the human body, and can also be derived from food items such as red meat, fish and dairy products [35]. Recently it became apparent that the capacity to form acetylcarnitines is vital for preserving metabolic flexibility, and thereby metabolic health in general [36-39]. Acetylcarnitines are generated in the mitochondria by the conjugation of acetyl-CoA and free carnitine [39] (Figure 2). The formation of acetylcarnitines can thereby buffer acetyl-CoA, and prevent its accumulation in the mitochondria. This is crucial for sustaining the glycolytic flux, as acetyl-CoA is a potent inhibitor of pyruvate dehydrogenase [40]. In addition, acetyl-CoA accumulation can lead to increased acetylation of many enzymes, thereby modulating their activity and influencing metabolism at many levels [36]. Since free carnitine is essential for buffering acetyl-CoA, decreased carnitine availability may be underlying the development of metabolic disturbances. Animal studies seem to confirm this hypothesis [38, 41], but human clinical trials investigating this principle are lacking. Supplying carnitine as a food supplement could potentially be a novel strategy to improve metabolic flexibility and thereby insulin sensitivity.

Figure 2. Buffering mechanism of carnitine, preventing accumulation of acetyl-coA and thereby preserving metabolic flexibility.

Thesis Outline

The aim of this thesis is to investigate if the food supplements resveratrol and carnitine can improve metabolic health in overweight and obese individuals. The focus is on insulin sensitivity, mitochondrial function, ectopic fat accumulation and substrate utilisation. To explore this, a review was written, four human clinical trials were performed and a combined data analysis was conducted.

Chapter 2 reviews animal studies and human clinical trials that investigated the potential of resveratrol in alleviating obesity-induced metabolic disturbances. In **chapter 3** the effects of resveratrol supplementation on insulin sensitivity and mitochondrial function are explored in patients with T2D, where resveratrol was administered on top of their regular treatment for diabetes. Patients received 30 days of resveratrol and 30 days of placebo, and thereby served as their own controls. **Chapter 4** focusses on prevention of T2D; in this study men at increased risk of developing T2D were supplied for 30 days with resveratrol and for 30 days with placebo. The study set-up was the same as for the patients with T2D, with the emphasis on insulin sensitivity and mitochondrial function, to verify effects in an insulin resistant population without glucose-lowering medication. In addition, effects on brown adipose tissue were also taken into account. Activation of brown adipose tissue has been associated with positive metabolic health effects and animal studies have indicated that resveratrol could stimulate brown adipose tissue. So far, these effects had not been investigated in humans yet. In **Chapter 5** is investigated which participant characteristics predict effectiveness of resveratrol. To this extend, the data of the two human clinical trials of chapter 3 and 4 is combined and analysed, together with a previously performed clinical trial by our group with the food supplement resveratrol in healthy obese men. In these three studies the same study set-up was applied in different participant populations,

namely: healthy obese men, patients with T2D (see also chapter 3) and men at risk of developing T2D (see also chapter 4). In this chapter is explored why resveratrol is effective in some, but not all individuals. **Chapter 6** is designed to investigate the effects of long-term treatment with resveratrol. To this end, a mixed group of overweight men and women received resveratrol or placebo for 6 months. At the beginning and end of the intervention period general parameters of metabolic health were assessed. Chapter 6 displays an interim-analyses of the results. Next to resveratrol, other promising food supplements are described in literature. Therefore, in **Chapter 7** is investigated if the food supplement L-carnitine can improve insulin sensitivity and metabolic flexibility. Overweight men and women with disrupted glucose homeostasis received carnitine supplementation and placebo both for a period of 36 days. A group of individuals with normal glucose homeostasis was added as a control group. Finally, in **Chapter 8** the major results of the different studies are summarised, and the results are debated in a broader perspective.

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CHAPTER 2

Resveratrol and obesity:
can resveratrol relieve metabolic disturbances?

Marlies de Ligt, Silvie Timmers and Patrick Schrauwen

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ABSTRACT

There is an increasing need for novel preventive and therapeutic strategies to combat obesity and related metabolic disorders. In this respect, the natural polyphenol resveratrol has attracted significant interest. Animal studies indicate that resveratrol mimics the effects of calorie restriction via activation of sirtuin 1 (SIRT1). SIRT1 is an important player in the regulation of cellular energy homeostasis and mitochondrial biogenesis. Rodent studies have shown beneficial effects of resveratrol supplementation on mitochondrial function, glucose metabolism, body composition and liver fat accumulation. However, confirmation of these beneficial effects in humans by placebo-controlled clinical trials remains relatively limited. This review will give an overview of pre-clinical and clinical studies examining the effects of resveratrol on obesity-induced negative health outcomes.

INTRODUCTION

The prevalence of obesity is increasing tremendously worldwide. A recent systematic analysis for the Global Burden of Disease Study reported a worldwide increase in overweight and obesity between 1980 and 2013 from 28.8% to 36.9% in men and 29.8% to 38.0% in women [1]. Obesity presents a health risk, partly due to ectopic fat accumulation; fat accumulation in non-adipose tissue such as liver and skeletal muscle. Accumulation of fat in the liver, when unrelated to alcohol intake, is a strong independent marker of dyslipidaemia and insulin resistance. Insulin resistance in turn predisposes to the development of type 2 diabetes (T2D) [2-4]. Impaired mitochondrial function is also often seen in obese and/or T2D patients [5]. Mitochondria play a central role in energy homeostasis and substrate metabolism. Therefore, reduced mitochondrial function has substantial effects on glucose and lipid metabolism, deteriorating metabolic health.

The rise in obesity prevalence is predominantly caused by changes in lifestyle, such as decreased physical activity and increased intake of energy-dense food. The primary solution for this obesity epidemic, and its related negative effects on public health, should therefore also be sought in changing lifestyle. Increasing the amount of physical activity or decreasing energy intake are proven effective therapeutic strategies to positively influence health outcomes related to obesity. Correspondingly, restricting calorie intake for six months leads to an improvement in insulin sensitivity [6], which in turn is accompanied by an increase in muscle mitochondrial biogenesis [7]. However, people in general have difficulties following strict exercise training or dieting regimes. Alternative treatments are therefore highly sought after. This has led to the search for compounds that can initiate beneficial health effects similar to those from exercise training or calorie restriction.

Resveratrol

Resveratrol (3, 5, 4' trihydroxystilbene) is a polyphenol naturally present in and produced by several plants. The richest source of natural resveratrol is *Polygonum cuspidatum*, a plant known from traditional Chinese and Japanese medicine [8]. Smaller amounts of resveratrol can also be found in peanuts, grapes, red wine and mulberries [9]. In 2003, Howitz et al. [9] identified resveratrol as a small-molecule activator of sirtuin 1 (SIRT1). SIRT1, like all members of the sirtuin family, requires nicotinamide adenine dinucleotide (NAD⁺) for its deacetylating activity [10]. The dependence of SIRT1 on NAD⁺ strongly links its activity to cellular energy levels. SIRT1 is induced both by calorie restriction and exercise [11] and plays an important role in the regulation of lipid and glucose homeostasis [12]. The fact that SIRT1 is closely connected to cellular energy levels and energy homeostasis makes it an interesting molecular target for treatment of metabolic disorders such as obesity. Considering resveratrol has been identified as a small-molecule activator of SIRT1, it is not surprising that resveratrol has been said to have calorie restriction-like effects [13-16]. However, there is debate whether resveratrol activates SIRT1 directly [9, 17,

18] or rather via activation of AMP-activated protein kinase (AMPK) [19, 20]. AMPK and SIRT1 both play a crucial role in energy homeostasis and their activity is closely interrelated. It is therefore difficult to identify whether resveratrol activates SIRT1 or AMPK or both, either direct or indirect. Recently, Park et al. [21] proposed that the metabolic effects of resveratrol might result from competitive inhibition of cAMP-degrading phosphodiesterases, leading to elevated cAMP levels. Consequently, through a cascade of effects, this could lead to activation of AMPK, followed by an increase in NAD⁺ and finally an increase in SIRT1 activity [21]. Unfortunately, the exact mechanism is still unknown. Despite the mechanism of action, resveratrol is a promising candidate for treatment and prevention of metabolic diseases by mimicking calorie restriction-like effects.

The aim of this review is to evaluate the potential effects of resveratrol on obesity-related health outcomes mainly in humans, both in an experimental and clinical setting (see table 1 for an overview of published peer-reviewed clinical trials on resveratrol and obesity-related health outcomes). Studies that used grape extract containing resveratrol or other formulas with multiple components are not taken into account. Effects of resveratrol on mitochondrial function, body composition, energy expenditure, insulin resistance and liver fat accumulation will be evaluated.

Effects of Resveratrol on Muscle Mitochondrial Function

Excessive energy intake and a low level of physical activity will lead to accumulation of fat in adipose tissue. In turn, this excessive fat accumulation can lead to lipid overflow and accumulation of fat in non-adipose tissue, such as the liver and skeletal muscle [22, 23]. In general, muscle fat accumulation correlates negatively with insulin sensitivity [24] especially when mitochondrial fat oxidative capacity is low. Indeed, T2D patients and people at high risk of developing T2D are characterised by high intramyocellular lipid levels and a decreased mitochondrial fatty acid oxidative capacity [25, 26]. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is a crucial regulator of mitochondrial metabolism and biogenesis, and a downstream effector of the AMPK-SIRT1 signalling pathway [27]. Several studies have reported a reduction in PGC-1α gene expression in T2D patients [28, 29]. This disrupted gene expression pattern can also be seen in non-diabetic offspring of T2D patients [29]. It has been suggested that a low PGC-1α gene expression can lead to reduced generation of mitochondrial proteins, resulting in loss of mitochondrial capacity and decreased insulin sensitivity [29]. Exercise training and calorie restriction are proven effective strategies to improve muscle mitochondrial oxidative capacity, paralleled by improvements in whole body insulin sensitivity [7, 30, 31]. Thus, six months of calorie restriction has been shown to increase expression of AMPK, PGC-1α and SIRT1, increase mitochondrial DNA content and reduce fasting insulin levels [7]. Therefore, improving muscle oxidative capacity appears to be an effective strategy for counteracting obesity-induced insulin resistance and T2D.

A couple of animal studies have actually investigated the effects of resveratrol on muscle mitochondrial capacity. A rodent study by Lagouge et al. [32] included four different intervention groups: male C57BL/6J mice on a high-fat diet (HFD) with or without a dose of 400 mg/kg/day (mpk) of resveratrol or on a chow diet with or without resveratrol (400 mpk). The intervention period was 15 weeks. They found that mitochondria in non-oxidative muscle fibres of resveratrol-treated HFD mice were larger and denser, and mitochondrial DNA content increased compared to HFD animals that did not receive resveratrol. Um et al. [20], who also treated male C57BL/6J mice while on a HFD with 400 mpk resveratrol, reported increased mitochondrial content (measured by cytochrome C protein levels and mitochondrial DNA) compared to no resveratrol-treatment after an intervention period of 13 weeks. In addition, they measured a decrease in the content of the fatty acid intermediates diacylglyceride and ceramide in skeletal muscle. To further investigate mitochondrial activity, both studies measured PGC-1 α expression in skeletal muscle. PGC-1 α mRNA [20, 32] and protein [32] significantly increased in resveratrol-treated animals. Additionally, resveratrol treatment increased physical endurance of mice, as evidenced by increased running time [20, 32]. A recent study by Price et al. [17], using two different doses of resveratrol (25-30 mpk and 215-300 mpk), also found beneficial effects of resveratrol on mitochondrial biogenesis and function. The mice were fed a HFD of a standard diet for eight months. The HFD led to significantly impaired function of mitochondria isolated from skeletal muscle. Supplementation with either of the two resveratrol doses for eight months prevented the HFD-induced mitochondrial dysfunction. Thus, increases were measured compared to HFD animals without resveratrol supplementation in: ADP-stimulated respiration (state 3), FCCP-induced maximal oxidative respiration (state u), mitochondrial membrane potential, and cellular ATP levels. These levels were comparable to mice fed a standard diet. In addition, treatment with resveratrol resulted in a fibre type switch towards more oxidative muscle fibre types, and prevented the HFD-induced decline in mitochondrial content (measured by citrate synthase activity and by mitochondrial DNA content). Interestingly, when SIRT1 knockout mice were used none of the above-mentioned effects of resveratrol were observed. Moreover, SIRT1 overexpression resulted in similar effects as resveratrol treatment in wild type mice. The authors therefore concluded that SIRT1 plays a crucial role in improving mitochondrial function by resveratrol supplementation (25-30 mpk). Chen et al. [33] performed a study with male Sprague Dawley rats fed a normal diet, HFD or HFD with resveratrol (100 mpk by intragastric administration) and found results comparable to Price et al. [17]. Hence, resveratrol-treatment increased SIRT1 activity and mitochondrial biogenesis, compared to a HFD without resveratrol. Furthermore, resveratrol reverted the decline in subsarcolemmal mitochondrial citrate synthase and electron transport chain activities and decreased IMCL content. Pearson et al. [34] investigated effects of resveratrol on muscle mitochondrial function in a non-obese animal model. One-year old male C57BL/6NIA mice received a chow diet, both diets with and without resveratrol added (~ 30.9 mpk). An additional group of mice were fed every-other-day, which is a form of calorie restriction. They concluded

Table 1. Summary of peer-reviewed clinical trials in the field of obesity.

Author, year, country [ref]	Population (n)	Design	Dose of resveratrol	Duration	Objective	Outcome
Bhatt et al. 2012, India [43]	T2D men and women on oral hypoglycaemic treatment (57)	Randomised, prospective, open-label control trial	250 mg once daily	3 mo	Glycaemic control and associated risk factors	Resveratrol significantly improved mean HbA1c, systolic blood pressure, total cholesterol and total protein. No changes in body weight and high-density lipoprotein and low-density lipoprotein cholesterol.
Brasnyo et al. 2011, Hungary [59]	T2D men on oral glucose lowering medication (19)	Randomised, placebo-controlled, double-blind parallel design	5 mg twice daily	4 wks	Insulin sensitivity and oxidative stress	Resveratrol significantly decreased insulin resistance (measured by HOMA-IR) and urinary ortho-tyrosine excretion (as a measure of oxidative stress), while it increased the pAkt:Akt ratio in platelets.
Chachay et al. 2014, Australia [55]	Overweight/obese men with NAFLD (20)	Randomised, placebo-controlled parallel design	3000 mg once daily	8 wks	Insulin resistance, hepatic steatosis and abdominal fat distribution	Resveratrol did not reduce insulin resistance (measured by a hyperinsulinemic euglycemic clamp), steatosis, or abdominal fat distribution. No change was observed in plasma lipids or antioxidant activity. Levels of ALT and AST increased significantly among patients in the resveratrol group until week 6
Crandall et al. 2012, USA [44]	Elderly men and women with impaired glucose tolerance (10)	Open-label study	1, 1.5 or 2 g once daily	4 wks	Glucose metabolism and vascular function	Resveratrol (1.5 and 2 g) decreased peak glucose and 3-h glucose AUC following a meal, and improved Matsuda Index for insulin sensitivity. Weight, blood pressure and lipids were unchanged. There was a trend toward improved post-meal reactive hyperaemia index
Dash et al. 2013, Canada [56]	Overweight/obese men with mild hyper-triglycercaemia (8)	Randomised, placebo-controlled, double-blind crossover study	1000 mg daily the first wk, 2000 mg daily the second wk	2 wks	Intestinal and hepatic lipoprotein turnover	Resveratrol did not significantly affect insulin sensitivity (measured by HOMA-IR), fasting or fed plasma triglyceride concentration. Reduction apoB-48 production without significant effect on fractional catabolic rate. Resveratrol reduced apoB-100 and fractional catabolic rate.
Elliott et al. 2009 [58]	T2D patients		2.5 or 5 g once daily	28 d	Insulin sensitivity	Resveratrol (5 g) decreased fasting and postprandial glucose and insulin

Magyar et al. 2012, Hungary [57]	Male and female patients with stable coronary artery disease (40)	Randomised, placebo-controlled, double-blind trial	10 mg once daily	3 mo	Cardio protective effects	Resveratrol improved left ventricle diastolic function, endothelial function, lowered LDL-cholesterol level and protected against unfavourable hemorheological changes. No significant difference in HbA1c.
Movahed et al. 2013, Iran [46]	T2D men and women on oral hypoglycaemic treatment (66)	Randomised, placebo-controlled, double-blinded parallel clinical trial	500 mg twice daily	45 d	Anti-hyperglycemic effects	Resveratrol significantly decreased systolic blood pressure, fasting blood glucose, HbA1c, insulin, and insulin resistance (measured by HOMA-IR), while HDL was significantly increased. Liver and kidney function markers were unchanged
Poulsen et al. 2013, Denmark [45]	Healthy obese men (24)	Randomised, placebo-controlled, double-blind parallel design. Resveratrol	500 mg thrice daily	4 wks	Metabolic effects	Resveratrol did not change insulin sensitivity (measured by a hyperinsulinemic euglycemic clamp), blood pressure, resting energy expenditure, lipid oxidation rates, ectopic or visceral fat content, or inflammatory or metabolic biomarkers
Timmers et al. 2011, The Netherlands [13]	Healthy obese men (11)	Randomised, placebo-controlled, double-blind crossover design	75 mg twice daily	30 d	Metabolic effects	Resveratrol improved metabolic profile: resveratrol reduced sleeping and resting metabolic rate. In muscle resveratrol activated the AMPK-SIRT1-PGC1α axis. A reduction in blood glucose and insulin levels and liver fat content were measured, and improved muscle mitochondrial function and reduced inflammation markers in the blood.
Yoshino et al. 2012, USA [15]	Non-obese post-menopausal woman with normal glucose tolerance (29)	Randomised, placebo-controlled, double-blind parallel design	75 mg once daily	12 wks	Metabolic effects	Resveratrol did not change resting metabolic rate, body composition, inflammatory markers or plasma lipids. Resveratrol did not increase liver, skeletal muscle, or adipose tissue insulin sensitivity (measured by a hyperinsulinemic euglycemic clamp). Resveratrol did not affect AMPK, SIRT1, NAMPT, or PGC1α in either skeletal muscle or adipose tissue.

ref, reference; T2D, type 2 diabetes mellitus; wks, weeks; mo, months; d, days; NAFLD, non-alcoholic fatty liver disease

that resveratrol shifts muscle mitochondrial gene expression patterns in mice on a standard diet toward those on a calorie restriction diet.

Together, these findings from animal studies indicate that resveratrol can influence mitochondrial biogenesis via activation of the AMPK-SIRT1-PGC-1 α axis. This has led to the generally accepted idea that improving mitochondrial function by resveratrol supplementation is a promising strategy for improving metabolic health in humans. In accordance with findings from rodent studies, we have demonstrated in a previous clinical trial that resveratrol-treatment leads to activation of AMPK, increases SIRT1 and PGC-1 α protein levels and increases citrate synthase activity [13]. Thus, Timmers et al. [13] studied 11 obese but otherwise healthy males receiving a dose of 150 mg resveratrol or placebo per day for 30 days, in a double-blind cross over design. No difference was found in mitochondrial content, in contrast to animal studies [20, 32]. However, muscle mitochondrial fatty acid oxidative capacity was improved on a fatty acid-derived substrate (state 3 respiration), as determined by increased mitochondrial respiration. In contrast to data from animal studies, IMCL content in the vastus lateralis muscle was increased upon resveratrol-treatment. Combining the improvement in muscle fat oxidative capacity, increased IMCL content and other beneficial metabolic adaptations found in this study, the hypothesis emerged that resveratrol-treatment could have an endurance training-like effect [14, 30]. One other human intervention investigated the effect of resveratrol on mitochondrial function, although indirectly by examining gene expression pathways related to mitochondrial function [15]. Thus, Yoshino et al. [15] studied non-obese postmenopausal women with normal glucose tolerance. Fifteen women received resveratrol (75 mg per day) and fourteen women received placebo, for a period of 12 weeks. Contradicting Timmers et al. [13], they concluded that resveratrol did not have an effect on the phosphorylation of AMPK or SIRT1 gene expression in skeletal muscle. It must be noted that the dose used by Timmers et al. [13] was double the dose Yoshino et al. [15] used. Furthermore, our trial included obese men, implying that resveratrol could have beneficial effects on people with compromised health but not on healthy non-obese humans. Unfortunately, no other data from human interventions are currently available regarding the effect of resveratrol on muscle mitochondrial function.

Effect of Resveratrol on Body Composition and Energy Expenditure

Rodent studies suggest that high doses of resveratrol (~400 mpk) can lead to a reduction in weight gain when animals are fed a HFD [32, 35]. Two rodent studies by Lagouge et al. [32] and Kim et al. [35] both used a similar set-up with male C57BL/BJ mice and a resveratrol dose of 400 mpk. The study by Kim et al. [35] included three different mice groups: HFD with or without resveratrol or chow diet without resveratrol, with an intervention period of 10 weeks. Both studies were consistent in reporting a blunting of body mass gain in the HFD-resveratrol group compared to the HFD group. The blunted weight gain was accounted for by a decrease in visceral fat-pad weights and smaller adipocytes in epididymal adipose tissue [32, 35]. The beneficial

effect of resveratrol on body mass and composition could not be attributed to decreased food intake, since both resveratrol-treated and non resveratrol-treated animals consumed an equal amount of calories. Lagouge et al. [32] measured energy expenditure of the mice by indirect calorimetry and recorded a higher basal energy expenditure (EE) accompanied by a decrease in locomotor activity. This suggests that resveratrol had a stimulating effect on energy expenditure. The latter results are in accordance with those found in a non-human primate model of obesity [36]. Six male grey mouse lemurs received a dose of ~200 mpk resveratrol for four weeks, during their winter body-mass gain period. Supplementation of resveratrol led to an increase in resting EE of 29% and a reduction in body-mass gain, compared to the control period. Contrary to the rodent studies, the grey mouse lemurs decreased their food intake upon resveratrol-treatment, thereby contributing to the decrease in weight gain. As a follow-up of this short-term non-human primate study a larger group of animals received the supplements (~200 mpk) for one year [37]. Overall, there were no differences in body weight, food intake or physical activity between resveratrol and control animals. Yet, at the beginning of the so-called long day period (spring/summer period) an increase in fat free mass was noted in the resveratrol-supplemented lemurs, which was accompanied by an increase in total daily EE and resting EE. Overall, findings from animal studies suggest that resveratrol could stimulate energy expenditure and protect against HFD-induced weight gain.

To further examine how resveratrol might increase energy expenditure, Lagouge et al. [32] examined thermogenesis during a cold test with the focus on brown adipose tissue (BAT). In mice the main contributor to heat production is BAT, which in contrast to white adipose tissue stores little fat and burns fat to produce heat and regulate body temperature; nonshivering thermogenesis [38]. It was found that mitochondria were significantly larger and also mitochondrial DNA content was increased in BAT of resveratrol-treated mice [32]. These changes were accompanied by an increase in gene expression of SIRT1, a decrease in PGC-1 α acetylation and an increase in PGC-1 α activity. These findings suggest that resveratrol may affect BAT metabolism. Accordingly, a significant increase in uncoupling protein 1 (UCP1) and SIRT1 gene expression in BAT was found in male mice treated with resveratrol for eight weeks (400 mpk) [39] and in Sprague-Dawley rats treated for six weeks (30 mpk) [40]. Also, gene expression of PGC-1 α and peroxisome proliferator-activated receptor β/δ (PPAR β/δ) was significantly higher in BAT of the resveratrol-supplemented rats. Bone Morphogenetic Protein 7 (BMP7) could also be involved in the effect of resveratrol on BAT, since BMP7 is a crucial factor in brown adipogenesis and 2 months of resveratrol-treatment (400 mpk) was shown to increase BMP7 expression in BAT of mice [39]. Previous studies demonstrated that BMP7 promotes brown pre-adipocyte differentiation [41]. In vitro treatment of brown pre-adipocytes with BMP7 induced PGC-1 α and increased gene expression of UCP1 and PPAR γ [41]. In addition, when gene expression of BMP7 was stimulated in vivo, this resulted in a significant increase in BAT mass accompanied by an increase in energy expenditure and a reduction in weight gain [41]. Taken together, the data

illustrate that resveratrol, via activation of SIRT1, UCP1 and potentially BMP7, could influence brown adipocyte differentiation. This in turn could lead to increased energy expenditure and eventually weight loss. Whether similar effects of resveratrol are present in humans unfortunately remains unclear. It is difficult to investigate molecular aspects of BAT metabolism in humans since BAT is located in regions of the human body that are difficult to reach. However, future human studies could investigate the effect of resveratrol on BAT activity using PET-CT scanning [42].

So far, no effect of resveratrol has been found in human trials concerning body weight [13, 15, 43-46] or body composition [15, 45]. It is however important to note that in the human studies performed so far, participants were generally instructed to consume their usual diet and were not challenged with a HFD or high-calorie diet (HCD) in contrast to the animal studies. Moreover, in human interventions, relatively low doses of resveratrol have been used ranging from 75 to 2000 mg/d. In that respect, it is interesting to mention that also in rodents a relatively low dose of resveratrol (~22.4 mpk) does not lead to a decrease in body weight [19]. In addition, the possibility cannot be excluded that metabolism of resveratrol differs between humans and animals. The duration of the resveratrol supplementation could also have been too short in the human intervention studies performed so far. Strikingly, we have previously demonstrated that 30 days of resveratrol supplementation (150 mg/day) leads to a *reduction* in energy expenditure in healthy obese males [13]. Thus, in sharp contrast to animal studies, resveratrol supplementation resulted in a lower sleeping metabolic rate as well as a lower postprandial energy expenditure [13]. Long-term studies in humans are needed to provide definitive answers as to whether resveratrol can affect energy expenditure or body composition in humans.

Effects of Resveratrol on Insulin Sensitivity

Obesity is one of the major determinants of insulin resistance and T2D. As described earlier in this review, improving muscle oxidative capacity can counteract obesity-induced insulin resistance [30]. Rodent studies have demonstrated that resveratrol can have beneficial effects on glucose homeostasis in animal models of obesity, diabetes and metabolic dysfunction [19, 20, 32, 35, 47-52]. These studies used variable doses of resveratrol, ranging from 2.5 to 400 mpk. In addition, the exposition time also varied from between 2 weeks and 16 months. Lagouge et al. [32] investigated mice on a HFD with or without resveratrol supplementation (400 mpk) for 15 weeks. A significant reduction in fasting insulin levels was found in resveratrol-treated animals, compared to HFD animals without resveratrol supplementation. This decrease in insulin was not accompanied by alterations in fasting glucose levels. In addition, insulin sensitivity was assessed by the hyperinsulinemic euglycemic clamp technique, which is regarded as the gold standard for measuring insulin sensitivity. Improved insulin sensitivity was found in the resveratrol group compared to the HFD group without resveratrol. Shang et al. [48] also used the hyperinsulinemic euglycemic clamp technique to determine insulin sensitivity. Resveratrol treatment (100 mpk for 10 weeks) in HFD-fed male Wistar rats resulted in a higher glucose infusion rate accompanied

by reduced fasting plasma insulin levels, compared to HFD animals that did not receive resveratrol. Sun et al. [52] used a relatively low dose of resveratrol, namely 2.5 mpk for 16 weeks, and found that resveratrol treatment significantly improved glucose tolerance (as determined by a glucose tolerance test) in HFD-fed male C57BL/6J mice with glucose disposal curves becoming comparable to mice fed a chow diet. In the same study, resveratrol administration also considerably improved insulin sensitivity as determined by an insulin tolerance test. These findings indicate that resveratrol has the potency to improve insulin sensitivity in diet-induced obesity in rodents, and that these effects are independent of dose and exposition time. The effect of resveratrol on insulin sensitivity has also been tested on non-human primates. Hence, Marchal et al. [53] performed a study with grey mouse lemurs fed a standard diet with or without resveratrol (200 mpk). Insulin sensitivity was determined with the oral glucose tolerance test and the homeostasis model assessment of insulin resistance (HOMA-IR index). They concluded that 33 months of resveratrol supplementation improved glucose tolerance and thereby affected insulin sensitivity without changes in basal insulin secretion. Jimenez-Gomez et al. [54] used male adult rhesus monkeys in their 2-year trial. Animals were randomised in one of three groups: high-fat high-sugar diet (HFS) with resveratrol (n=10), HFS without resveratrol (n=10) or a standard diet (n=4). The dose of resveratrol was 40 mg twice a day for the first year, and 240 mg twice a day during the second intervention year. Resveratrol supplementation for two years increased SIRT1 protein expression, decreased adipocyte size, and improved insulin sensitivity in visceral but not subcutaneous white adipose tissue. Regarding insulin sensitivity, an increase of insulin receptor substrate 1 protein levels, a decrease of Akt serine 473 phosphorylation, and an increase in the content of insulin-responsive GLUT4 glucose transporter was measured in visceral fat depots from fasted rhesus monkeys on a HFS diet supplemented with resveratrol as compared to animals on a HFS diet without resveratrol supplementation. These findings from animal studies led to the hypothesis that resveratrol could be a promising anti-diabetic agent for humans.

So far, eleven clinical trials have investigated effects of resveratrol on glucose homeostasis and insulin sensitivity. Three of these studies used the gold-standard hyperinsulinemic euglycemic clamp technique to determine insulin sensitivity [15, 45, 55]. Thus, Poulsen et al. [45] supplemented healthy obese men, in a parallel-group design, with a daily dose of 1500 mg resveratrol (n=12) or placebo (n=12) for four weeks. Subjects consumed the resveratrol supplements in doses of 500 mg three times a day. Insulin sensitivity was measured by hyperinsulinemic euglycemic clamp in conjunction with ³H-labelled glucose tracer infusion. No changes upon resveratrol supplementation were observed in endogenous glucose production, oxidative glucose disposal or non-oxidative glucose disposal. Yoshino et al. [15] also did not find a difference in insulin sensitivity in non-obese postmenopausal woman who received 75 mg resveratrol per day. Twelve weeks of resveratrol supplementation did not induce changes in liver, skeletal muscle or adipose tissue insulin sensitivity, as measured by the hyperinsulinemic euglycemic clamp in combination with stable isotope labelled tracer infusion. The third study, by Chachay et al.

[55], included 20 overweight or obese men diagnosed with non-alcoholic fatty liver disease (NAFLD). The participants were randomly assigned to groups given 3000 mg of resveratrol ($n = 10$) or placebo ($n = 10$) daily for eight weeks. Also in this study resveratrol administration did not improve insulin sensitivity, as measured by the clamp technique.

Studies that used other measures of glucose homeostasis or insulin sensitivity are also inconsistent in effects of resveratrol. Timmers et al. [13] did find positive metabolic changes in healthy obese men ($n=11$) supplemented with resveratrol. Thirty days of resveratrol supplementation significantly reduced blood glucose and insulin levels and improved the HOMA-IR index compared to the placebo condition. Crandall et al. [44] found beneficial effects of resveratrol in overweight and obese men and woman with impaired glucose tolerance. Ten subjects were enrolled in this 4-week open-label study. Resveratrol-treatment for four weeks, with either 1.5 or 2 g per day, led to decreased peak glucose and 3-hour glucose area under the curve following a meal. Furthermore, both 1.5 and 2 g of daily resveratrol supplementation led to an improvement of the Matsuda index, representing improved insulin sensitivity. It must be noted that this study did not include a placebo condition. Dash et al. [56], who investigated overweight or obese men with mild hypertriglyceridemia, did not observe differences in fasting plasma glucose or insulin levels. The participants ($n=8$) were studied in two experimental conditions: after treatment with resveratrol and after treatment with placebo in a randomized, double-blinded, crossover design. During the resveratrol condition participants received a daily dose of 1000 mg resveratrol for one week, followed by a daily dose of 2000 mg resveratrol during the second week. No significant improvement in HOMA-IR index was observed. One study investigated effects of resveratrol on HbA1c, in patients with stable coronary artery disease [57]. In total 26 male and 14 female patients were enrolled, receiving either 10 mg resveratrol per day ($n=20$) or a placebo ($n=20$) for three months. No significant change in HbA1c was found after three months of resveratrol-treatment compared to baseline. Unfortunately systemic conversion of resveratrol was not measured, so no statements can be made as to whether the low dose used (10 mg) was high enough to evoke an observable increase in resveratrol in blood plasma. Furthermore, the patients received medical therapy including platelet aggregation inhibitors, β -blockers, ACE-inhibitors and statins, which could have influenced the efficacy of resveratrol. Contrary to the previously mentioned studies, studies in T2D patients (without insulin treatment) have been more consistent in reporting beneficial effects of resveratrol supplementation on blood glucose levels [46, 58, 59], insulin levels [46, 58], insulin resistance (defined by HOMA-IR index) [46, 59] and HbA1c [43, 46]. Hence, Elliott et al. [58] measured a decrease in fasting and postprandial glucose and insulin after a daily administration of 5 g of resveratrol for 28 days to T2D patients. Brasnyó et al. [59] performed a double-blind, placebo-controlled trial with male T2D patients supplemented with a dose of two times 5 mg resveratrol per day ($n=10$) or placebo ($n=9$) for four weeks. Mean changes in insulin sensitivity (defined by HOMA-IR index), blood glucose and insulin were compared between the placebo and resveratrol group. Resveratrol

significantly decreased insulin sensitivity (defined by HOMA-IR index) and decreased blood glucose levels in T2D patients on standard oral glucose lowering medication. No difference was found in serum insulin levels between the resveratrol and placebo groups at any time during the trial. Bhatt et al. [43] investigated the effects of resveratrol on HbA1c in T2D patients. Fifty-seven T2D patients, males and females combined, received a daily dose of 250 mg resveratrol (n=28) or received no interventional-treatment (n=29). The results revealed that three months of resveratrol supplementation significantly improved average HbA1c in T2D patients on oral hypoglycemic treatment (metformin and/or glibenclamide) compared to their baseline values. A limitation of this study is the lack of a placebo group. The fourth clinical trial in T2D patients was executed by Movahed et al. [46]. A total of 64 T2D patients, consisting of both males and females on standard diabetic treatment, completed this randomised placebo-controlled double-blinded parallel clinical trial. The intervention group was supplemented with resveratrol at a dose of 500 mg twice a day (n=33), and the control group (n=31) received placebo tablets both for a period of 45 days. Resveratrol-treatment significantly decreased fasting blood glucose, HbA1c, insulin and insulin sensitivity (defined by HOMA-IR index), whereas in the placebo group fasting glucose levels increased slightly compared to their baseline values.

To conclude, most rodent studies show a decrease in plasma glucose [19, 35, 47, 49, 51], a decrease in plasma insulin [19, 32, 35, 47-49] or improved insulin sensitivity/glucose tolerance [20, 32, 48, 50, 52] upon resveratrol administration. Clinical trials in T2D patients imply an anti-diabetic effect of resveratrol, but studies in non-diabetic patients are less consistent. Studies in T2D patients investigating effects of resveratrol on insulin sensitivity by means of the gold-standard hyperinsulinemic euglycemic clamp technique are highly demanded.

Effect of Resveratrol on Liver Fat Accumulation

Excessive body weight and obesity can exert negative metabolic health effects partly via accumulation of fat in the liver [60]. Indeed, the increase in obesity prevalence is accompanied by an increase in NAFLD. NAFLD itself does not explicitly evoke a risk to health but when inflammation occurs and NAFLD progresses into non-alcoholic steatohepatitis, it strongly correlates with morbidity and mortality rates [61]. It has been suggested that resveratrol can decrease liver fat accumulation through activation of AMPK and/or SIRT1. In the liver, deletion of SIRT1 leads to impaired signalling of the lipid-sensing transcription factor PPAR α resulting in decreased β -oxidation [62]. In addition, knockdown of hepatic SIRT1 results in inhibition of PGC-1 α leading to increased levels of hepatic free fatty acids [63]. Sterol regulatory element-binding protein 1c (SREBP-1c) is a transcription factor that regulates the expression of genes related to triglyceride and fatty acid synthesis [64, 65]. SIRT1 down-regulates SREBP-1c expression in the liver, leading to a decrease in lipid synthesis and fat storage [64, 65]. These findings lead to the idea that resveratrol could stimulate hepatic fatty acid oxidation through AMPK/SIRT1-mediated activation of PGC-1 α and PPAR α and inhibition of SREBP-1c.

Baur et al. [19] investigated resveratrol supplementation at a dose of ~22.4 mpk in one-year old male C57BL/6NIA mice. The mice received a HCD with or without resveratrol or a standard diet, for 6 months. It was found that resveratrol prevented an increase in size and weight of the liver as seen in the HCD group. In addition, histological staining showed that accumulation of large lipid droplets in the liver was present in the HCD group but not in the group on a HCD with resveratrol. The livers of the HCD resveratrol-treated mice also had substantially more mitochondria than those of HCD animals, and were not significantly different from those in the standard diet group. These findings were accompanied by increased phosphorylation of AMPK and decreased gene expression of fatty acid synthase in the liver. In a separate cohort of the one-year-old mice on the HCD, which had been treated with a dose of 186 mpk resveratrol for six weeks, acetylation of PGC-1 α was significantly lower, indicating increased activity, in resveratrol-fed animals compared to the diet matched controls. Shang et al. [48] performed a rat study with comparable results. Male Wistar rats fed a HFD developed abdominal obesity, NAFLD and insulin resistance, which was considerably improved by 10 weeks of resveratrol supplementation (100 mpk). Again, resveratrol-treatment promoted phosphorylation of AMPK in the liver, which in turn suppressed SREBP-1c and fatty acid synthase gene expression. Another rat study found that a low dose of resveratrol (30 mpk for six weeks) led to a decreased accumulation of fat in the liver and activated AMPK and PGC-1 α in male Sprague-Dawley rats [66]. However, they did not find a difference in mRNA expression of SREBP-1c, PPAR α , SIRT1 or PGC-1 α . Poulsen et al. [67] detected that male Wistar rats treated with 100 mg resveratrol daily for eight weeks increased hepatic mitochondrial content when on a HFD. In addition, hepatic uncoupling protein 2 (UCP2) gene expression was significantly increased compared to both control and HFD-fed animals, suggesting that a resveratrol-induced increase in mitochondrial number and UCP2 gene expression may contribute to normalising liver fat content in HFD-induced liver fat accumulation. Recently, Heebøll et al. [68] further reviewed the effects of resveratrol on experimental and clinical NAFLD. They concluded that rodent studies demonstrate a consistent decrease in cholesterol accumulation [35, 69-74], hepatic triglycerides [35, 47, 66, 69, 70, 72, 75-79] and liver weight [19, 48, 70, 73, 76, 77]. Also, several other improvements in liver fat content and anatomy were reported in rodents after resveratrol supplementation [19, 48-50, 67, 69-72, 74, 76-82]. For example, improvements were noted regarding steatosis, steatohepatitis and fibrosis. Taken together, resveratrol in rodents is at least partly able to prevent liver fat accumulation induced by HFD or HCD, probably via increasing fatty acid oxidation and decreasing lipogenesis. These effects could be mediated by activation of the AMPK-SIRT1 axis.

To date, only four clinical trials have been performed investigating effects of resveratrol on liver fat accumulation. One of these studies specifically focussed on patients suffering from NAFLD, whilst the other three studies involved healthy participants. In the study by Chachay et al. [55] 10 NAFLD patients received a daily dose of 3000 mg resveratrol and another group of 10

NAFLD patients received a placebo. One of the study outcomes was hepatic steatosis assessed by magnetic resonance spectroscopy (MRS) and imaging (MRI). Chachay et al. [55] concluded that eight weeks resveratrol administration did not significantly improve any features of NAFLD, compared with placebo. Furthermore, based on increases in liver enzymes ALT and AST, they concluded that there was an increase in hepatic stress in the resveratrol group. Timmers et al. [13] used a much lower dose of resveratrol for a shorter period of time: 150 mg/d, for 30 days. The outcome of this study was that resveratrol supplementation led to a reduction in liver fat in healthy obese males, measured by MRS. In addition, a significant reduction in liver transaminases (ALT) was found suggesting beneficial effects of resveratrol on liver function. In contrast, Poulsen et al. [45], who also investigated healthy obese males, did not find effects of resveratrol (1500 mg/d) on liver fat content (measured by MRS) or liver transaminase levels after four weeks of supplementation. The fourth clinical trial, in non-obese postmenopausal women, also did not find significant differences in liver fat (measured by MRS) after 12 weeks of supplementation (75 mg/d) [15]. Taken together; although rodent studies have generally found a lowering effect of resveratrol on liver fat content, human studies have so far been less consistent. Again, differences in doses used or duration of resveratrol treatment may explain differences between human studies so far, and more clinical studies are required.

2

DISCUSSION AND FUTURE STUDIES

The effect of resveratrol on metabolic health has received much attention in the last decade. Pre-clinical studies have revealed promising results regarding beneficial effects of resveratrol on preventing and reversing obesity-induced metabolic disturbances. Specifically, beneficial effects have been observed in rodents supplemented with resveratrol with respect to mitochondrial function, insulin sensitivity and liver fat accumulation. These effects can be attributed to activation of the AMPK-SIRT1-PGC-1 α axis. Clinical trials performed so far have been less consistent. This inconsistency can most likely be explained by differences in duration and dose of resveratrol used between studies. Relative to animal studies, clinical trials used low doses of resveratrol. For example, a dose of 400 mpk is frequently used in rodents and would translate into a dose of 30 g resveratrol per day for an average human with a body weight of 75 kg. Despite using lower doses of resveratrol in human clinical trials, plasma resveratrol concentrations have been found comparable to those found in mice studies [13]. However, a direct comparison of circulating levels between animals and humans may be difficult as in vivo metabolism of resveratrol might differ between mice and humans. Human studies would be needed to determine the optimal dose of resveratrol to activate the AMPK-SIRT1 axis. In fact only few human studies so far have determined if resveratrol administration indeed resulted in activation of the AMPK-SIRT1 axis, leaving the possibility that suboptimal doses have been used so far. In addition, long-term studies in humans are needed to investigate the possibility of

cumulative effects of resveratrol on metabolic health. Finally, it is important to note that effects of resveratrol are mainly observed in animals fed a HFD or HCD diet, implying that resveratrol is particularly potent in reversing early stage metabolic disorders. The same could apply to humans, which is supported by the fact that so far only clinical trials in overweight/obese participants [13, 44] or T2D patients [43, 46, 58, 59] have found beneficial effects.

An interesting new venue for future research would be a focus on human BAT and so-called browning of white adipose tissue. In contrast to white adipose tissue, BAT stores little fat and instead burns fat to produce heat and regulate body temperature. This feature of BAT led to the hypothesis that stimulating BAT thermogenesis could initiate weight loss [83]. A few animal studies have been performed investigating potential effects of resveratrol on BAT thermogenesis and these studies were overall encouraging, indicating enhanced mitochondrial biogenesis [32], induction of UCP1 [32, 39, 40] and activation of BMP7 [40] (a promoter of brown preadipocyte differentiation) in BAT. Given the interest in the physiology of human BAT, it would be interesting to investigate if resveratrol can also activate BAT in humans.

To conclude, even though data from animal studies look promising, currently the number of clinical trials is too limited to make any firm statements regarding the effects of resveratrol on obesity-induced negative health outcomes in humans. More specifically, future research should primarily focus on dose, efficacy and chronic exposure effects of resveratrol. Finally, human intervention studies are necessary focusing on the potential of resveratrol to stimulate BAT thermogenesis.

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CHAPTER 3

Resveratrol as add-on therapy in subjects with well-controlled type 2 diabetes: a randomized controlled trial

Marlies de Ligt, Silvie Timmers, Esther Phielix, Tineke van de Weijer, Jan Hansen, Esther Moonen-Kornips, Gert Schaart, Iris Kunz, Matthijs K.C. Hesselink, Vera B. Schrauwen-Hinderling and Patrick Schrauwen

M.d.L and S.T. contributed equally to this work

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ABSTRACT

Methods

To determine whether resveratrol supplementation can improve insulin sensitivity and promote overall metabolic health on top of standard diabetes care.

Research design and methods

17 well-controlled subjects with type 2 diabetes (T2D) were treated with placebo and 150 mg/day resveratrol (resVida™) in a randomized double-blind crossover study for 30 days. The main outcome measure was insulin sensitivity by the hyperinsulinemic-euglycemic clamp technique.

Results

Hepatic and peripheral insulin sensitivity were not affected by resveratrol treatment. Intrahepatic lipid content also remained unaffected by resveratrol, however the change in intrahepatic lipid content correlated negatively with plasma resveratrol levels ($R=-0.68$, $p=0.03$). Intramyocellular lipid content increased in type 2 muscle fibers ($p=0.03$) and systolic blood pressure tended to decrease ($p=0.09$) upon resveratrol treatment. In addition, resveratrol significantly improved *ex vivo* mitochondrial function (state 3 and state U respiration upon malate + octanoyl-carnitine, $p<0.005$). Intriguingly, a correlation was found between plasma levels of a metabolite of resveratrol (dihydroresveratrol) and the patients' metformin dose ($R=0.66$, $p=0.005$), suggesting interaction between metformin and resveratrol. It could be speculated that the lack of a resveratrol-induced insulin sensitizing effect is caused by this interaction.

Conclusions

Resveratrol supplementation does not improve hepatic or peripheral insulin sensitivity. Our results question the generalized value of resveratrol as add-on therapy in the treatment of T2D, and emphasize the need to perform studies in drug-naïve patients with T2D or pre-diabetic subjects.

INTRODUCTION

Exercise and calorie restriction [1] are the primary treatment options for type 2 diabetes (T2D). Both target the cellular energy-sensing route, with activation of AMP-activated protein kinase (AMPK) and the sirtuin (SIRT) family of transcription factors resulting in stimulation of mitochondrial biogenesis and function [2]. Nutraceutical compounds such as resveratrol can also target these pathways [3]. Interest in resveratrol peaked when Howitz and colleagues identified this polyphenolic compound as a potent SIRT1 activator [4]. Since then, resveratrol has been postulated to alleviate metabolic consequences of consumption of energy-dense foods and physical inactivity, including T2D [5, 6]. Indeed, animal studies suggest that resveratrol may blunt high-fat-diet-induced metabolic complications [3, 7-9]. These encouraging findings have stimulated the application of resveratrol in clinical trials to investigate its potency in humans with chronic metabolic diseases. Some [10, 11], but not all studies [12-14] in obese humans have reported positive effects of resveratrol on measures of insulin sensitivity. In contrast to studies in obese humans, studies in subjects with T2D have been more consistent in reporting beneficial effect of resveratrol on blood glucose levels [15-17], insulin levels [16, 17], markers of insulin resistance such as HOMA-IR index [15, 17], and HbA1c [17, 18]. However, none of these studies examined the effect of resveratrol on peripheral and hepatic insulin sensitivity by the gold-standard hyperinsulinemic-euglycemic clamp technique.

Hence, we performed a randomized, double-blinded, placebo-controlled crossover study to examine if 30 days of resveratrol supplementation leads to an improvement in peripheral and hepatic insulin sensitivity, in subjects with well-controlled T2D.

RESEARCH DESIGN AND METHODS

The study protocol was approved by the institutional Medical Ethical Committee and registered on ClinicalTrials.gov (NCT01638780). All study participants were informed about the nature and risks of the experimental procedures before their written informed consent was obtained.

Participants

Seventeen men (age 40-70 years, BMI 27-35 kg/m², body fat percentage >25%) with well-controlled T2D (HbA1c <8.0% (<64 mmol/mol)) participated in the study. Sixteen participants were treated with the oral glucose-lowering medication metformin, of whom six in combination with sulphonylurea derivatives (SUDs) (Supplementary Table 1). The majority of the participants received additional cholesterol-lowering medication (n=11) and/or blood pressure lowering medication (n=12). Exclusion criteria were unstable body weight (weight gain or loss >3 kg in the previous three months), engagement in programmed exercise >2 hours per week, impaired renal

and/or kidney function, intake of dietary supplements (except vitamins and minerals), alcohol consumption >20 g/day, diabetes co-morbidities and insulin treatment.

Outcomes

The primary outcome measure was the effect of resveratrol treatment on insulin sensitivity in comparison to placebo. Secondary outcome measures were: intrahepatic lipid content (IHL), intramyocellular lipids (IMCL), mitochondrial function (*in vivo* and *ex vivo*), blood pressure and cardiac function.

Clinical Study Design

The study was conducted at Maastricht University Medical Center, The Netherlands, between June 2012 and June 2014. In randomized order, participants underwent two experimental trials: a placebo and a resVida® (150 mg/day *trans*-resveratrol [99.9%; provided by DSM Nutritional Products Ltd.]) condition, with a washout period of at least 30 days. The dose of resveratrol was based on our previous study in healthy obese subjects in which we found activation of the energy-sensing pathway AMPK-SIRT1-PGC1 α in combination with metabolic improvements [10]. Randomization was carried out according to standard procedures as described in Statistical Methods, by William [19].

Participants were instructed to take the first supplement on the day of the baseline measurements (day 0) and the last supplement in the evening of day 29, and to abstain from food and beverages containing substantial amounts of resveratrol (e.g. wine, grapes, peanuts and berries) and from food supplements. In addition, instructions to maintain their normal living, activity and sleeping patterns were given. At the start (day 0) and end (day 30) of both intervention periods, blood samples were analyzed for general safety parameters (creatinine, AST, ALT, gamma-GT, bilirubin), blood pressure was measured after an overnight fast as previously described [10] and a 12-lead ECG was performed. Body fat percentage was determined by DXA on day 0 of the first intervention period for patient characterization. Each experimental trial lasted 30 days, and the participants came to the university on a weekly basis (days 0, 7, 14, 21 and 29). The weekly checkup took place in the morning in overnight fasted state and included body weight measurement and drawing of a blood sample for analysis of resveratrol (parental and metabolites) to confirm compliance to the protocol. On day 29 at 4PM, subjects came to the university for cardiac function measurements by M-mode, two-dimensional and Doppler echocardiography, using a Vivid 7 ultrasound system (GE Healthcare, Milwaukee, WI, USA) with 3.5 MHz cardiac transducer. Results were interpreted according to the criteria of the American Societies of Echocardiography. Subsequently, proton magnetic resonance spectroscopy (¹H-MRS) measurement of the liver took place to quantify IHL content, as described earlier [20], on a 3T whole-body scanner (Achieva Tx, Philips Healthcare, Best, The Netherlands) using an echo time of 32.5 ms. Spectra were fitted with a home-written script [21] in MATLAB R2014b (Mathworks, Natick, MA, USA). Values are

given as T2-corrected ratios [22] of the CH_2 peak, relative to the unsuppressed water resonance (as percentage). Hereafter, post-exercise phosphocreatine (PCr) recovery was assessed by ^{31}P -MRS to estimate *in vivo* mitochondrial function in vastus lateralis muscle on a 3T whole-body scanner (Achieva Tx, Philips Healthcare, Best, The Netherlands) as described elsewhere [21]. To standardize food intake before these measurements, subjects consumed a standardized lunch at a fixed time and afterwards stayed fasted until completion of the measurements.

After the MRS measurements, participants entered the respiration chamber for 12h starting at 7PM to allow measurement of sleeping metabolic rate (SMR) [23]. Before starting the respiration chamber measurement, a standardized evening meal (pasta Bolognese and fruit yogurt) was provided. The energy provided was based on individual daily energy requirements, calculated with the Harris and Benedict equation. In the morning of day 30, participants left the respiration chamber at 7AM in the overnight fasted state and a muscle biopsy was taken from the vastus lateralis muscle under local anesthesia (2% lidocaine), as previously described [24]. A portion of the muscle tissue was directly frozen in melting isopentane for immunohistochemistry and stored at -80°C until assayed. Another portion (~30 mg) was immediately placed in ice-cold preservation medium for determination of *ex vivo* mitochondrial respiration. From the muscle tissue in the preservation medium permeabilized muscle fibers were immediately prepared [24]. The permeabilized muscle fibers (~2.5 g wet weight) were analyzed for mitochondrial function using an oxygraph (OROBOROS Instruments, Innsbruck, Austria) [10]. Fresh cryosections (5 μm) were stained for IMCL by Oil Red O staining combined with fiber typing and immunolabeling of the basal membrane marker laminin to allow quantification of IMCL [25]. Mitochondrial DNA copy number, and protein expression of oxidative phosphorylation (OXPHOS) and peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC1 α) by western blot were performed according to standard procedures as described previously [10].

After taking the muscle biopsy, a two-step hyperinsulinemic-euglycemic clamp was performed to assess peripheral and hepatic insulin sensitivity. Three days before the clamp, subjects were instructed to refrain from strenuous activities, and to continue their anti-diabetic medication, with the last dose on the morning of the test. The clamp started by giving the subjects a primed-continues infusion of D-[6,6- $^2\text{H}_2$]glucose (0.04 mg/kg/min) to determine rates of endogenous glucose production (EGP), glucose appearance (R_a) and glucose disposal (R_d) rates [26]. After 120 minutes, low insulin infusion was started (10 mU/m ^{2} /min) for three hours after which high insulin infusion was started (40 mU/m ^{2} /min) for two hours. During the last 30 minutes of each insulin-infusion step (0, 10 and 40 mU/m ^{2} /min) blood samples were collected and substrate utilization was measured by indirect calorimetry. Steele's single pool non-steady state equations were used to calculate glucose R_a and R_d [27]. Volume of distribution was assumed to be 0.160 l/kg for glucose.

Compliance

To check compliance, resveratrol metabolites were measured by mass spectroscopy in plasma on days 0, 7, 14, 21 and 30 [10]. In addition, unused capsules were counted.

Sample Size

The sample size was determined based on demonstrating the statistical superiority of resveratrol on insulin stimulated glucose disposal in muscle compared to placebo treatment. A total of 14 subjects were estimated to be required to achieve 80% power, with an assumed treatment difference of 1.4 mg/kg FFM/min after 30 days and an assumed SD of 1.7 mg/kg FFM/min for a hyperinsulinemic clamp. A dropout of 20% was taken into account, so a total of 17 subjects were recruited. The expected effect size and SD of a hyperinsulinemic-euglycemic clamp was based on a previous study in our research group with a pre-and post-intervention clamp in subjects with T2D [28].

Statistical Analysis

Results are presented as means $\pm$ SEM when normally distributed, and median (95% CI) when this was not the case (using Shapiro-Wilk normality test). Student's paired t-test was used to compare placebo and resveratrol supplementation in normally distributed data; otherwise Wilcoxon signed-rank test was used. Treatment comparisons of plasma parameters measured at beginning and end intervention were assessed by two-way repeated measures ANOVA. Linear regression analyses were conducted to identify correlations between variables. On normally distributed data Pearson correlation was used, otherwise Spearman correlation was used. Potential carry-over effect between treatment and period was examined by unpaired t-test analyses according to Pocock et al. [29]. A p-value <0.05 was considered statistically significant. Analyses were performed using SPSS 22.0 for Mac OS X.

RESULTS

Study Design and Plasma Biochemistry

A total of 17 well-controlled male subjects with T2D participated in this study (baseline characteristics, Supplementary Table 1). To check compliance with the study protocol and to confirm systemic conversion of resveratrol to dihydroresveratrol (DHR), parental resveratrol and dihydroresveratrol (aglycone + glucuronide conjugates) levels were determined on a weekly basis in the plasma. While no resveratrol or DHR could be detected during the placebo treatment, both compounds were present in the plasma of all resveratrol-supplemented subjects. Plasma levels were 378.59 ± 40.65 ng/ml for parental resveratrol and 444.69 ± 101.81 ng/ml for DHR at day 30 of the resveratrol treatment. These levels are representative for the whole study period.

In addition, no carry-over effect was found between treatment and period for the primary study outcome, confirming the washout period was sufficient.

Insulin Sensitivity and Substrate Kinetics assessed by Hyperinsulinemic-euglycemic Clamp

Plasma glucose profiles during the hyperinsulinemic-euglycemic clamp procedure were similar for both the placebo and the resveratrol condition (Fig. 1A). Both during the low and high-insulin state, glucose levels were clamped ~ 5 mmol/l (Fig. 1A). Insulin-stimulated glucose disposal, expressed as the change in R_d from basal to either low or high-insulin state, was not significantly changed by resveratrol supplementation (Fig. 1B, $p_{\text{basal}}=0.65$, $p_{\text{low insulin}}=0.89$, $p_{\text{high insulin}}=0.66$). Also, basal EGP, reflecting hepatic glucose output, and EGP under low-insulin stimulated conditions were similar between both conditions, suggesting that hepatic insulin sensitivity was unchanged (Fig. 1C, $p_{\text{basal}}=0.22$). In fact, EGP was similarly suppressed during the low-insulin state of the hyperinsulinemic-euglycemic clamp (Fig. 1C, $p_{\text{low insulin}}=0.84$, $p_{\text{high insulin}}=0.74$), and EGP suppression was nearly complete upon high-insulin infusion in both conditions (Fig. 1D, $p_{\text{low insulin}}=0.64$, $p_{\text{high insulin}}=0.73$). In line with the absence of resveratrol-induced changes in R_d and EGP, non-oxidative glucose disposal (NOGD) also remained unaffected by 30 days of resveratrol supplementation (Supplementary Table 2). In addition, resveratrol did not exert an effect on circulating insulin levels (Fig. 1E, $p_{\text{basal}}=0.68$, $p_{\text{low insulin}}=0.37$, $p_{\text{high insulin}}=0.67$), thereby excluding an effect of resveratrol on insulin clearance.

As expected, carbohydrate oxidation increased during the clamp at the expense of free fatty acid (FFA) oxidation (Supplementary Table 2). No statistically significant changes in carbohydrate or FFA oxidation rates were observed when comparing basal and insulin-stimulated oxidation rates between placebo and resveratrol (Supplementary Table 2). At the end of the hyperinsulinemic-euglycemic clamp, suppression of FFA was similar between both conditions (Fig. 1F, $p=0.36$).

In accordance with the lack of effect of resveratrol on insulin sensitivity, no improvement in fasting glucose, insulin or HbA1c levels were found compared to placebo (Supplementary Table 3). We did observe a time effect for HbA1c, however this was found in both treatment conditions. Similarly, no resveratrol-effect on other markers of metabolic health was found (Supplementary Table 3).

Sleeping Metabolism

Sleeping metabolic rate, measured in a whole-body respiratory chamber during the last night of the intervention, decreased upon resveratrol supplementation in the majority (82%) of subjects. However, the difference failed to reach statistical significance (7.81 ± 0.20 MJ/day vs. 7.55 ± 0.16 MJ/day, for placebo and resveratrol respectively, $p=0.14$). During the night, substrate utilization (reflected by respiratory quotient) was similar upon both treatments (0.81 (0.81 - 0.88) vs. 0.82 (0.80 - 0.85), for placebo and resveratrol, $p=0.45$).

Figure 1. Effect of resveratrol on peripheral and hepatic insulin sensitivity

After 30 days of resveratrol and placebo, peripheral and hepatic insulin sensitivity was assessed with a two-step hyperinsulinemic-euglycemic clamp ($t=0-120$ min: D-[6,6- $^2\text{H}_2$]glucose tracer infusion; $t=120-300$ min: low-insulin infusion; $t=300-420$ min: high-insulin infusion). Due to technical problems, data is only available for 14 subjects (in one subject we had malfunctioning equipment, in another subject the line with D-[6,6- $^2\text{H}_2$]glucose tracer leaked, and in the last subject aspiration of the venous line was no longer possible in the late phase of the clamp, which is necessary for regular blood sampling). (A) Plasma glucose levels during the last 30 minutes of the basal, low- and high-insulin state of the clamp. Glucose levels were clamped ~ 5 mmol/l, (B) Insulin-stimulated glucose disposal, expressed as the rate of disappearance of the glucose (R_d glucose) and (C) endogenous glucose production (EGP) were calculated for the last 30 minutes of the basal, low- and high-insulin state, (D) EGP suppression upon low- and high-insulin infusion. Data are presented as mean $\pm$ SEM. R_d , rate of disappearance; EGP, endogenous glucose production; NOGD, non-oxidative glucose disposal.

Ectopic Lipid Storage

Lipid area fraction, measured *ex vivo* in muscle biopsies by Oil Red O staining, increased significantly in type 2 muscle fibers (Fig. 2A, $p=0.03$), and a similar tendency was observed for total lipid content (Fig. 2A, $p=0.08$).

Figure 2. Effect of resveratrol on ectopic lipid storage

(A) Muscle biopsy sections from a representative study participant, stained for intramyocellular lipids with Oil Red O staining (in red), muscle laminin (in blue) and type 1 muscle fibers (in green). Intramyocellular lipid content is quantified as the percentage area of a muscle fiber that is covered by lipids ($n=17$). (B) IHL content quantified by ^1H -MRS after 29 days of resveratrol and placebo supplementation ($n=10$). (C) Δ IHL in relation to total plasma resveratrol concentration ($n=10$). Data are presented as means $\pm$ SEM. * $P<0.05$.

IHL content, determined by ^1H -MRS, remained unaffected by 30 days of resveratrol supplementation (Fig. 2B). Interestingly, linear regression analysis showed that the difference in IHL content between resveratrol and placebo negatively correlated with the parental plasma resveratrol concentration (Fig. 2C, $R=-0.68$, $p=0.03$), indicating that a decrease in IHL may be dependent on plasma resveratrol concentrations achieved.

Mitochondrial Function

Mitochondrial state 3 respiration on a lipid-derived substrate (malate + octanoyl-carnitine) and upon parallel electron input to both complex I and II (malate + octanoyl-carnitine + glutamate + succinate) (Fig. 3A), and maximal FFCP-induced uncoupled respiration (Fig. 3B, $p=0.001$) were significantly higher after resveratrol supplementation. However, in the absence of a lipid-derived substrate, no resveratrol effect was observed in state 3 respiration upon complex I and II-linked substrates (Supplementary Fig. 1A). In addition, State 4o respiration upon addition of oligomycin (reflecting mitochondrial proton leak) was similar between the resveratrol and the placebo

Figure 3. Effect of resveratrol on *ex vivo* mitochondrial function

After 30 days of resveratrol and placebo, a muscle biopsy was obtained from the vastus lateralis muscle. Part of the muscle biopsy was used for evaluation of *ex vivo* mitochondrial function (n=17). (A) ADP-stimulated respiration (state 3) upon a lipid-like substrate, and upon parallel electron input into complex I and II. (B) Maximally uncoupled respiration upon FCCP. Data are presented as means $\pm$ SEM. * P < 0.05. M, malate; O, octanoyl-carnitine; G, glutamate; S, succinate.

condition (Supplementary Fig. 1B). Mitochondrial DNA (mtDNA) copy number (2251 ± 162 AU vs. 2234 ± 152 AU, respectively, $p=0.91$), mean protein content of the OXPHOS complexes, PGC1 α protein content and pAMPK/AMPK ratio remained unaffected by resveratrol supplementation (Supplementary Fig. 1C-E). Mean PCr recovery half-time, a measure of *in vivo* mitochondrial function, was unchanged by resveratrol compared to placebo (Supplementary Fig. 1F). In line, $\text{VO}_{2\text{max}}$, a measure of cardiorespiratory fitness, was not affected by resveratrol supplementation (23.88 ± 1.10 ml.kg⁻¹.min⁻¹ vs. 23.40 ± 1.34 ml.kg⁻¹.min⁻¹, for placebo and resveratrol respectively, $p=0.29$).

Cardiac Function

Systolic blood pressure, measured in overnight fasted state on day 30, tended to decrease by resveratrol supplementation ($p=0.09$), whereas diastolic blood pressure remained unchanged (Supplementary Table 4). Echocardiography revealed a marginal reduction in left ventricular end systolic diameter upon resveratrol supplementation (Supplementary Table 4, $p=0.04$). As the values remain well within the normal range they are not interpreted as clinically significant. No change in stroke volume, cardiac output or left ventricular ejection fraction was found. Parameters of diastolic function of the heart remained unaffected by resveratrol. Furthermore, no structural changes were observed in the heart by resveratrol (Supplementary Table 4).

Plasma Resveratrol Levels and Metformin Dose

A strong correlation was found between plasma DHR concentration and metformin dose after resveratrol treatment at day 30 (Fig. 4A, $R=0.66$, $p=0.005$), suggesting an interaction between metformin and the metabolism of resveratrol. Post-hoc analysis revealed that total plasma DHR levels were higher during 30 days of resveratrol supplementation in subjects taking a relatively high daily dose of metformin (>1000 mg/day ($n=9$), average metformin dose 2188 mg/day) compared to patients treated with a lower dose (≤ 1000 mg/day ($n=8$), average metformin dose 639mg/day) (Fig. 4B), whereas the daily dose of metformin did not seem to influence plasma levels of parental resveratrol (Fig. 4C).

Adverse Events

Resveratrol was well tolerated by the participants and no adverse events occurred. Measurement of parameters for kidney and liver function (creatinine, AST, ALT, gamma-GT and bilirubin) indicated that resveratrol was well tolerated (Supplementary table 3). Creatinine showed a significant, albeit very modest, decline from day 0 to day 30 in both treatment conditions, but no time*treatment interaction was found (Supplementary table 3).

Figure 4. Possible interaction of resveratrol with metformin

Post-hoc analysis revealed a possible interaction of metformin with the metabolism of resveratrol. (A) Correlation of the daily metformin dose used by the type 2 diabetic patients and plasma DHR levels. Subsequent sub-analysis was performed in which type 2 diabetic patients were separated in a group using high daily doses of metformin (>1000 mg/day ($n=9$), average metformin dose 2188 mg/day) versus a group using low daily doses of metformin (≤ 1000 mg/day ($n=8$), average metformin dose 639mg/day). (B) Total plasma DHR levels and (C) total resveratrol levels for the high- versus low-metformin dose groups. Data are presented as mean $\pm$ SEM. DHR, dihydroresveratrol.

DISCUSSION

Preclinical research has suggested that resveratrol could be used to prevent the metabolic consequences of high-fat feeding, including insulin resistance, by improving mitochondrial function. Previously, we observed in obese, normoglycemic subjects that resveratrol supplementation exerted beneficial metabolic effects accompanied by an improvement in mitochondrial function. The present study did not find a stimulatory effect of resveratrol on peripheral or hepatic insulin sensitivity in well-controlled T2D subjects on top of standard diabetes care. Other markers of metabolic health also largely remained unaffected by resveratrol, although improvements in *ex vivo* muscle oxidative capacity were detected. Overall, our results suggest that resveratrol may not be of added value as adjunct therapy for subjects with T2D receiving the oral glucose-lowering medication metformin.

The metabolic actions of resveratrol are mainly ascribed to activation of the energy-sensing pathway AMPK-SIRT1-PGC1 α [1]. Several downstream targets of this pathway (e.g. mitochondrial biogenesis and glucose and lipid homeostasis) are interesting targets for treatment of T2D. No positive effect of 30 days resveratrol supplementation on endogenous hepatic glucose production, (non)oxidative skeletal muscle glucose disposal, circulating insulin levels, and substrate utilization was detected. Also, fasting glucose, insulin and HbA1c values remained unaffected by resveratrol. It should be noted that we used an add-on approach, in which resveratrol was supplemented on top of the patients' normal blood-glucose lowering treatment, mainly metformin. It is well known that treatment with the oral glucose-lowering medication metformin leads to phosphorylation of the threonine residue 172 (thr172) in the α -subunit of AMPK [30]. Accordingly, measurement of AMPK phosphorylation by western blot revealed no significant differences between the resveratrol and placebo condition. Potentially, a daily dose of 150 mg of resveratrol may not have been able to further activate AMPK in subjects with T2D receiving metformin, thereby explaining the lack of effect of resveratrol on insulin sensitivity. In addition to this explanation, our data indicate that metformin may interact with the metabolism of resveratrol. This potential interaction could have influenced the efficacy of the active component, thereby accounting for the lack of a resveratrol-induced improvement of insulin sensitivity.

Interestingly, Movahed et al. [17] recently reported that subjects with T2D treated with metformin only did not show an improvement in circulating insulin or HbA1c levels upon resveratrol administration, whereas patients receiving SUDs only, or metformin in combination with SUDs demonstrated a resveratrol-induced improvement in both plasma makers of insulin sensitivity. Together with our study results, these findings reinforce the importance of investigating the relationship between resveratrol and metformin in future research. A potential mechanism that may, in part, explain the interaction of metformin with resveratrol can be sought in the recently

appreciated influence of metformin on gut microbiota composition [31, 32]. The gut microbiota is one of the major sites of metabolism of *trans*-resveratrol to the metabolite dihydroresveratrol [33]. Although it is beyond the scope of this research, alterations in gut microbiota composition by metformin could have affected the metabolism of our *trans*-resveratrol. It would therefore be worthwhile to examine if resveratrol could improve insulin sensitivity in drug-naïve subjects with T2D, or pre-diabetic subjects.

Despite the lack of effect of resveratrol on AMPK activity and insulin sensitivity, we did observe an improvement of *ex vivo* mitochondrial function. These results are in line with Price et al. [34], who recently demonstrated that effects of resveratrol on mitochondrial metabolism are not always paralleled by an effect on AMPK. Furthermore, we found that resveratrol increased IMCL content, consistent with our previous findings in healthy, obese subjects [10]. These findings may suggest that the beneficial effects of resveratrol on insulin sensitivity, as found by others, are not entirely dependent on the effects on skeletal muscle mitochondrial function. Alternatively, these results may suggest that the beneficial effects of resveratrol on muscle mitochondrial function may be too small to affect insulin sensitivity, or that the duration of the treatment may have been too short to affect glycemic control. In that respect, recent work of Thazhath et al. [35] in subjects with T2D controlled by diet-only, also failed to improve glycemic control when treated with 500 mg resveratrol twice daily for five weeks.

Intrahepatic lipid content also remained unaffected by resveratrol in subjects with T2D, which was in contrast to our previous study in which we noted a significant decrease in healthy, obese subjects [10]. However, we did observe a correlation between plasma parental resveratrol levels and changes in IHL content, suggesting that higher doses of resveratrol may be able to reduce IHL content also in subjects with T2D. However, further clinical studies are needed to test this hypothesis.

Resveratrol supplementation did not affect systolic and diastolic function of the heart, but tended to decrease systolic blood pressure. A decrease in systolic blood pressure has previously been observed with resveratrol. For example, a recent meta-analysis of six randomized controlled trials investigated the effect of resveratrol on blood pressure and reported that high doses of resveratrol (≥ 150 mg/day) significantly reduced systolic blood pressure, while lower doses had no effect on blood pressure [36].

In conclusion, 30 days of resveratrol supplementation did not improve hepatic- or peripheral insulin sensitivity in subjects with T2D treated with oral glucose-lowering medication. Intriguingly, levels of a metabolite of resveratrol were affected by the dose of metformin used, suggesting that the lack of effect of resveratrol on insulin sensitivity may have been affected by metformin use in these patients. These results question the generalized value of resveratrol

as an add-on therapy for treatment of T2D, and emphasize the need to explore the possible interaction between resveratrol and metformin. Studies in pre-diabetic subjects are needed to examine if resveratrol can improve insulin sensitivity when not combined with oral glucose-lowering drug therapy.

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V.B.S.H., M.K.C.H. and P.S. contributed to the design of the study, analyzed and interpreted the data, and reviewed and edited the manuscript. I.K. delivered the resVida and placebo capsules and performed the analysis of plasma resveratrol and dihydroresveratrol levels. All authors approved the final version of the manuscript.

S.T., M.d.L., E.P., T.v.d.W., J.H., E.M.K., G.S., I.K., M.K.C.H., V.B.S., P.S. have no potential conflicts of interest to report regarding this article. P.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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SUPPLEMENTARY DATA

Table 1. Baseline subject characteristics

Parameter	
Age, years	64.00 (59.19 – 67.28)
Type 2 diabetes diagnosis, years	6.76 ± 0.96
Height, m	1.77 ± 0.01
Fat, %	25.87 ± 1.11
Oral glucose-lowering medication, n	16
Metformin + SUD, n	6
Metformin only, n	10
Diet only, n	1

Data are presented as means ± SEM when normally distributed, otherwise median and 95% CI (n=17). Medication use did not change during the study protocol. SUD, sulphonylurea derivatives.

Table 2. Insulin sensitivity and substrate kinetics assessed by hyperinsulinemic euglycemic clamp

	Placebo	Resveratrol	P-value
Rd glucose ($\mu\text{mol.kg}^{-1}\text{body weight.min}^{-1}$)			
Basal	12.18 $\pm$ 0.72	12.60 $\pm$ 0.86	0.65
Low insulin	9.98 $\pm$ 0.87	9.84 $\pm$ 0.53	0.89
High insulin	21.10 $\pm$ 1.69	20.52 $\pm$ 1.92	0.66
Delta (Rd low insulin - Rd basal)	-2.07 $\pm$ 0.96	-2.69 $\pm$ 0.78	0.56
Delta (Rd high insulin - Rd basal)	9.23 $\pm$ 1.75	8.25 $\pm$ 1.95	0.52
EGP ($\mu\text{mol.kg}^{-1}\text{body weight.min}^{-1}$)			
Basal	11.71 $\pm$ 0.77	12.49 $\pm$ 1.11	0.22
Low insulin	6.73 $\pm$ 0.52	6.67 $\pm$ 0.66	0.84
High insulin	0.77 $\pm$ 0.28	0.88 $\pm$ 0.38	0.74
% suppression (low insulin)	39.79 $\pm$ 5.19	41.88 $\pm$ 6.93	0.64
% suppression (high insulin)	92.45 $\pm$ 2.73	91.26 $\pm$ 3.90	0.73
Carbohydrate oxidation ($\mu\text{mol.kg}^{-1}\text{body weight.min}^{-1}$)			
Basal	5.02 $\pm$ 0.62	5.19 $\pm$ 0.58	0.85
Low insulin	7.27 (5.75 - 8.65)	7.23 (6.12 - 9.54)	0.51 ^a
High insulin	9.32 (8.27 - 11.55)	8.22 (7.77 - 12.30)	0.88 ^a
Plasma insulin (pmol.L ⁻¹)			
Basal	97 (78 - 126)	94 (79 - 129)	0.68 ^a
Low insulin	212 (200 - 244)	223 (207 - 264)	0.59 ^a
High insulin	963 $\pm$ 28	945 $\pm$ 37	0.67
NOGD ($\mu\text{mol.kg}^{-1}\text{body weight.min}^{-1}$)			
Basal	7.02 $\pm$ 0.86	7.39 $\pm$ 1.10	0.79
Low insulin	3.06 (0.43 - 6.27)	2.40 (1.89 - 3.97)	0.70 ^a
High insulin	10.16 (7.61 - 14.76)	8.48 (5.63 - 14.05)	0.64 ^a
Delta (NOGD low insulin - NOGD basal)	-3.59 $\pm$ 1.58	-4.78 $\pm$ 1.30	0.53
Delta (NOGD high insulin - NOGD basal)	5.15 (0.42 - 8.71)	1.44 (-2.73 - 8.76)	0.86 ^a
FFA oxidation ($\mu\text{mol.kg}^{-1}\text{body weight.min}^{-1}$)			
Basal	3.54 $\pm$ 0.19	3.63 $\pm$ 0.19	0.74
Low insulin	2.90 $\pm$ 0.16	2.69 $\pm$ 0.12	0.31
High insulin	2.42 $\pm$ 0.15	2.31 $\pm$ 0.21	0.57
Respiratory quotient			
Basal	0.77 $\pm$ 0.01	0.78 $\pm$ 0.01	0.88
Low insulin	0.81 $\pm$ 0.009	0.82 $\pm$ 0.009	0.35
High insulin	0.86 (0.83 - 0.87)	0.84 (0.82 - 0.88)	0.78 ^a

Substrate metabolism assessed by a two-step hyperinsulinemic euglycemic clamp after 30 days of placebo and resveratrol supplementation. The glucose oxidation, lipid oxidation, and respiratory quotient were calculated by means of indirect calorimetry. Non-oxidative glucose disposal (NOGD) is calculated by subtracting the rate of glucose oxidation from the rate of disappearance of the glucose (Rd glucose). Data are presented as mean $\pm$ SEM when normally distributed, otherwise median and range are shown. Due to technical problems, the data is only available for 14 subjects (in one subject we had malfunctioning equipment, in another subject the line with D-[6,6-²H₂]glucose tracer leaked, and in the last subject aspiration of the venous line was no longer possible in the late phase of the clamp, which is necessary for regular blood sampling). ^a P-value relates to non-parametric Wilcoxon Signed Rank test. Rd, rate of disappearance; EGP, endogenous glucose production; NOGD, non-oxidative glucose disposal.

Table 3. Subject characteristics before and after 30 days of resveratrol and placebo

	Placebo	Resveratrol	P-value
Weight, kg	n = 17	n = 17	
0 days	95.31 ± 2.36	95.57 ± 2.24	0.86
30 days	93.61 ± 2.32	93.98 ± 2.24	
BMI, kg/m²	n = 17	n = 17	
0 days	30.52 ± 0.60 ^a	30.48 ± 0.56 ^a	0.53
30 days	29.96 ± 0.57 ^a	30.06 ± 0.58 ^a	
Systolic blood pressure, mmHg	n = 17	n = 17	
0 days	142 ± 3.9	139 ± 4.2	0.96
30 days	141 ± 2.8	138 ± 2.9	
Diastolic blood pressure, mmHg	n = 17	n = 17	
0 days	87 ± 2.7	85 ± 2.7	0.13
30 days	86 ± 1.9	86 ± 1.9	
Glucose, mmol/l	n = 17	n = 17	
0 days	8.29 ± 0.37	8.11 ± 0.37 ^a	0.28
30 days	7.70 ± 0.41	7.80 ± 0.39	
HbA1c, %	n = 17	n = 17	
0 days	6.78 ± 0.21	6.77 ± 0.19	0.12
30 days	6.61 ± 0.19	6.80 ± 0.21	
HbA1c, mmol/mol	n = 17	n = 17	
0 days	50.59 ± 2.33	50.53 ± 2.06	0.12
30 days	48.12 ± 2.06	50.35 ± 2.23	
Insulin, pmol/l	n = 16	n = 16	
0 days	87.29 ± 6.30	99.53 ± 12.70	0.31
30 days	90.61 ± 10.99	90.45 ± 9.41	
Creatinine, μmol/l	n = 16	n = 16	
0 days	86.09 ± 3.41	90.69 ± 4.98	0.64
30 days	80.88 ± 3.50	83.68 ± 4.07	
Gamma-GT, U/l	n = 17	n = 17	
0 days	31.97 ± 2.43	33.29 ± 2.22	0.99
30 days	30.59 ± 3.50	31.94 ± 2.57	
AST, U/l	n = 17	n = 17	
0 days	26.78 ± 1.66	26.29 ± 1.72	0.62
30 days	26.94 ± 1.91	27.41 ± 1.51	
ALT, U/l	n = 17	n = 17	
0 days	34.54 ± 2.94	32.53 ± 2.42	0.07
30 days	32.18 ± 3.56	34.71 ± 2.55	
Bilirubin, μmol/l	n = 15	n = 15	
0 days	10.15 ± 1.65	9.52 ± 1.55	0.79
30 days	10.34 ± 1.62	9.89 ± 1.26	
Cholesterol, mmol/l	n = 17	n = 17	
0 days	4.15 ± 0.19	4.15 ± 0.17 ^a	0.32
30 days	3.87 ± 0.18	4.05 ± 0.19 ^a	
HDL-cholesterol, mmol/l	n = 17	n = 17	
0 days	1.18 ± 0.08 ^a	1.18 ± 0.10 ^a	0.41
30 days	1.06 ± 0.07	1.09 ± 0.07 ^a	

LDL-cholesterol , mmol/l	n = 17	n = 17	
0 days	2.17 ± 0.19	2.16 ± 0.18	0.37
30 days	1.93 ± 0.15	2.05 ± 0.16	
Triglycerides , mmol/l	n = 17	n = 17	
0 days	2.04 ± 0.40 ^a	2.18 ± 0.41 ^a	0.49
30 days	2.07 ± 0.35	2.09 ± 0.26	

Data are mean ± SEM. P-value reflects time*treatment effect by two-way repeated measures ANOVA. Plasma values are obtained after an overnight fast. ^a Data is not normally distributed but residuals from the ANOVA fit are normally distributed. n.d., not detectable.

Figure 1. Effect of resveratrol on *in vivo* and *ex vivo* mitochondrial function

After 30 days of resveratrol and placebo, a muscle biopsy was obtained from the vastus lateralis muscle. Part of the muscle biopsy was used for evaluation of *ex vivo* mitochondrial function (n=17). (A) ADP-stimulated respiration (state 3) upon parallel electron input into complex I and II. (B) Mitochondrial respiration uncoupled from ATP synthesis (state4o). The other part of the biopsy was used for Western blotting. Total protein extracts were made from the vastus lateralis muscle as previously described [1], and 50 µg of protein was used to determine protein expression of important mitochondrial proteins (n=15). Representative subsets for all mitochondrial proteins are shown. (C) Protein content of the individual complexes of the mitochondrial respiratory chain was quantified by using an antibody cocktail that detects all five complexes (MS601, MitoSciences, Eugene, OR). (D) PGC1α protein content was measured using a polyclonal antibody (Santa Cruz, Heidelberg, Germany). (E) Total and the phosphorylated subunit α of AMPK (#2531 and #2532 Cell Signalling Technology, Inc., Beverly MA, USA) were detected as described [2]. The relative expression of actin was used as loading control for the Western blotting (n=15). (F) Muscle oxidative capacity expressed as PCR half-time measured by ³¹P-MRS after 29 days of resveratrol and placebo supplementation (n=13). Data are presented as means ± SEM. * P < 0.05. M, malate; G, glutamate; S, succinate.

Table 4. Cardiac function with echocardiography

	Placebo	Resveratrol	P-value
Characteristics			
HR , bpm	70.00 ± 2.30	74.31 ± 3.14	0.11
Systolic blood pressure , mmHg	140 ± 2.84	138 ± 2.87	0.09
Diastolic blood pressure , mmHg	86 ± 1.87	86 ± 1.86	1.00
LV systolic function			
SV , ml	78.35 (76.88 – 102.02)	78.35 (72.79 – 92.61)	0.14 ^a
CO , L/min	5.90 ± 0.28	5.81 ± 0.37	0.85
LVEDD , mm	50.00 (48.78 – 52.85)	48.50 (46.95 – 51.17)	0.08 ^a
LVESD , mm	34.88 ± 0.82	33.13 ± 0.93	0.04
LVFS , %	30.94 ± 0.82	32.41 ± 0.80	0.19
LVEF , %	58.81 ± 1.29	60.25 ± 1.17	0.35
LV diastolic function			
E top , cm/s	63.19 ± 3.48	61.06 ± 2.11	0.41
A top , cm/s	78.63 ± 3.55	80.69 ± 3.79	0.53
E/A	0.80 (0.71 – 0.89)	0.75 (0.67 – 0.87)	0.36 ^a
E/E'	8.32 ± 0.48	8.23 ± 0.55	0.84
Structure			
LPVW , mm	9.00 (8.71 – 9.92)	10.00 (9.11 – 9.89)	0.64 ^a
LV mass , gram	181.29 ± 10.50	175.57 ± 8.43	0.50
LV mass index , gram/m ²	85.07 ± 4.53	82.36 ± 4.00	0.49

Left ventricular systolic and diastolic function measured at day 29 of the placebo and resveratrol intervention (n=16). Data are presented as means ± SEM. ^a P-value relates to non-parametric Wilcoxon Signed Rank test. HR, heart rate; SV, stroke volume; CO, cardiac output; LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; LVFS, left ventricular fractional shortening; LVEF, left ventricular ejection fraction; LPVW, left ventricular posterior wall thickness; LV mass, Left ventricular mass.

SUPPLEMENTAL REFERENCES

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CHAPTER 4

Resveratrol improves *ex vivo* mitochondrial function but does not affect insulin sensitivity or brown adipose tissue in first degree relatives of patients with type 2 diabetes: a randomised controlled trial

Marlies de Ligt, Yvonne M.H. Bruls, Jan Hansen, Marie-Fleur Habets, Bas Havekes, Emmani B. M. Nascimento, Esther Moonen-Kornips, Gert Schaart, Vera B. Schrauwen-Hinderling, Wouter van Marken Lichtenbelt and Patrick Schrauwen

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GRAPHICAL ABSTRACT

ABSTRACT

Objective

Resveratrol supplementation improves metabolic health in healthy obese men, but not in patients with type 2 diabetes (T2D) when given as add-on therapy. Therefore, we examined whether resveratrol can enhance metabolic health in men *at risk* of developing T2D. Additionally, we examined if resveratrol can stimulate brown adipose tissue (BAT).

Methods

Thirteen male first degree relatives (FDR) of patients with T2D, received resveratrol (150 mg/day) and placebo for 30 days in a randomized placebo controlled cross-over trial.

Results

Resveratrol significantly improved *ex vivo* muscle mitochondrial function on a fatty acid-derived substrate. However, resveratrol did not improve insulin sensitivity, expressed as the rate of glucose disposal during a two-step hyperinsulinemic-euglycemic clamp. Also, intrahepatic and intramyocellular lipid content, substrate utilization, energy metabolism and cold-stimulated ^{18}F -FDG glucose uptake in BAT (n=8) remained unaffected by resveratrol. *In vitro* experiments in adipocytes derived from human BAT confirmed the lack of effect on BAT.

Conclusions

Resveratrol stimulates muscle mitochondrial function in FDR males, which is in concordance with previous results. However, no other metabolic benefits of resveratrol were found in this group. This could be attributed to subject characteristics causing alterations in metabolism of resveratrol and thereby affecting resveratrol's effectiveness.

ClinicalTrials.gov ID: NCT02129595

INTRODUCTION

Resveratrol, a polyphenol present in various foods, has been proposed as a promising treatment in the prevention and treatment of type 2 diabetes (T2D) [1]. Beneficial effects of resveratrol are strongly related to the activation of sirtuin 1 (SIRT1) and AMP-activated protein kinase (AMPK) [2]. Both proteins are important regulators of cellular fuel metabolism and mitochondrial function, thereby making them interesting targets for combating T2D [3]. Previously, we have shown that in healthy obese men 150 mg resveratrol per day for 30 days exerted beneficial health effects, among which improvements in *ex vivo* muscle mitochondrial function, energy metabolism, hepatic lipid content, and blood glucose levels [4]. However, we did not find comparable results when the same dose of resveratrol was administered to patients with T2D [5]. Although we confirmed beneficial effects of resveratrol on mitochondrial function, no improvements in insulin sensitivity or related health parameters were achieved. However, since these patients were using the oral-glucose lowering drug metformin, which may have similar targets as resveratrol, we proposed that the use of metformin might have interfered with the effectiveness of resveratrol on glucose homeostasis. To determine if resveratrol could still have a role in the prevention of diabetes, there is a need for studies in drug-naïve humans with compromised metabolic health. First-degree relatives (FDR) of T2D patients display decreased beta cell and mitochondrial function, and have reduced insulin sensitivity [6-10]. Thereby, they are at increased risk of developing T2D and may specifically benefit from resveratrol treatment.

Positive effects of resveratrol on metabolic health could also be related to stimulation of brown adipose tissue (BAT) thermogenesis, as represented by increased expression of uncoupling protein 1 (UCP1), SIRT1 and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) in BAT of animals receiving resveratrol [11-13]. The main role of BAT is generation of heat, mediated via UCP1 [14]. Activation of brown adipose tissue has been associated with positive metabolic health effects [15], but so far the effect of resveratrol on BAT in humans is unknown.

Therefore, in this placebo-controlled crossover study we investigated if 30 days of resveratrol supplementation can improve insulin sensitivity and muscle mitochondrial function in males at increased risk of developing T2D. In a sub-set of the participants, as well as in human primary brown adipocytes, we examined if resveratrol is able to stimulate BAT.

MATERIAL AND METHODS

Participants

Thirteen overweight males at increased risk of developing T2D participated in the study (Table S1). The protocol was reviewed and approved by the institutional medical ethics committee (NL 47018.068.13; ClinicalTrials.gov ID: NCT02129595). An increased risk for T2D was defined as having at least one first-degree relative with T2D, BMI between 27-35 kg/m², and disrupted glucose homeostasis determined by a 2-hour oral glucose tolerance test (OGTT). From the OGTT, glucose clearance was calculated using the oral glucose insulin sensitivity model [16] {Mari, 2001 #320}. Participants with a glucose clearance ≤ 350 ml/kg/min were regarded as having disrupted glucose homeostasis. A sub-set of eight participants was enrolled for BAT measurements. The study was carried out at Maastricht University and Medical Centre, the Netherlands. A flow chart of the enrollment process can be found in Figure S1.

Study Design

Subjects participated in a double-blind, crossover trial with two conditions: a placebo and a resveratrol condition (150 mg/day *trans*-resveratrol [99.9%; provided by DSM Nutritional Products Ltd.]), with a washout period of at least 30 days. Each experimental condition lasted 30 or 34 days; the 34-day protocol applied only to the sub-set of eight participants who underwent BAT measurements. Participants were instructed to take the first supplement on the day baseline measurements were performed (day 0), and the last supplement in the evening before the last test day (day 29 or day 33; depending on participation in the BAT measurement). At each visit, the participants returned unused capsules. Participants were instructed to abstain from food containing substantial amounts of resveratrol (e.g. red wine, grapes and peanuts), and to maintain their normal eating, activity and sleeping pattern. Participants came to the university on a weekly basis (days 0, 7, 14, 21, 29 and 34), in the morning in overnight fasted state, for measuring body weight and drawing blood samples for the analysis of plasma resveratrol. The latter was analyzed by mass spectroscopy, as previously described [4]. Potential changes in physical activity level were evaluated by performing a maximal aerobic capacity test (VO₂max) on a cycle ergometer on day 27 of both treatment periods.

Biochemical Measures

General health parameters

On day 0 and 30 of both the resveratrol and placebo period, fasting blood samples were drawn for analysis of general safety parameters (creatinine, bilirubin, aspartate aminotransferase, alanine aminotransferase and γ -glutamyl transferase). In addition, heart rate and blood pressure were measured in triplicate in the resting condition (Omron Healthcare, Hamburg, Germany), and a 12-lead ECG was made. Maximal aerobic capacity (VO₂max) was measured on a cycle ergometer on day 27, to verify cardiorespiratory fitness (Omnicol, Maastricht Instruments, Maastricht University).

Body fat percentage was determined only once by dual energy X-ray absorptiometry (DXA, Hologic, the Netherlands) on day 0 of the first intervention period.

Cardiac function

On day 29, in the afternoon, myocardial function was measured by Doppler ultrasound. M-mode, two-dimensional and Doppler echocardiography were performed, using a Vivid 7 ultrasound system with 3.5 MHz cardiac transducer (GE Healthcare, Milwaukee, WI, USA).

Magnetic resonance spectroscopy

On day 29 the ultrasound was followed by a proton magnetic resonance spectroscopy (¹H-MRS) measurement to quantify intrahepatic lipid (IHL) content. Proceeded by a post-exercise phosphocreatine (PCr) recovery rate measurement, by ³¹P-MRS, to estimate *in vivo* mitochondrial function in vastus lateralis muscle. MRS scans were performed on a 3T whole body scanner (Achieva Tx; Philips Healthcare, Best, The Netherlands). In short, for IHL a PRESS sequence was used with a repetition time of 4 s and an echo time of 32.5 ms. Values are given as T2 corrected ratios of the CH₂ peak relative to unsuppressed water resonance, expressed as percentage (for further details see [17]). For *in vivo* mitochondrial function, the PCr recovery half-time in seconds was determined after submaximal knee-extension exercise performed in the scanner (for details see [18]). To standardize food intake, subjects had lunch with the same food items in the two conditions and stayed fasted until completion of the MRS scans. Afterwards a standardized evening meal was provided and subjects stayed in a respiration chamber during 10 hours to allow measurement of sleeping metabolic rate [19].

Muscle biopsy

In the morning of day 30 a biopsy was taken from the vastus lateralis muscle. A portion of the muscle was directly frozen in melting isopentane for determination of protein expression of oxidative phosphorylation (OXPHOS) by Western blotting (AB110411, abcam, Cambridge, UK), and intramyocellular lipid content (IMCL) by Oil red O staining combined with fiber typing via immunolabeling of myosin heavy chain type I (see [20]). Another portion of ~50 mg was immediately placed in ice-cold preservation medium for determination of *ex vivo* mitochondrial respiration by oxygraph (OROBOROS Instruments, Innsbruck, Austria), as previously described [4].

Hyperinsulinemic-euglycemic clamp

On day 30, after the muscle biopsy, a two-step hyperinsulinemic-euglycemic clamp was performed to assess whole-body and liver-specific insulin sensitivity. The clamp started with a primed continuous infusion of D-[6,6-²H₂]glucose (0.04 mg/kg/min) to determine rates of endogenous glucose production (EGP), glucose appearance (Ra), and glucose disposal (Rd), as described earlier [5]. After 120 minutes, low dose insulin infusion was started (10 mU/m²/min) for 3 hours, followed by high dose insulin infusion (40 mU/m²/min) for 2.5 hours. During the last 30

minutes of each insulin infusion phase (0, 10, and 40 mU/m²/min), blood samples were collected, and substrate utilization was measured by indirect calorimetry (Omnicol, Maastricht Instruments, Maastricht). Steele's single pool non-steady state equations were used to calculate glucose Ra and Rd [21]. The distribution volume of glucose was assumed to be 0.160 L/kg.

Brown adipose tissue

After the clamp, a sub-set of eight participants continued supplement intake for another four days. On day 34 these participants underwent an 18-Fluoro-Deoxy-Glucose (¹⁸F-FDG) PET/CT scan, upon acute cold exposure, to measure glucose uptake in the BAT region as described earlier [22]. The protocol started with a thermoneutral period at 36°C for 45 minutes, followed by an individualized 2-hour cooling protocol using a water-perfused suit. Water temperature was decreased by 4°C every 15 minutes, until shivering occurred. When shivering occurred, subjects were warmed-up again for 5 minutes to diminish shivering. Finally, the suit temperature was set at 2°C above the temperature at which shivering started, for a minimum of 30 minutes. During this mild cold stimulation ~74 MBq of ¹⁸F-FDG was intravenously injected. One hour after injection imaging started with a low-dose CT scan (120 kV, 30 mAs), and a static PET scan (6-7 bed positions, 6 min per bed position) (Gemini TF PET-CT, Philips). The PET image was used to determine the ¹⁸F-FDG uptake, and the CT image for PET attenuation correction and localization of the ¹⁸F-FDG uptake sites. Scans were analyzed using PMOD software (version 3.0; PMOD Technologies). PET-active areas, defined as a standard uptake value (SUV) ≥ 1.5, were selected in the supraclavicular adipose tissue region (-200 and -10 Hounsfield units) to establish the presence and volume of active BAT. The fixed-volume method [22] was used to determine the average SUV and maximum SUV of several tissues. Fixed volumes of 2.67 cm³ were placed and analyzed in supraclavicular region for BAT, subcutaneous white adipose tissue (WAT), skeletal muscle, and liver glucose uptake. The same anatomic locations were used in the analysis between the two experimental conditions.

To examine *in vitro* effects of resveratrol on BAT oxidative capacity, BAT and WAT biopsies were obtained from patients undergoing thyroid surgery. The protocol was reviewed and approved by the institutional ethics committee (NL31367.068.10). From the collected biopsies, the stromal vascular fraction was isolated and grown to confluence before differentiation was initiated as described previously with slight modifications [23]: bFGF and BMP4 were removed from the differentiation protocol. *In vitro* differentiated adipocytes were incubated for 24 hours with *trans*-resveratrol (0, 5 and 50 μM) prior to measuring oxygen consumption in the XF96 bioanalyzer from Seahorse Bioscience. Basal oxygen consumption rate and norepinephrine (NE)-induced mitochondrial uncoupling were measured. For the latter, 1 μM NE was added after blocking ATPase by addition of 2 μM oligomycin.

Statistical Analysis

Results are presented as means $\pm$ SEM when normally distributed, and as median and range when this was not the case. Shapiro-Wilk normality test was performed to evaluate normal distribution. Student's paired t-test was used to compare placebo and resveratrol treatment in normally distributed data, otherwise Wilcoxon signed-rank. Treatment comparisons of parameters measured at the beginning and end of the intervention periods were assessed by two-way repeated measures ANOVA. Linear regression analyses were conducted to identify correlations between variables. A p-value <0.05 was considered statistically significant. Statistical analyses were performed using SPSS 22.0 for Mac OS.

RESULTS

Study Compliance

Compliance was confirmed by analysis of plasma levels of resveratrol (free + conjugated) and dihydro-resveratrol (DHR), a metabolite of resveratrol, on a weekly basis. Resveratrol and DHR levels were below detection during the placebo period, while during the resveratrol period both were elevated, with resveratrol levels of 412 ± 63 ng/mL and DHR levels of 541 ± 80 ng/mL on day 30 (Figure 1). Participants were instructed to remain their habitual physical activity level and not to loose bowy weight throughout the entire study. This was confirmed by VO_2max (27 ± 1.5 mL/kg bw/min upon placebo vs. 28 ± 1.7 upon resveratrol, $p=0.823$) and body weight on day 30 (90.2 ± 1.67 kg after placebo vs. 89.9 after resveratrol, $p=0.524$).

Insulin Sensitivity and Substrate Kinetics

Insulin sensitivity was measured by two-step hyperinsulinemic euglycemic clamp. Basal, low- and high-insulin phase R_d were not affected by resveratrol (Figure 2A). Insulin-stimulated glucose uptake, as expressed by the change in glucose disposal (ΔR_d) from basal to the low or high-

Figure 1. Total plasma resveratrol (RSV) and dihydro-resveratrol (DHR) values during the 30 days of resveratrol treatment period. Data are presented as mean $\pm$ SEM ($n = 13$).

insulin phase, was not different between resveratrol and placebo (Figure 2B-C; Table 1). Indirect calorimetry, performed during the different stages of the clamp, revealed no effect of resveratrol on insulin-induced glucose oxidation or suppression of fat oxidation (Table 1). Also, non-oxidative glucose disposal remained unchanged (Table 1). Insulin-mediated suppression of endogenous glucose production (%EGP suppression) was similar after resveratrol and placebo, both during low and high-insulin infusion (Figure 2D; Table 1). In accordance with a lack of effect of resveratrol on substrate utilization during the clamp, resveratrol did not alter sleeping metabolic rate (5.1 ± 0.09 kJ/min after placebo, versus 5.0 ± 0.08 kJ/min after resveratrol) or respiratory exchange ratio, both measured overnight in a respiration chamber. Furthermore, fasting plasma FFA levels were comparable between treatments and were similarly suppressed by insulin (Table 1). In conformity, no changes in fasting insulin, glucose, HbA1c levels or other plasma markers related to metabolic health were found upon resveratrol (Table S2).

Figure 2. Effect of resveratrol (RSV) on peripheral and hepatic insulin sensitivity.

After 30 days of resveratrol and placebo, peripheral and hepatic insulin sensitivity were assessed by a two-step hyperinsulinemic-euglycemic clamp ($t = 0$ -120 min: D-[6,6- $^2\text{H}_2$]glucose tracer infusion; $t = 120$ -300 min: low-insulin infusion; $t = 300$ -420 min: high-insulin infusion). Insulin-stimulated glucose disposal, expressed as the Rd and EGP were calculated for the last 30 min of the basal, low- and high-insulin state. (A) Rd and (B-C) difference in Rd compared to basal; (D) EGP suppression (delta EGP %) upon low- and high-insulin infusion. Data are presented as individual data points, mean $\pm$ SEM ($n = 13$). Rd, rate of disappearance; EGP, endogenous glucose production.

Table 1. Insulin sensitivity and substrate kinetics

	Placebo	Resveratrol	P-value
Rd ($\mu\text{mol/kg}$ body weight/min)			
Basal	8.0 (6.86 – 9.42)	7.9 (6.86 – 8.92)	0.972 ^a
Low insulin	10.2 $\pm$ 0.44	9.4 $\pm$ 0.56	0.258
High insulin	32.0 $\pm$ 2.17	32.0 $\pm$ 2.03	0.965
Delta (Rd low insulin - Rd basal)	1.5 (0.67 – 3.37)	0.9 (0.04 – 3.02)	0.507 ^a
Delta (Rd high insulin - Rd basal)	23.8 $\pm$ 2.04	24.1 $\pm$ 1.90	0.810
EGP ($\mu\text{mol/kg}$ body weight/min)			
Basal	7.9 (4.48 – 9.16)	8.5 (7.29 – 9.54)	0.937 ^a
Low insulin	3.0 (2.32 – 5.18)	3.3 (2.36 – 4.41)	0.875 ^a
High insulin	-0.3 (-1.19 – -0.08)	-0.63 (-0.92 – -0.03)	0.695 ^a
% suppression (low insulin)	62 (40.0 – 70.7)	58 (42.0 – 72.1)	>0.99 ^a
% suppression (high insulin)	103 (99.8 – 113.1)	107 (100.4 – 114.6)	0.814 ^a
NOGD ($\mu\text{mol/kg}$ body weight/min)			
Basal	2.1 $\pm$ 0.92	2.0 $\pm$ 0.65	0.894
Low insulin	2.2 $\pm$ 0.78	1.0 $\pm$ 0.88	0.350
High insulin	18.1 $\pm$ 2.11	17.6 $\pm$ 5.91	0.742
Carbohydrate oxidation ($\mu\text{mol/kg}$ body weight/min)			
Basal	5.3 (4.60 – 7.47)	5.9 (4.27 – 7.38)	0.944 ^a
Low insulin	7.9 $\pm$ 0.53	8.4 $\pm$ 0.76	0.630
High insulin	13.9 $\pm$ 0.51	14.4 $\pm$ 0.95	0.478
FFA oxidation ($\mu\text{mol/kg}$ body weight/min)			
Basal	3.5 $\pm$ 0.24	3.6 $\pm$ 0.13	0.978
Low insulin	2.9 $\pm$ 0.17	2.7 $\pm$ 0.14	0.527
High insulin	1.6 $\pm$ 0.13	1.5 $\pm$ 0.18	0.757
Plasma FFA ($\mu\text{mol/L}$)			
Basal	552 $\pm$ 40.2	609 $\pm$ 59.9	0.333
Low insulin	167 $\pm$ 12.2	174 $\pm$ 19.7	0.652
High insulin	66 $\pm$ 5.3	65 $\pm$ 6.3	0.550
Respiratory exchange ratio			
Basal	0.77 (0.765 – 0.811)	0.78 (0.766 – 0.805)	0.916 ^a
Low insulin	0.82 $\pm$ 0.008	0.82 $\pm$ 0.009	0.634
High insulin	0.90 $\pm$ 0.008	0.90 $\pm$ 0.011	0.833

Substrate metabolism assessed by a two-step hyperinsulinemic euglycemic clamp after 30 days of placebo and resveratrol supplementation. Glucose oxidation, lipid oxidation, and respiratory quotient were determined by means of indirect calorimetry. Non-oxidative glucose disposal (NOGD) is calculated by subtracting the rate of glucose oxidation from the rate of disappearance of the glucose (Rd glucose). Data are presented as mean $\pm$ SEM when normally distributed, otherwise median and range are shown (n = 13). ^aP-value relates to non-parametric Wilcoxon Signed Rank test. Rd, rate of disappearance; EGP, endogenous glucose production; NOGD, non-oxidative glucose disposal; FFA, free fatty acid.

Skeletal Muscle Mitochondrial Function

Resveratrol is expected to act on whole body metabolism via improvement of mitochondrial function. Therefore, mitochondrial state 3 respiration on a lipid-derived substrate (malate + octanoyl-carnitine, 3MO) was measured. State 3 respiration was significantly higher after resveratrol supplementation (32 ± 1.8 after placebo vs. 37 ± 1.9 pmol/mg wet weight/s after resveratrol, $p < 0.001$; Figure 3A). Also, state 3 respiration with parallel electron input to both complex I and II (malate + octanoyl-carnitine + glutamate + succinate, 3MOGS) was significantly higher after resveratrol (73 ± 3.7 after placebo vs. 78 ± 4.3 pmol/mg wet weight/s after resveratrol,

Figure 3. Effect of resveratrol (RSV) on *ex vivo* and *in vivo* mitochondrial function.

After 30 days of resveratrol and placebo, a muscle biopsy specimen was obtained from the vastus lateralis muscle. Part of the specimen was used for evaluation of *ex vivo* mitochondrial function ($n = 10$). **A:** ADP stimulated respiration (state 3) upon a lipid-like substrate and upon parallel electron input into complex I and II. **B:** Maximally uncoupled respiration upon FCCP (carbonyl cyanide p-(trifluoro-methoxy)-phenylhydrazide). **C:** the protein content of the individual complexes of the electron transport chain is quantified by western blotting in vastus lateralis muscle. An antibody cocktail that detects all five complexes was used ($n = 10$). **D:** *in vivo* mitochondrial function expressed as PCr half-time ($n = 9$). Data are presented as individual data points, means $\pm$ SEM. * $P < 0.05$ compared to placebo. M, malate; O, octanoyl-carnitine; G, glutamate; S, succinate; ww, wet weight; OXPHOS, oxidative phosphorylation; PCr, phosphocreatine recovery

$p=0.050$; Figure 3A). Maximal FCCP-induced uncoupled respiration (state u) also seemed to increase upon resveratrol, but failed to reach statistical significance ($p=0.218$, Figure 3B). In the absence of octanoyl-carnitine, no effects of resveratrol were observed in state 3 (3MG, 3MGS) or state 4o respiration with complex I- and II-linked substrates, consistent with our previous reports [4, 5]. Protein content of the structural components of the individual OXPHOS complexes remained unaffected, except for complex IV that was significantly lower expressed after resveratrol supplementation ($p=0.033$, Figure 3C). Finally, *in vivo* mitochondrial function, determined by PCR recovery half-time, was unchanged (Figure 3D).

Ectopic Fat Accumulation

Resveratrol has been shown to be able to affect ectopic fat accumulation. However, IHL content, determined by ^1H -MRS, remained unaffected by resveratrol (Figure 4A). Furthermore, IMCL, analyzed *ex vivo* in muscle biopsies, revealed no change in lipid area fraction in neither type I or type II muscle fibers (Figure 4B).

Cardiac Function

Animal studies have suggested that resveratrol may improve cardiovascular function. Nevertheless, systolic and diastolic blood pressure were unaltered by resveratrol (Table S3). Also, no changes in stroke volume, cardiac output, left ventricular end systolic diameter, or left ventricular ejection fraction were found (Table S3). Parameters of diastolic function and parameters representing structural changes of the heart also remained unchanged (Table S3).

Figure 4. Effect of resveratrol (RSV) on ectopic lipid storage.

A: Intrahepatic lipid content quantified by ^1H -MRS after 29 days of resveratrol and placebo supplementation. Box plot represents minimum, first quartile, median, third quartile, and maximum ($n = 10$). B: Muscle biopsy sections for the vastus lateralis muscle were stained for intramyocellular lipid content by Oil Red O staining. Intramyocellular lipid content is quantified as the percentage area of a muscle fiber that is covered by lipids. Data are presented as individuals data points, means $\pm$ SEM ($n = 9$).

Brown Adipose Tissue

Rodent studies indicated that resveratrol could activate BAT. Here, we examined if resveratrol treatment increases cold-activated BAT activity using PET-CT scanning. Only one participant showed ^{18}F -FDG uptake in BAT above the threshold of 1.5 SUV in both periods, as can be clearly seen in Figure 5A as the top line, indicating that these FDR participants are characterized by low levels of cold-induced BAT activity. Using the fixed volume method, we found that in the BAT region both SUVmax (1.6 ± 0.82 and 1.9 ± 0.61 , Figure 5A) and SUVmean (1.1 ± 0.62 and 1.0 ± 0.38 after placebo and resveratrol respectively, Figure 5B) were not changed after resveratrol. Likewise, ^{18}F -FDG uptake did not change in skeletal muscle, subcutaneous WAT or liver (Figure 5B-E). In accordance with a lack of effect of resveratrol on BAT, resveratrol treatment did not influence cold-induced metabolic rate (5.3 ± 0.22 after placebo, versus 5.4 ± 0.22 kJ/min after resveratrol).

In our clinical trial, the treatment duration may have been too short or the dose of resveratrol too low to accomplish effects on BAT. Therefore, to further investigate if resveratrol can stimulate human BAT activity, we examined mitochondrial uncoupling in adipocytes derived from human BAT and WAT. NE-stimulated mitochondrial uncoupling was specifically increased in adipocytes derived from human BAT. Illustrating the characteristic capacity of BAT to induce mitochondrial uncoupling. Nevertheless, incubating the cells with 5 or 50 μM *trans*-resveratrol for 24h did not alter NE-stimulated mitochondrial uncoupling in adipocytes derived from BAT or WAT (Figure 5F-G).

Figure 5. Effects of resveratrol (RSV) on *in vivo* and *in vitro* brown adipose tissue.

A-B: ^{18}F -FDG uptake in BAT, WAT, muscle, and liver after 34 days of placebo and resveratrol intervention ($n = 8$). BAT SUV max (A) and BAT, SM, WATsc and liver SUV mean (B-E) were measured under cold-stimulated conditions. (F-G) Respiration was measured in oligomycin (OG)-treated brown adipocytes ($n=6$) following $1 \mu\text{M}$ norepinephrine. Data are presented as individual data points, mean $\pm$ SEM. BAT, brown adipose tissue; WAT, white adipose tissue; SM, skeletal muscle; SUV, standard uptake value; OCR, oxygen consumption rate.

DISCUSSION

Resveratrol has been suggested to be beneficial in the prevention and treatment of T2D. Here, we investigated the effects of 30 days of resveratrol supplementation, 150 mg/day, in men at increased risk of developing T2D. However, in this population we did not see an improvement in insulin sensitivity after 30 days of resveratrol compared to placebo. Neither hepatic nor peripheral insulin sensitivity were improved, albeit we did demonstrate that 150 mg resveratrol per day was sufficient to improve *ex vivo* muscle mitochondrial oxidative capacity. Other markers of metabolic health and BAT metabolism also remained unchanged. Our data indicates that low-dose resveratrol supplementation does not seem to have beneficial metabolic effects in men at increased risk of developing T2D.

The potential role resveratrol could play in prevention and treatment of metabolic diseases, such as T2D, has been widely studied over the past decades. Studies in rodents show clear improvement upon resveratrol administration on among others insulin sensitivity, mitochondrial function, liver fat accumulation and indications towards increased BAT metabolism [24]. Nevertheless, human clinical trials have given inconsistent results. Some studies show clear improvement in parameters related to metabolic health [4, 25-29], while others see no or barely any effects [5, 30-36].

Here, we used the same dose and treatment duration of resveratrol as in our first study in healthy obese volunteers. In that study, we found beneficial effects of resveratrol on several metabolic parameters, and most importantly activation of the SIRT1-PGC-1 α pathway in skeletal muscle leading to improved mitochondrial function [4]. Illustrating that resveratrol at this dose is indeed capable of affecting this pathway in humans. However, three other studies, with comparable resveratrol dose and treatment duration, failed to achieve improvements in glucose control [5, 35, 36]. One can therefore debate whether the dose of resveratrol used might be too low or the treatment duration too short to elicit effects on glucose metabolism in patients or subjects with disrupted glucose homeostasis. A recent clinical trial by Kjaer et al. [37] used a high (1000 mg/day) and low dose (150 mg/day) of resveratrol for a period of six months but also did not measure effects on glucose or lipid metabolism in obese men with moderate insulin resistance. It can therefore be speculated that differences in subject characteristics, such as age, body weight, gut microbiota composition, and metabolic status are more important influential factors for effects of resveratrol on metabolic health.

It is known that resveratrol is rapidly metabolized into its glucuronidated and sulfated form in the intestine and liver, resulting in a very low bioavailability [38]. In addition, a recent study suggest that unconjugated resveratrol, also called free resveratrol, is the active form of resveratrol [39]. Therefore, measuring plasma concentrations of free and conjugated resveratrol could shed

light on why resveratrol is effective in some but not in other participants. Unfortunately, few clinical trials - focussed on improving metabolic health - have actually measured free resveratrol concentrations. Therefore we compared our plasma total resveratrol (free + conjugated) concentrations to two of our previous studies that used the same dose and duration of resveratrol treatment: a study with healthy obese men [4] and a study with T2D patients [5]. In all three studies, we observed positive effects of resveratrol on mitochondrial metabolism. Whereas the study with healthy obese men gave positive effects on glucose metabolism and other measures of metabolic health, our current study in FDRs and our previous study in T2D patients did not. Strikingly, in the study with healthy obese men, total resveratrol concentrations were markedly lower than those measured in the study with T2D patients and in our current FDR study (healthy obese: 183 ± 30 ng/mL; T2D patients: 379 ± 41 ng/mL; FDRs: 412 ± 63 ng/mL, $p < 0.01$). Higher plasma levels of resveratrol with the same intake of resveratrol could be caused by several factors: increased uptake of resveratrol from the gut, increased conjugation, diminished excretion by the kidneys and/or lower uptake of resveratrol from the plasma by the tissues. Over the 30-day resveratrol treatment period no increase was observed in total resveratrol or DHR, indicating no accumulation occurred in the plasma. This suggests the excretion by the kidneys was not hampered. Whether potential differences in the active form of resveratrol or uptake of resveratrol in the tissues can explain differences between studies can however not be deduced from the current study. Of note, it is also important to note that in our first study in healthy obese men, we did not determine insulin sensitivity, but only had data on fasting plasma glucose and insulin concentrations, and thus HOMA-IR.

In our current study, we found significant improvements in mitochondrial state 3 respiration on a lipid-derived substrate. These results are comparable to previous clinical trials [4, 5, 44]. We noted these improvements without changes in OXPHOS protein content, except for a decrease in complex IV, indicating that resveratrol affects predominantly mitochondrial efficiency and not abundance. An alternative explanation for the lack of effect of resveratrol on metabolic health parameters is that merely improving mitochondrial function is not sufficient to improve whole body insulin sensitivity or metabolic health in general. This suggestion is supported by findings of Hoehn et al. [45], who concluded that acute or chronic up-regulation of mitochondrial fatty acid oxidation has no net effect on whole-body energy expenditure or adiposity. In addition, Wong et al. [46] previously reported that overexpressing PGC-1 α in mouse skeletal muscle did not prevent high-fat diet induced insulin resistance, despite improved mitochondrial function. Only when mice were stimulated to become physical active, the PGC-1 α induced increase in mitochondrial function was also accompanied by improved insulin sensitivity. This may suggest that just increasing mitochondrial function is not sufficient to stimulate insulin sensitivity and it could be speculated that physical activity levels in our T2D patients [4] or FDR subjects were too low to benefit from the small increase in resveratrol-induced mitochondrial function. Future

studies could test the concept that resveratrol, in addition to a physical activity program, could have beneficial effects in volunteers with disturbed glucose homeostasis.

Brown adipocytes contain a high density of mitochondria and have been suggested to also be a target of resveratrol. This hypothesis has, to our knowledge, never been examined *in vivo* in humans. In general our FDRs display low levels of uptake of ^{18}F -FDG in BAT, which could be attributed to the sex, age and body weight of the participants since BAT activity declines with age and is decreased in males and overweight [47]. Despite the low starting levels of BAT, 34 days of supplementation with resveratrol did not increase BAT activity as expressed by cold-stimulated ^{18}F -FDG uptake. Correspondingly, when *in vitro* cultured adipocytes derived from human BAT were incubated with 5 or 50 μM *trans*-resveratrol this did not increase NE-stimulated mitochondrial uncoupling.

CONCLUSION

The current study indicates that resveratrol supplementation does not affect insulin sensitivity, substrate utilization, energy expenditure, ectopic fat accumulation or cardiac function, and does not stimulate BAT in men at increased risk of developing T2D. However, the data do support our previous findings that low-dose resveratrol supplementation can improve muscle mitochondrial oxidative capacity. Future studies should unravel why there are inconsistencies in effectiveness of resveratrol when supplied to different populations, which may include investigating physical activity level of participants and bioavailability and active compounds of resveratrol.

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Authors' contributions

M.d.L. designed and performed the experiments, analyzed the data, and wrote the manuscript. Y.B., J.H., M-F.H., B.H., E.N., E.M-K., and G.S. assisted during the experiments and reviewed and edited the manuscript. V.S., W.M-L., and P.S. contributed to the design of the study, interpretation of the data, and reviewed and edited the manuscript.

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Conflict of interest

All authors approved the final version of the manuscript. None of the authors have a potential conflict of interest to report regarding this article. P.S. is the guarantor of this work. Thus, he has full access to all the data of the study, and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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SUPPLEMENTARY DATA

Figure S1. Participant enrollment flow chart

Table S1. Baseline characteristics

Parameter	
Age, years	66 (59.2 – 67.6)
Height, m	1.77 ± 0.012
Body weight, kg	90.9 ± 1.43
BMI, kg/m ²	28.9 ± 0.38
Body fat, %	29.0 ± 0.87
Glucose clearance, ml/kg/min	334 (303.0 – 340.3)
First-degree relatives patients with T2D, n	1 ± 0.2

Subject's characteristics at baseline. Values are given as means ± SEM for normal distributed data, and as median (95% CI) when this is not the case (n = 13)

Table S2. - Plasma biochemistry before and after 30 days of resveratrol and placebo

	Placebo	Resveratrol	P-value
Glucose, mmol/l			
baseline	5.7 ± 0.14 ^a	5.9 ± 0.18	0.408
30 days	5.5 ± 0.17	5.6 ± 0.17	
HbA1c, %			
baseline	5.7 ± 0.09 ^a	5.6 ± 0.10	0.326
30 days	5.7 ± 0.09	5.6 ± 0.07	
HbA1c, mmol/mol			
baseline	39 ± 0.9	37 ± 1.1	0.354
30 days	38 ± 1.0	38 ± 0.8	
Insulin, pmol/l			
baseline	71.8 ± 8.24	78.2 ± 5.85	0.948
30 days	76.3 ± 9.33	83.3 ± 10.15	
Potassium			
baseline	4.46 ± 0.05	4.45 ± 0.05	0.976
30 days	4.25 ± 0.06	4.23 ± 0.05	
Urea			
baseline	5.3 ± 0.28	5.5 ± 0.30	0.293
30 days	4.7 ± 0.15	4.5 ± 0.18	
Creatinine, µmol/l			
baseline	89 ± 2.3	87 ± 1.8 ^a	0.764
30 days	84 ± 1.8	84 ± 2.1	
GGT, U/l			
baseline	26 ± 2.7	23 ± 1.9	0.033
30 days	23 ± 3.1 ^a	22 ± 2.2 ^a	
AST, U/l			
baseline	24 ± 1.9	23 ± 1.7 ^a	0.208
30 days	22 ± 2.1	22 ± 1.6	
ALT, U/l			
baseline	22 (19.6 – 34.4) ^b	21 (18.3 – 27.8) ^b	0.173 ^b
30 days	17 (15.7 – 30.5) ^b	23 (16.4 – 30.3) ^b	
Bilirubin, µmol/l			
baseline	9.1 ± 0.97	9.2 ± 1.11	0.959
30 days	10.5 ± 0.97	10.6 ± 0.79	
Cholesterol, mmol/l			
baseline	5.8 ± 0.29	5.5 ± 0.28	0.012
30 days	5.3 ± 0.23	5.5 ± 0.27	
HDL-cholesterol, mmol/l			
baseline	1.3 ± 0.09	1.3 ± 0.09	0.614
30 days	1.2 ± 0.09	1.2 ± 0.08	
LDL-cholesterol, mmol/l			
baseline	3.9 ± 0.22	3.6 ± 0.24	0.120
30 days	3.4 ± 0.16	3.5 ± 0.27	

Table S2. - Continued

	Placebo	Resveratrol	P-value
Triglycerides, mmol/l			
baseline	1.40 (1.20 – 1.77)	0.98 (0.93 – 1.70) ^b	0.30 ^b
30 days	1.29 (1.12 – 1.81)	1.32 (1.19 – 2.04)	
Free fatty acids, mmol/l			
baseline	0.50 ± 0.07	0.52 ± 0.08	0.731
30 days	0.70 ± 0.06	0.75 ± 0.09	

Data are presented as mean ± SEM when normally distributed, otherwise median and range are shown (n = 13). P-value reflects time*treatment effect by two-way repeated measures ANOVA. Plasma values are obtained after an overnight fast. ^aData has a non-normal distribution, but residuals from the ANOVA fit are normally distributed. ^bData and residuals are not normal distributed, analyses performed on log transformed data. GGT, γ-glutamyl transferase; AST, aspartate transaminase; ALT, alanine transaminase.

Table S3. Cardiac function with echocardiography

	Placebo	Resveratrol	P-value
Blood pressure and heart rate			
HR , bpm	61 ± 1.9	62 ± 2.2	0.653
Systolic blood pressure , mmHg	136 ± 3.0	138 ± 2.2	0.391
Diastolic blood pressure , mmHg	86 ± 1.8	87 ± 2.1	0.776
LV systolic function			
SV , ml	86 ± 7.8	84 ± 6.7	0.832
CO , L/min	6.0 ± 0.64	5.8 ± 0.42	0.707
LVEDD , mm	49 ± 1.4	49 ± 1.2	>0.99
LVESD , mm	33 ± 0.9	33 ± 1.1	>0.99
LVEF , %	60 ± 1.8	60 ± 1.3	0.742
LV diastolic function			
E-wave , cm/s	60 ± 3.6	61 ± 3.6	0.707
A-wave , cm/s	73 ± 2.8	70 ± 3.5	0.381
E/A ratio	0.8 (0.67 - 1.01)	0.8 (0.72 - 1.08)	0.509 ^a
Structure			
LPVW , mm	9 (8.2 - 9.4)	9 (8.7 - 9.6)	0.340 ^a
LV mass , gram	159 ± 5.9	159 ± 9.9	>0.99
LV mass index , gram/m ²	73 (70.0 - 80.5)	70 (65.7 - 81.2)	0.635 ^a

Left ventricular systolic and diastolic function measured at day 29 of the placebo and resveratrol intervention (n = 12). Data are presented as mean ± SEM when normally distributed, otherwise median and range are shown. ^aP-value relates to nonparametric Wilcoxon Signed Rank test. HR, heart rate; SV, stroke volume; CO, cardiac output; LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; LVEF, left ventricular ejection fraction; LPVW, left ventricular posterior wall thickness; LV mass, Left ventricular mass.

CHAPTER 5

Why resveratrol is effective in some but not all individuals: a combined data-analysis

Marlies de Ligt, Esther Phielix, Bas Havekes, Vera B. Schrauwen-Hinderling and Patrick Schrauwen

In preparation

ABSTRACT

Background

Results from pre-clinical studies have suggested that resveratrol may have metabolic health effects and can prevent insulin resistance. However, the translation from animal to human studies turned out to be challenging. Even with similar treatment duration and dose of resveratrol, the efficacy differs between participant populations. Here we investigated which subject characteristics predict efficaciousness of resveratrol on a number of metabolic health parameters in humans.

Methods

Data from three placebo-controlled, cross-over human clinical trials was combined. In all three clinical trials the same dose (150 mg/d) and treatment duration (30 days) was applied, but the populations varied from healthy obese men, to men at risk of type 2 diabetes (T2D) and to patients with T2D. Paired sample t-test was applied to evaluate effects of resveratrol treatment on a whole group basis. Pearson correlation coefficient analysis followed by stepwise backwards linear regression was used to detect which baseline participant characteristics are determinants of resveratrol-induced changes in metabolic health outcome parameters.

Results

Overall, resveratrol had beneficial effects on systolic blood pressure (137 ± 1.8 upon placebo vs. 134 ± 1.8 mmHg upon resveratrol $p=0.038$), *ex vivo* mitochondrial function ($p<0.001$, for all mitochondrial states) and sleeping metabolic rate (7.71 ± 0.12 upon placebo vs. 7.51 ± 0.10 MJ/day upon resveratrol; $p=0.029$), but not on plasma glucose or HOMA index. Remarkably, despite similar dosing, circulating plasma resveratrol levels were markedly different between the three groups ($p=0.008$), but plasma resveratrol levels did not predict outcome. Baseline serum bilirubin was found to significantly predict resveratrol-induced change in diastolic blood pressure ($p=0.044$), ALT ($p=0.030$), intrahepatic lipid content ($p=0.020$) and plasma glucose ($p=0.004$). The latter was especially profound when also baseline serum albumin levels were taken into account ($p=0.002$).

Discussion

Baseline serum bilirubin was identified as most significantly related to resveratrol-induced changes in metabolic health outcome parameters. Thus, higher baseline serum bilirubin levels were associated with improvements in metabolic health upon resveratrol treatment. It has been suggested that bilirubin is a surrogate of UGT1A1 activity, which is an enzyme involved in glucuronidation of *trans*-resveratrol. Although speculative, our data may suggest that bilirubin levels reflect activity of glucuronidation enzymes, implying glucuronidation of resveratrol is important for efficacy. Further studies are needed to investigate the importance of glucuronidation of resveratrol.

INTRODUCTION

Interest in resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) increased after it was identified as a biologically active polyphenol. Initial research focused on potential cardio protective effects, and revealed anticoagulant activities of resveratrol, inhibiting platelet aggregation [1]. Subsequent *in vitro* and animal studies indicated that resveratrol can also have anti-inflammatory, anti-oxidant and anti-carcinogenic properties [2]. Although the results from pre-clinical studies were promising, the translation from animal to human studies turned out to be challenging. The number of clinical trials failing to show beneficial effects of resveratrol, almost outbalance the number of studies that do show positive effects. Major underlying problems are that it is still unknown what the optimal dose and treatment duration are of resveratrol, and what the exact mechanisms of action are.

In addition, even when studies used similar treatment duration and dose, the efficaciousness differs between (patient) populations. For example, when 150 mg/day of resveratrol was supplied for 30 days to a group of healthy obese men (HOM) [3], to patients with type 2 diabetes (T2D) [4] and male first degree-relatives (FDRs) of patients with T2D at risk of developing T2D [5], different outcomes were reported. Thus, in HOM administration of resveratrol improved several parameters related to metabolic health, such as a decrease in plasma glucose, insulin, triglyceride (TG), alanine-aminotransferase (ALT), inflammatory markers, systolic blood pressure and intrahepatic lipid (IHL) content and HOMA index was improved [3]. In contrast, when the same protocol was implement in a group of patients with T2D [4] and a group of FDRs [5], these improvements remained absent. Interestingly, *ex vivo* muscle mitochondrial function consistently improved in all three clinical trials.

Therefore, beside unravelling the optimal dose and treatment duration, it is of importance to identify why resveratrol is beneficial for some populations but not for others. It seems sensible to assume that participant characteristics play an important role in effectiveness of resveratrol. Considering resveratrol has a poor bioavailability [6], due to rapid metabolism and excretion, physiological factors related to the absorption, distribution and metabolism of the compound in the human body could be of interest for efficaciousness of resveratrol.

To shed light on which subject characteristics are related to efficaciousness of resveratrol - in relation to metabolic health - we combined data from three placebo-controlled, cross-over human clinical trials with the same study design and dose of resveratrol, and investigated which subject characteristics are effect modifiers for the response to resveratrol treatment.

RESEARCH DESIGN AND METHODS

Participants

Data from three placebo-controlled clinical trials was combined and analysed. Studies were approved by the Medical Ethical Review Committee of Maastricht University Medical Centre. The combined study covered 41 male participants: 11 HOM, 17 male patients with T2D and 13 male FDRs at risk of developing T2D.

Study Design

All three studies were performed according to a similar study set-up, by the same research group but in three distinct participant populations. All subjects participated in a double-blind, crossover, randomized trial with two conditions: a placebo and a resveratrol condition (150 mg/day *trans*-resveratrol [99.9%; provided by DSM Nutritional Products Ltd.]). Both treatment periods lasted 30 days, and there was a washout period in between of at least 30 days. Participants were instructed to take the first supplement on day 0 and the last supplement in the evening of day 29. During the 30-day intervention periods participants came to the facilities on a weekly basis, and the main study parameters were determined on days 29-30. All three studies were designed to investigate the effects of 30 days resveratrol supplementation on glucose homeostasis, mitochondrial function and ectopic lipid accumulation.

Compliance

To check compliance, unused capsules were counted and plasma levels of resveratrol (free + conjugated) and dihydro-resveratrol (DHR) were measured by mass spectrometry on days 0, 7, 14, 21, 29, and 30 as described previously [3].

General Health Parameters

On day 30 of both the resveratrol and placebo period, fasting blood samples were drawn to check kidney and liver function. Thus, creatinine, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and γ -glutamyl transferase (gamma-GT) were measured in blood serum. Furthermore, plasma free fatty acids (FFA) and triglycerides (TG) were measured as markers related to dyslipidemia, and plasma glucose and insulin, combined with HOMA-IR, for estimating insulin sensitivity. In addition, blood pressure was measured in triplicate in the resting condition (Omron Healthcare, Hamburg, Germany), and body fat percentage was determined by dual energy X-ray absorptiometry (DXA, Hologic, the Netherlands). Body fat percentage was determined to characterize participants, and therefore measured only once: on day 0 of the first intervention period. Maximal aerobic capacity ($\text{VO}_{2\text{max}}$) was measured on different days in the three studies, but according to the same protocol [7].

Muscle Mitochondrial Function

Muscle mitochondrial function was determined both *in vivo* and *ex vivo*. For estimating *in vivo* mitochondrial function, phosphocreatine (PCr) recovery rate was measured in the vastus lateralis muscle by phosphorus magnetic resonance spectroscopy (^{31}P -MRS). PCr recovery half-time in seconds was determined after submaximal knee-extension exercise performed in a MRI scanner (for details see [8]). The measurement was performed in the evening of day 28 on a 1.5T scanner for HOM, and day 29 on a 3T scanner for the T2D patients and FDRs. For assessment of *ex vivo* muscle mitochondrial function, a biopsy was taken from the vastus lateralis muscle in the morning of day 30. A portion of the muscle (30-50 mg) was immediately placed in ice-cold preservation medium for determination of *ex vivo* mitochondrial respiration by oxygraph (OROBOROS Instruments, Innsbruck, Austria), as previously described [3]. Mitochondrial function was expressed as oxygen consumption per picomoles per second per mg wet weight muscle tissue.

Ectopic Lipid

IHL content was quantified by proton magnetic resonance spectroscopy (^1H -MRS) on a whole-body 3T scanner (Achieva, Philips Healthcare, Best, The Netherlands). In the study with HOM the measurement took place in the evening of day 28 (for details see [3, 9]), and in the T2D and FDR studies quantification took place in the evening of day 29 (for details see [10, 11]). To minimize food intake effects, the participants had lunch with the same food items in the two conditions, afterwards they remained fasted until completion of the MRS scans around 6 PM.

Intramyocellular lipid content (IMCL) was determined in a portion of the vastus lateralis muscle biopsy taken in the morning of day 30. Directly after obtaining the biopsy, a portion of the muscle was directly frozen in melting isopentane. By a combination of Oil red O staining and fibre typing via immunolabelling of myosin heavy chain type I, IMCL was determined in type I and type II fibres (see [12]). Since ORO-based determination of IMCL cannot be easily compared between studies, relative changes in IMCL upon resveratrol are calculated (relative change in IMCL upon resveratrol compared to placebo). Therefore, IMCL is only taken into account for identifying predictors of effectiveness of resveratrol, and not for identifying effects of resveratrol on a whole group basis.

Sleeping Metabolic Rate

Participants stayed in a metabolic chamber to determine sleeping metabolic rate (SMR) according to [13]. Depending on the study protocol, the participants remained in these chambers for 12 till 36 hours. The SMR of the first night was analysed and used for calculations, and was corrected for fat free mass.

Statistical Analysis

Results are presented as means $\pm$ SEM when normally distributed, and as median and range when this was not the case. First, effects of resveratrol treatment were evaluated on a whole group basis. Data of the three studies was combined, and student's paired t-test was used to compare placebo versus resveratrol treatment in normally distributed data. If data was not normally distributed Wilcoxon signed-rank was used. Potential carryover and sequence effects were examined by unpaired t-test analyses according to Pocock [14]. In the second analysis, differences between the three studies in baseline characteristics were identified by one-way ANOVA combined with Tukey's post hoc test. Outcome parameters obtained during the placebo treatment period were regarded as 'baseline characteristics' and were used for these analyses. Finally, to identify the influence of baseline characteristics on response to resveratrol treatment, Pearson correlation coefficient analysis followed by stepwise backward linear regression was performed. For the linear regression analysis, resveratrol-induced changes in outcome parameters (delta: resveratrol-placebo) were entered as dependent variables, and relevant baseline characteristics as predictors. Baseline characteristics that were significantly different between the studies – as identified in the second analysis – and showed significant correlations with resveratrol-induced changes in outcome parameters, were regarded as relevant and entered as potential predictors of response to resveratrol treatment.

A p-value <0.05 was considered statistically significant. Statistical analyses were performed using SPSS 24.0 for Mac OS.

RESULTS

Effects of Resveratrol Treatment on Whole Group Basis

When combining the data from all three studies, a significant reduction in systolic blood pressure (137 ± 1.8 mmHg upon placebo vs. 134 ± 1.8 mmHg upon resveratrol; $p=0.038$); a decrease in SMR (7.71 ± 0.12 upon placebo vs. 7.51 ± 0.10 MJ/day upon resveratrol; $p=0.029$); and an increase in *ex vivo* muscle mitochondrial function upon a fatty acid substrate (Table 1; $p<0.001$ for all mitochondrial states) was found upon resveratrol treatment. Markers of insulin sensitivity, such as plasma insulin and glucose levels as well as calculated HOMA-IR, were not different upon resveratrol treatment. Also, no effect was found on intrahepatic lipid content. In addition, no carryover or sequence effects were found for the main study outcome parameters confirming the washout period was sufficient.

Table 1. Whole group analysis resveratrol treatment effects

	Variable	Treatment		p-value
		Placebo	Resveratrol	
General	Body weight, kg	94.2 ± 1.6	94.1 ± 1.5	0.870
	Diastolic bp, mmHg	85 ± 1.2	85 ± 1.3	0.624
	Systolic bp, mmHg	137 ± 1.8	134 ± 1.8	0.038
Blood biochemistry	HOMA-IR,	1.9 ± 0.1	1.9 ± 0.1	0.978
	Glucose, mmol/L	6.2 ± 0.2	6.2 ± 0.2	0.775
	Insulin, pmol/L	82.7 ± 5.6	82.8 ± 5.5	0.984
	Creatinine, µmol/L	80 ± 1.9	81 ± 2.1	0.398
	Bilirubin, µmol/L	11.8 ± 0.8	11.4 ± 0.7	0.412
	Albumin, g/L	38.0 ± 0.9	37.3 ± 0.6	0.179
	GammaGT, U/L	28 ± 2.1	28 ± 1.6	0.921
	AST, U/L	23 ± 1.2	24 ± 1.0	0.623
	ALT, U/L	29 ± 2.0	29 ± 1.7	0.937
	TG, mmol/L	1.93 ± 0.17	1.94 ± 0.14	0.797
	FFA, mmol/L	0.71 ± 0.04	0.74 ± 0.04	0.605
Energy metabolism	SMR, MJ/day	7.71 ± 0.12	7.51 ± 0.10	0.029
Mitochondrial function[#]	PCr t ½, s	24 ± 1.4	24 ± 1.4	0.351
	3MO, pmol/s/mg ww	28.17 ± 0.93	31.70 ± 1.12	<0.001
	3MOG, pmol/s/mg ww	44.44 ± 1.33	49.03 ± 1.32	<0.001
	3MOGS, pmol/s/mg ww	63.91 ± 2.03	70.28 ± 2.03	<0.001
	State U, pmol/s/mg ww	85.23 ± 3.22	93.34 ± 3.15	0.001
Ectopic lipid	IHL, %	4.8 ± 0.9	5.0 ± 1.0	0.529

Outcome parameters measured after 30 days of resveratrol or placebo supplementation. Data are presented as mean ± SEM (n=41) [#] *In vivo* mitochondrial function: expressed as PCr half-time. *Ex vivo* mitochondrial function: ADP stimulated respiration (state 3) upon a fatty acid substrate and upon parallel electron input into complex I and II, expressed as pmol/ second / mg muscle fibre wet weight. bp, blood pressure; ALT, alanine-aminotransferase; AST, aspartate aminotransferase; TG, triglycerides; FFA, free fatty acids; SMR, sleeping metabolic rate; PCr, phosphocreatine recovery; M, malate; O, octanoyl-carnitine; G, glutamate; S, succinate; IHL, intra-hepatic lipid content

Differences in Baseline Characteristics Between the Three Studies

The HOM were significantly younger than the T2D and FDRs (p= 0.001 between-groups; Table 2). Between the groups there was a significant difference in body weight (p= 0.043) and body mass index (p= 0.019), because the FDRs had a significantly lower body weight and body mass index compared to HOM and T2D. By definition, due to the inclusion criteria of the studies, HOMA-IR values were significantly different between the three studies (p<0.001), with lowest values in FDRs (Table 2). Other general subject characteristics were comparable between the groups (Table 2).

Table 2. Differences between studies in baseline participant characteristics

	Variable	Study			p-value between groups
		HOM n=11	T2D n=17	FDR n=13	
General	Age, y	53 ± 2.1	63 ± 1.9	63 ± 1.9	0.001
	Body weight, kg	100.3 ± 3.8	93.6 ± 2.3	90.2 ± 1.7	0.043
	Height, m	1.78 ± 0.02	1.77 ± 0.01	1.77 ± 0.01	0.872
	BMI, kg/m ²	31.6 ± 0.9	30.0 ± 0.6	28.7 ± 0.6	0.019
	Total body fat, %	26.4 ± 0.5	25.9 ± 1.1	29.0 ± 0.9	0.066
	VO ₂ max, mL/kg/min	25 ± 1.3	24 ± 1.1	27 ± 1.5	0.139
	Diastolic bp, mmHg	81 ± 2.8	86 ± 1.9	86 ± 1.8	0.060
	Systolic bp, mmHg	131 ± 2.7	140 ± 2.8	136 ± 3.0	0.243
Blood biochemistry	HOMA-IR	2.80 ± 0.20	1.87 ± 0.17	1.27 ± 0.14	<0.001
	Glucose, mmol/L	5.4 ± 0.2	7.2 ± 0.3	5.5 ± 0.2	<0.001
	Insulin, pmol/L	83.2 ± 6.8	87.7 ± 10.7	76.0 ± 8.6	0.677
	Creatinine, µmol/L	76 ± 4.0	80 ± 3.4	84 ± 1.8	0.271
	Bilirubin, µmol/L	14.9 ± 0.9	9.7 ± 1.5	9.1 ± 1.0	0.008
	Albumin, g/L	38.0 ± 0.8	36.2 ± 0.6	34.3 ± 0.6	0.002
	gammaGT, U/L	30 ± 3.9	31 ± 3.5	23 ± 3.1	0.278
	AST, U/L	19 ± 1.5	27 ± 1.9	22 ± 2.1	0.015
	ALT, U/L	32 ± 2.2	32 ± 3.6	23 ± 3.3	0.115
	TG, mmol/L	2.27 ± 0.21	2.07 ± 0.35	1.46 ± 0.16	0.152
	FFA, mmol/L	0.50 ± 0.07	0.83 ± 0.07	0.70 ± 0.06	0.009
Energy metabolism	SMR, MJ/day [§]	7.74 ± 0.18	7.78 ± 0.13	7.58 ± 0.17	0.641
Mitochondrial function[#]	PCr t ½, sec	23 ± 2.9	26 ± 2.2	22 ± 2.5	0.509
	3MO, pmol/s/mg ww	28.66 ± 1.11	25.51 ± 1.38	32.13 ± 1.79	0.011
	3MOG, pmol/s/mg ww	47.62 ± 2.12	40.72 ± 1.89	47.25 ± 2.57	0.038
	3MOGS, pmol/s/mg ww	67.68 ± 3.22	56.21 ± 2.32	72.85 ± 3.68	0.001
	State U, pmol/s/mg ww	97.68 ± 6.72	72.41 ± 3.06	93.34 ± 4.59	<0.001
Ectopic lipid	IHL, %	2.2 ± 0.7	8.9 ± 1.5	2.5 ± 0.6	<0.001

Outcome parameters measured at baseline or after 30 days of placebo supplementation. Data are presented as mean ± SEM. [#] *In vivo* mitochondrial function: expressed as PCr half-time. *Ex vivo* mitochondrial function: ADP stimulated respiration (state 3) upon a lipid-like substrate and upon parallel electron input into complex I and II, expressed as pmol/second/mg muscle fibre wet weight. [§] Adjusted for fat free mass, kg; HOM, healthy obese men; T2D, patients with type 2 diabetes; FDRs, first-degree relatives of patients with T2D; BMI, body mass index; VO₂max, maximum rate of oxygen consumption; bp, blood pressure; RSV, resveratrol (free + conjugated); DHR, dihydro-resveratrol; ALT, alanine-aminotransferase; AST, aspartate aminotransferase; TG, triglycerides; FFA, free fatty acids; SMR, sleeping metabolic rate; PCr, phosphocreatine recovery; M, malate; O, octanoyl-carnitine; G, glutamate; S, succinate; IHL, intrahepatic lipid content

Blood biochemistry analyses revealed that T2D had significantly higher plasma glucose, FFA and AST. Also, IHL was significantly higher in the T2D ($p < 0.001$, between groups), and *ex vivo* mitochondrial function was lowest in the T2D patients ($p < 0.05$, between groups). Furthermore, significant differences were measured between groups in serum total bilirubin and albumin levels. Bilirubin was highest in the HOM ($p = 0.008$, between groups), but still within the normal range, whereas albumin was lowest in the FDRs ($p = 0.002$, between groups) and highest in the HOM.

In addition to evaluating baseline characteristics, plasma resveratrol (free + conjugated) and plasma DHR values measured during the resveratrol treatment period were compared (Figure 1A-B). Plasma resveratrol values, were significantly lower in the HOM study when compared to those measured in the T2D patients and FDRs ($p = 0.008$ between groups). Plasma resveratrol values were 183 ± 96 in HOM, 379 ± 41 in T2D and 412 ± 63 ng/mL in FDR at day 30 of the resveratrol treatment period. Plasma DHR levels were not significantly different at day 30 ($p = 0.232$ between groups). Plasma dihydro-resveratrol values on day 30 were 289 ± 94 in HOM, 445 ± 102 in T2D and 541 ± 80 ng/mL in FDRs.

Figure 1. Plasma resveratrol and dihydro-resveratrol measured throughout the studies. HOM, healthy obese men; T2D, patients with type 2 diabetes; FDR, first-degree relatives of patients with T2D

Influence of Participant Characteristics on Efficaciousness of Resveratrol

Next, we investigated the correlation between baseline participant characteristics and the response to resveratrol treatment using Pearson correlation coefficient analysis. Based on the analyses of the previous section, the following baseline characteristics were selected as potential predictors of response to resveratrol treatment: age, body weight, serum total bilirubin, serum albumin and HOMA-IR. In addition, although not a baseline characteristic, plasma resveratrol values are taken into account.

Pearson Correlation Coefficient Analysis

Plasma resveratrol values – upon resveratrol supplementation – correlated with baseline age and body weight (Table 3), but not with any of the resveratrol-induced outcome parameters. Plasma DHR levels correlated with baseline serum albumin levels ($r = -0.397$, $p = 0.012$).

Several correlations were found between baseline participant characteristics and resveratrol-induced changes in outcome parameters (Table 3). A negative correlation was observed between age and resveratrol-induced relative change in IMCL content ($r = -0.420$, $p = 0.012$). A positive correlation was found between body weight and resveratrol-induced changes in IMCL ($r = 0.380$, $p = 0.027$). Furthermore, significant correlations were found between baseline HOMA-IR and resveratrol-induced change in systolic blood pressure, HOMA-IR, IHL and IMCL. Especially, a strong correlation was found between HOMA-IR and resveratrol-induced change in IHL ($r = -0.509$, $p = 0.008$). There were several significant correlations between baseline serum total bilirubin levels and resveratrol-induced changes in metabolic parameters. Thus, baseline serum bilirubin correlated with resveratrol-induced changes in: diastolic blood pressure ($r = -0.359$, $p = 0.031$), plasma glucose ($r = -0.484$, $p = 0.002$), AST ($r = -0.333$, $p = 0.047$) and ALT ($r = -0.380$, $p = 0.022$). Furthermore, baseline bilirubin levels correlated significantly with resveratrol-induced change in IHL ($r = -0.480$, $p = 0.015$) (see also Table 3).

Table 3. Pearson correlation coefficients between participant characteristics assessed at baseline or after 30 days of placebo supplementation (columns) and resveratrol-induced changes in outcome parameters (rows)

		Characteristic					
	Parameter	Plasma RSV, ng/mL	Age, years	Weight, kg	HOMA-IR	Albumin, g/L	Bilirubin, μ mol/L
General	Plasma RSV, ng/mL	n.a.	0.379*	-0.430**	-0.292	-0.185	0.003
	Plasma DHR, ng/mL	-0.067	0.336*	-0.159	-0.243	-0.397*	-0.306
	Δ Diastolic bp, mmHg	0.161	0.218	-0.222	-0.288	-0.004	-0.359*
	Δ Systolic bp, mmHg	0.267	0.122	-0.094	-0.336*	0.021	-0.307
Blood biochemistry	Δ HOMA-IR	0.121	0.025	-0.225	-0.328*	0.115	-0.253
	Δ Glucose, mmol/L	0.035	0.262	-0.277	-0.166	0.152	-0.484**
	Δ Insulin, pmol/L	0.144	-0.128	-0.102	-0.321	0.062	0.017
	Δ AST, U/L	-0.074	-0.080	0.087	0.156	-0.089	-0.333*
	Δ ALT, U/L	0.010	0.150	-0.167	-0.130	-0.163	-0.380*
	Δ TG, mmol/L	-0.047	0.006	-0.107	-0.078	-0.156	-0.103
	Δ FFA, mmol/L	<0.01	-0.327*	0.179	-0.079	0.085	0.142
Energy metabolism	Δ SMR, MJ/day	-0.039	-0.197	-0.130	-0.161	0.264	0.079
Mitochondrial function*	Δ PCr t $\frac{1}{2}$, sec	0.120	0.138	0.041	-0.007	-0.066	0.037
	Δ 3MO, pmol/mg ww/s	-0.252	-0.004	0.233	-0.003	-0.117	0.006
	Δ 3MOG, pmol/mg ww/s	-0.299	0.076	-0.093	-0.024	-0.074	-0.167
	Δ 3MOGS, pmol/mg ww/s	-0.144	0.039	-0.025	-0.098	0.029	-0.152
	Δ State U, pmol/mg ww/s	-0.050	-0.073	-0.120	-0.150	0.032	-0.028
Ectopic fat accumulation	Δ IHL, %	0.240	0.294	-0.252	-0.509**	-0.179	-0.480*
	Δ rel. IMCL	-0.263	-0.420*	0.380*	0.373*	0.081	0.323

*Correlation is significant at the 0.05 level (two-tailed), **correlation is significant at the 0.01 level (two-tailed) # *In vivo* mitochondrial function: expressed as PCr half-time. *Ex vivo* mitochondrial function: ADP stimulated respiration (state 3) upon a lipid-like substrate and upon parallel electron input into complex I and II, expressed as pmol/mg muscle fibre wet weight/second; RSV, resveratrol (free+conjugated); DHR, dihydro-resveratrol; bp, blood pressure; ALT, alanine-aminotransferase; AST, aspartate aminotransferase; TG, triglycerides; FFA, free fatty acids; SMR, sleeping metabolic rate; PCr, phosphocreatine recovery; M, malate; O, octanoyl-carnitine; G, glutamate; S, succinate; IHL, intrahepatic lipid content; IMCL, intra-myocellular lipid content

Table 4. Stepwise linear regression of resveratrol-induced changes in outcome parameters and participant characteristics

	Variable	Predictor	R2	Adjusted R2	B	Beta	p-value
General	Plasma RSV, ng/mL	(Constant)			1230.79		
		Body weight	0.20	0.17	-9.43	-0.45	0.007
	Plasma DHR, ng/mL	(Constant)			2399.44		
		Albumin	0.18	0.16	-53.84	-0.43	0.012
	Δ Diastolic bp, mmHg	(Constant)			3.48		
		Bilirubin	0.12	0.09	-0.31	-0.35	0.044
	Δ Systolic bp, mmHg	None					
Blood bio-chemistry	Δ HOMA-IR	None					
	Δ Glucose, mmol/L	(Constant)			-1.43		
		Bilirubin + Albumin	0.32	0.28			0.002
		Bilirubin			-0.061	-0.52	0.001
		Albumin			0.060	0.31	0.041
		(Constant)			0.67		
		Bilirubin	0.23	0.20	-0.06	-0.47	0.004
	Δ AST, U/L	None					
	Δ ALT, U/L	(Constant)			8.00		
		Bilirubin	0.14	0.11	-0.64	-0.37	0.030
	Δ FFA, mmol/L	(Constant)			1.07		
		Age	0.13	0.10	-0.02	-0.35	0.040
Ectopic fat	Δ IHL, %	(Constant)			2.00		
		Bilirubin	0.22	0.19	-0.16	-0.47	0.020
	Δ rel. IMCL	(Constant)			-185.96		
		Body weight	0.14	0.11	2.43	0.37	0.041

Results of stepwise linear regression analyses are displayed. Resveratrol-induced changes in outcome parameters are entered as dependent variables. Independent variables included in each regression analyses were: age, body weight, HOMA-IR, serum albumin and serum bilirubin. The best individual predictor is displayed, as well as the best overall model (a combination of variables that explain the greatest amount of variation in the dependent variable). [#] *In vivo* mitochondrial function: expressed as PCr half-time. *Ex vivo* mitochondrial function: ADP stimulated respiration (state 3) upon a lipid-like substrate and upon parallel electron input into complex I and II, expressed as pmol/mg muscle fibre wet weight/second; RSV, resveratrol (free+conjugated); DHR, dihydro-resveratrol; bp, blood pressure; ALT, alanine-aminotransferase; AST, aspartate aminotransferase; TG, triglycerides; FFA, free fatty acids; SMR, sleeping metabolic rate; PCr, phosphocreatine recovery; M, malate; O, octanoyl-carnitine; G, glutamate; S, succinate; IHL, intrahepatic lipid content; IMCL, intra-myocellular lipid content

Figure 2. Correlations are displayed between significant predictors of resveratrol-induced changes in metabolic health outcome parameters and resveratrol-induced changes in outcome parameters. DHR, dihydro-resveratrol; FFA, free fatty acids; IMCL, intramyocellular lipid

Stepwise Backwards Linear Regression Analysis

To investigate which of the above characteristics are potential effect modifiers of resveratrol-induced changes in outcome parameters, stepwise backwards linear regression analyses was performed. Body weight was the only significant predictor of resveratrol plasma values ($p = 0.007$, Figure 2A). Furthermore, baseline albumin levels were identified as a predictor of plasma DHR values ($p = 0.012$, Figure 2B), accounting for 16% of the variance in plasma DHR levels (Table 4).

Age was identified as a predictor of resveratrol-induced change in plasma FFA levels ($p = 0.040$, Figure 2C), but not as a predictor of any of the other metabolic health outcome parameters (Table 4). Although, a negative correlation existed between age and resveratrol-induced change in IMCL content, stepwise linear regression analyses did not identify age as a significant predictor. Body weight however, was identified as a predictor of resveratrol-induced changes in relative IMCL ($p = 0.041$, Figure 2D). Furthermore, HOMA-IR was not regarded a significant predictor for any of the resveratrol-induced changes in outcome parameters.

Figure 3. Correlations are displayed between the significant predictor serum bilirubin – measured after 30 days of placebo - and resveratrol-induced changes in metabolic health outcome parameters. ALT, alanine-aminotransferase.

Baseline serum bilirubin was found to significantly predict resveratrol-induced change in diastolic blood pressure ($p = 0.044$), ALT ($p = 0.030$), IHL content ($p = 0.020$) and plasma glucose ($p = 0.004$) (Figure 3A-D). The latter was especially profound when also baseline albumin levels were taken into account ($p = 0.002$). Combined, baseline serum bilirubin and albumin levels account for 28% of the variance in resveratrol-induced change in plasma glucose. Bilirubin had a higher impact than albumin, established by comparing the standardized coefficients ($\beta = -0.52$, versus $\beta = 0.31$ respectively). Finally, even though a negative correlation existed between baseline bilirubin and resveratrol-induced change in AST, bilirubin was not regarded a significant predictor of change in AST.

DISCUSSION

Previous research has shown that resveratrol can improve metabolic health in some but not all (patient) populations. Here, we combined data from three human clinical trials - including diverse participant populations – in an attempt to unravel why resveratrol is effective in some but not all individuals. First, effects of resveratrol treatment were evaluated on a whole group basis. Secondly, we determined which baseline characteristics were different across the three studies, as these characteristics may be effect modifiers of response to resveratrol. Finally, Pearson correlation coefficient analysis followed by stepwise linear regression was employed to explore the relationship between baseline participant characteristics and resveratrol-induced changes in metabolic health outcome-parameters. This analysis revealed that serum total bilirubin was the main predictor of response to resveratrol treatment. Thus, baseline serum bilirubin was identified as significantly related to resveratrol-induced change in systolic blood pressure, plasma glucose, ALT and IHL.

One of the findings was that, independent of the study population, administration of 150mg resveratrol for 30 days consistently improved *ex vivo* muscle mitochondrial function upon a lipid-like substrate, decreased systolic blood pressure and lowered SMR. This is in line with other reports that also found robust improvements in *ex vivo* mitochondrial function [15] and, albeit less robust, improvement in systolic blood pressure [16, 17]. The effect of resveratrol on SMR is less well investigated. In fact, the clinical trials included in the present analysis are the only studies that investigated if resveratrol affects SMR in humans.

Several differences in baseline participant characteristics were identified between the three studies included in this analysis, which may potentially explain the difference in response to resveratrol across the groups. The T2D patients had the lowest *ex vivo* muscle mitochondrial oxidative capacity. The decreased mitochondrial function in patients with T2D has also been previously established by us and others [18]. Although age and body weight were different among the groups, stepwise linear regression analysis revealed that age did not predict response to resveratrol treatment for any of the metabolic health outcome-parameters, except resveratrol-induced change in FFA. For body weight, relevant correlations were found with plasma resveratrol (free + conjugated) levels and resveratrol-induced change in IMCL. Body weight accounted for 17% of the variance in plasma resveratrol, with higher body weight resulting in lower plasma resveratrol levels. This relationship could imply a higher volume of distribution, leading to lower plasma values. Considering resveratrol supplementation gave most improvements in metabolic health in the HOM participants, who had the highest body weight, the higher volume of distribution does not negatively seem to affect effectiveness of resveratrol. Body weight did not influence plasma DHR levels, although a tendency was observed towards higher DHR plasma levels in the T2D and specifically FDRs.

Plasma resveratrol values (free + conjugated resveratrol combined) were not associated with any of resveratrol-induced changes in the outcome parameters. Since it is generally known that resveratrol is rapidly metabolized into its glucuronidated and sulfated form [19], measuring plasma concentrations of free, glucuronidated and sulfated resveratrol separately could shed light on which form of resveratrol actually induces beneficial effects on metabolic health. In turn, this could provide additional information on why resveratrol is more effective in certain individuals than others, and which subject characteristics play a role herein. Unfortunately, in these three clinical trials plasma concentrations of separately measured free-resveratrol were below detection level. The lack of information on the concentrations of the different forms of resveratrol separately, could explain why we did not find any relationships between plasma resveratrol levels and resveratrol-induced changes in the outcome parameters.

Remarkably, we found distinctive values of baseline serum albumin and total bilirubin between the three studies. In general, HOM had the highest values of both serum albumin and bilirubin, and the participants of the FDR study the lowest, albeit all values were within the normal physiological range. Stepwise linear regression analyses revealed that serum albumin was a negative predictor of plasma DHR values, accounting for 16% of the variance in plasma DHR. It has been shown previously that resveratrol can bind to serum proteins, specifically albumin [20-22], and it has been hypothesised that resveratrol might get trapped in serum in so-called resveratrol-albumin complexes [23]. In addition, higher baseline serum albumin levels negatively affected resveratrol-induced change in plasma glucose, but only when also baseline bilirubin levels were taken into account. Thus, lower baseline serum albumin levels, combined with higher baseline bilirubin levels led to stronger resveratrol-induced improvements of plasma glucose levels. The combination of the predictive effect of baseline serum albumin and bilirubin, and previously established binding capacity of resveratrol to serum proteins, suggest that albumin and bilirubin could both play a role in the bioavailability and distribution of circulating resveratrol [24].

Interestingly, baseline serum bilirubin levels were related to several resveratrol-induced improvements in metabolic health outcome parameters. Thus, changes in diastolic blood pressure, plasma glucose, ALT and IHL were all bigger if baseline serum bilirubin levels were higher. To our knowledge this relationship has not been observed before. Interestingly, bilirubin is a substrate of UDP-glucuronosyltransferase 1A1 (UGT1A1). UGT1A1 is a gene belonging to a family of genes that generate enzymes involved in glucuronidation of among others *trans*-resveratrol [25]. It has been suggested that bilirubin can be seen as a surrogate for UGT1A1 activity [26, 27]. In that context, higher serum bilirubin levels - as seen in our HOM - could indirectly imply a higher UGT1A1 activity, suggesting that UGT1A1 activity may be a factor involved in the activation of resveratrol. Although speculative, this could imply that glucuronidation - by UGT1A1 - of *trans*-resveratrol is important for effectiveness of resveratrol.

Figure 4. Proposed mechanism of de-conjugation of resveratrol, initiated by mitochondrial stress. 1) Resveratrol-glucuronides bind to the cell surface. 2) Resveratrol-glucuronide is de-conjugated into its aglycone by β -glucuronidase. The de-conjugation activity of β -glucuronidase is promoted by acidification instigated by mitochondrial dysfunction. 3) The aglycone passes through the cell membranes where it activates PGC-1 α and thereby initiates improvement in mitochondrial biogenesis.

Furthermore, it is believed that resveratrol conjugates, in particular glucuronic acid conjugates, can serve as a pool in the body from which active resveratrol can be released locally by tissue β -glucuronidases [19]. Resveratrol glucuronides can become deconjugated at the target sites of action, thereby releasing the aglycone for eliciting biological effects [19]. β -glucuronidase is an important enzyme for deconjugation, and it has been shown that mitochondrial stress can actually stimulate the enzymatic activity of β -glucuronidase [28]. It could thus be speculated that acidification induced by mitochondrial stress stimulates β -glucuronidase enzyme activity leading to de-conjugation of resveratrol-glucuronide thereby releasing its active aglycone. In Figure 4 this potential mechanism is schematized. Potentially this could also explain why despite inconsistencies in achieving effects on glucose homeostasis, there is consistency in achieving improvement in *ex vivo* muscle mitochondrial function, considering the participants had relatively low mitochondrial function [29, 30]. This may suggest that mitochondrial stress at the level of the muscle in fact stimulates the conversion of resveratrol conjugates to its active form in all three groups, and may explain why the effects on mitochondrial function are very consistent and less influenced by other factors. The finding that none of the baseline characteristics from our study participants were predictive for resveratrol-induced improvement of mitochondrial function would fit with this hypothesis of an alternative mechanism of action, although it is still also possible that the effects of resveratrol on mitochondrial function were too small to induce whole body metabolic health effects.

Study Limitations

Caution should be taken when interpreting the results of this combined-data analysis. Resveratrol treatment – all three studies combined – was compared to placebo using a paired t-test. In this analysis method study and treatment sequence cannot be accounted for. If a linear mixed model analysis had been conducted treatment sequence and study effects could have been taken into account, which in general gives the most robust results. However, the sample size was regarded too small for this type of analyses. In continuation, only parameters that were significantly different between the three studies were used for conducting the linear regression analyses. Again, this approach was chosen because of the relatively small sample size. Due to this approach, potential effect modifiers of resveratrol might have been missed. The same applies to the stepwise regression analyses that followed the linear regression analyses: effect modifiers could have been missed by only incorporating a selection of variables. Furthermore, it would be valuable to also take study and treatment sequence into consideration in the regression analyses. Thus, to be able to make more robust statements about effect modifiers of resveratrol, increasing the total sample size is essential. Incorporating the current combined-data analysis into a broader meta-analysis may therefore be worthwhile.

CONCLUSION

To summarise, baseline serum bilirubin levels were identified as the most significant effect modifiers of resveratrol-induced change in metabolic health outcome parameters. Higher baseline serum bilirubin levels were associated with improvements in metabolic health upon resveratrol treatment. We hypothesise that differences in bilirubin levels could represent differences in activity of glucuronidating enzymes such as UGT1A1, implying glucuronidation plays a role in the activation of resveratrol. Future studies are needed to test this hypothesis and to investigate the importance of glucuronidation of resveratrol.

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CHAPTER 6

A randomised controlled trial of long-term
resveratrol use: an interim-analysis

Marlies de Ligt, Bas Havekes, Vera B. Schrauwen-Hinderling, Patrick
Schrauwen

In preparation

ABSTRACT

Background

Resveratrol has been reported to promote metabolic health in animal studies and short-term human clinical trials, but long-term studies are lacking. Therefore, the aim of the present study was to investigate the effects of 6-months of 150 mg/day resveratrol supplementation – previously shown to improve muscle mitochondrial function – on metabolic health outcome parameters. Here, we report the results of an interim-analysis of this study.

Methods

Twenty participants (15 men, 5 women) with a mean age of 63 ± 1.6 years completed the study and data was analysed for this interim-analysis. The participants were overweight to obese, with a mean body mass index of 29 ± 0.4 kg/m². The study was carried out in parallel design and participants were randomised to receive either 150 mg/day of resveratrol or placebo for 6 months. The primary outcome of the study is insulin sensitivity, as assessed by an oral glucose tolerance test, but was not analysed here to preserve integrity of the study until completion. Secondary outcomes measures were intrahepatic liver (IHL) content measured by Magnetic Resonance Spectroscopy and expressed as percentage of the water resonance, body composition, resting energy metabolism, blood pressure, plasma markers and physical performance. The change in outcome parameters from baseline to month 6 (delta: Month 6 - baseline) was used to evaluate differences between the two treatment groups by independent samples t-test.

Results

At baseline, no differences were observed between the two intervention groups. No significant effect of resveratrol treatment was found for change in any of the plasma markers, except for HbA1c (%). HbA1c decreased upon resveratrol ($\Delta -0.11 \pm 0.06\%$) while increasing upon placebo ($\Delta 0.13 \pm 0.06\%$), and this difference was statistically between the groups ($p=0.016$). Change in IHL content over the 6 months tended to be different between the groups ($p=0.066$), with IHL content decreasing slightly upon resveratrol ($\Delta -0.55 \pm 0.58\%$) and increasing within the placebo group ($\Delta 1.00 \pm 0.49\%$). There was a tendency towards a greater improvement in the stand-sit time after 6 months of resveratrol compared to placebo ($p=0.098$, between groups). Finally, no differences in change in body composition, blood pressure or energy utilisation were found between the groups.

Conclusion

This interim-analysis suggests that 6-months of 150mg resveratrol per day lowers HbA1c, tends to decrease IHL content and tends to decrease stand-sit time. Considering this is an interim-analysis, and the primary study outcome parameter is not taken into account in this analysis, no definitive conclusions can be drawn.

INTRODUCTION

In our western society, the number of people suffering from age-related chronic diseases such as obesity and type 2 diabetes mellitus (T2D) increases progressively [1]. One of the most potent non-pharmacological interventions known to alleviate these deleterious conditions is calorie restriction. Although short-term dietary restriction in humans has metabolic effects such as improving insulin sensitivity [2] and reducing cardiovascular risk markers [3], eating less in favour of creating a desirable metabolic profile is not likely to gain widespread compliance. As such, dietary restriction mimetics are being explored, that evoke some of the beneficial effects of calorie restriction without an actual reduction in calorie intake. Resveratrol, a natural polyphenol present in various food items, was identified as a promising activator of sirtuin 1 (SIRT1) [4], a member of a family of NAD⁺-dependent histone deacetylases that play a central role in health span regulation by mediating effects in metabolic stress situations such as high-fat diet-induced obesity [5]. Given the growing number of patients suffering from metabolic diseases, compounds that activate SIRT1 directly or indirectly might offer protection against the onset of metabolic damage and promote healthy ageing.

The potential of resveratrol has been extensively studied in animals. These studies confirm the hypothesis that resveratrol is indeed beneficial for improving metabolic health, such as improving insulin sensitivity and decreasing liver fat accumulation, via an improvement of muscle mitochondrial function [6]. However, results from human clinical trials are inconsistent. Some studies show clear enhancements in parameters related to metabolic health [7-12], while others see no or barely any effects [13-20]. On the contrary, there has been consistency in those studies that have employed invasive measurements of muscle metabolism in finding improvements in muscle mitochondrial oxidative capacity upon several weeks of low-dose resveratrol supplementation [7, 13, 21, 22] despite discrepancies in finding improvements in glucose homeostasis in these trials. Why some studies do, and others do not find effects of resveratrol on metabolic parameters is so far unknown. It may involve factors like dosage of resveratrol, as well as the duration of the supplementation period. One of the explanations for a lack of effect on more clinically-relevant parameters, may be the treatment duration applied in human clinical trials. The treatment duration may have been too short for improvements in for example mitochondrial function to translate into effects on whole body metabolic health. Long-term supplementation with resveratrol could provide an important bridge between findings from animal and human studies. Therefore, the aim of the present study was to investigate the effects of 6 months of 150 mg/day resveratrol supplementation – previously shown to improve muscle mitochondrial function – on metabolic health outcome parameters in relatively healthy overweight and obese men and women. Here, we report the results of an interim analysis of this study.

RESEARCH DESIGN AND METHODS

Participants

Overweight and obese (BMI 27-35 kg/m²) men (40-70 years) and postmenopausal women (50-70 years) were recruited. Additional inclusion criteria were stable body weight (no weight gain or loss >5 kg in the past three months), and willingness to limit consumption of resveratrol-containing food products or supplements. Exclusion criteria were uncontrolled hypertension, HbA1c >6.5%, T2D, alcohol consumption >20 g/d, use of medication known to interfere with glucose homeostasis/metabolism, and participation in another biomedical study within one month prior to start of the intervention.

Study Overview

In this parallel group, double-blind, randomised, clinical trial, 6 months of resveratrol supplementation was compared to 6 months of placebo. The protocol was reviewed and approved by the medical ethics committee of Maastricht University and Medical Centre (NCT02565979). The data presented in this manuscript represent the results of an interim analysis. Eligible subjects started with a 2-week run-in period during which they were instructed to stop consuming resveratrol-containing food items such as grapes, red wine and peanuts. This was continued during the whole intervention period. After the run-in period participants were randomly assigned to 6 months of 150 mg/day of *trans*-resveratrol (resVida™, 99.9%; provided by DSM Nutritional Products Ltd.) or 6 months of placebo. At the beginning of the treatment period, on two separate days, baseline measurements were performed, and these measurements were repeated again at the end of the 6 months on two separate days. The general experimental design is presented schematically in Figure 1. During the intervention period, participants came to the university once a month in the morning in overnight fasted state. During these visits body weight and blood pressure (Omron Healthcare, Hamburg, Germany) were measured and a blood sample was obtained for the analysis of plasma resveratrol. The latter was analysed by mass spectroscopy, as previously described [7]. Finally, during these monthly visits the participants received a new container of supplements for the upcoming month.

In this interim-analyses not all measures will be included in the analyses to preserve data integrity until final completion of the study. Therefore, the main outcome parameter was not analysed, which implies results from the oral glucose tolerance test (OGTT) will not be shown.

Nutrition and Physical Activity Standardisation

To minimize meal effects on the metabolic health outcome parameters all subjects consumed the same standardized meal the evenings before the baseline and 6-month measurements (559kcal, 73g carbohydrates, 16g fat and 26g protein). They consumed the meals around the same time and remained fasted until completing of the OGTT. In addition, they were instructed to avoid intense physical activity two days prior to these visits.

Figure 1. Schematic presentation of the study design.

OGTT, oral glucose tolerance test; 6MWT, 6-minute walk test; TCST, Timed Chair-Stand Test; DXA, dual-energy X-ray absorptiometry

Blood Sampling

Blood samples were collected between 07:30 and 09:00hr after an overnight fast. At baseline and after 6 months, general safety parameters were determined: urea, creatinine, bilirubin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT). In addition, markers for dyslipidaemia (total cholesterol, HDL- and LDL-cholesterol, triglyceride and free fatty acid) and glucose homeostasis (glucose, insulin and HbA1c) were measured.

Insulin Sensitivity

At baseline and after 6 months, insulin sensitivity was calculated from a 2-hour oral glucose tolerance tests (OGTT). Both, before and after the 6-month intervention period OGTTs were performed twice, separated by 2-6 days, and outcomes were averaged to reduce inter-individual variation. A standard 2h 75g (Novolab NV, Geraadsbergen, Belgium) OGTT was performed, and blood samples were collected at $t=0$, 30, 60, 90 and 120 for measurement of plasma glucose and insulin. During the OGTT participants remained seated without moving, eating or drinking. The Matsuda Index was calculated as estimate of insulin sensitivity, using glucose and insulin values of time points $t=0$, 60 and 120 [23]. The average of the duplicate Matsuda Index values was used for statistical analysis. The outcome of the OGTTs is not taken into account in the results or discussion of this manuscript.

Intrahepatic Lipid Content

At baseline and after 6 months, intrahepatic lipid (IHL) content was quantified by proton magnetic resonance spectroscopy (¹H-MRS). The MRS scans were performed on a 3T whole body scanner (Achieva Tx; Philips Healthcare, Best, The Netherlands) in the morning in the overnight fasted state. In short, a PRESS sequence was used with a repetition time of 4 s, an echo time of 32.5 ms. A voxel of 20 x 20 x 20 mm was placed in the lower right hepatic lobe. Values are given as T2 corrected ratios of the CH₂ peak relative to unsuppressed water resonance, expressed as percentage (for further details see [13]).

Body Composition

Total mass, fat mass, lean mass and fat percentage were measured by dual energy X-ray absorptiometry (DXA) using the same Hologic Discovery scanner (Hologic, The Netherlands) at baseline and after 6 months of treatment.

Resting Energy Metabolism

At baseline and after 6 months, energy expenditure and substrate utilisation were measured in the overnight fasted state. Gas exchange was measured by open circuit respirometry with an automated ventilated hood system (Omnical, Maastricht Instruments, Maastricht University) for 45 minutes. Data from the first ten and last five minutes was omitted. The Weir equation [24] was used to calculate whole body resting energy expenditure from measurements of VO₂ consumption and VCO₂ production. Carbohydrate and fat oxidation rates were calculated using the non-protein equations by Péronnet and Massicotte [25].

Physical Performance

In the early afternoon, after consumption of a light lunch, two physical performance tests were performed: the 6-minute walk test (6MWT) and the Timed Chair-Stand Test (TCST). During the 6MWT participants were asked to walk as far as possible within 6 minutes, without running, on a 10m flat track. For the TCST participants had to complete 10 chair-rise repetitions as fast as possible and time was recorded.

Statistical Analysis

Statistics were performed using SPSS 24.0 for Mac OS and p<0.05 was considered statistically significant. Shapiro-Wilk normality test was used to evaluate normality distribution. Data is presented as mean and standard error of the mean in text and tables, and as minimum, first quartile, median, third quartile and maximum in graphs. The change in outcome parameters from baseline to month 6 (delta: Month 6 - baseline) was used to evaluate differences between the two treatment groups by independent samples t-test. If data were not normally distributed, Mann-Whitney U test was used and data presented as median and range. Because of randomization, it is expected that confounders will be divided equally. Within group differences were explored by paired sample t-test.

RESULTS

Study Compliance

Compliance was confirmed by capsule counting and analysis of plasma levels of resveratrol (free + conjugated) and dihydro-resveratrol (DHR), a metabolite of resveratrol, on a monthly basis. Resveratrol and DHR levels were below detection in the placebo group at all time points, while in the resveratrol group both were elevated, with resveratrol levels of 521 ± 105 ng/mL and DHR levels of 569 ± 127 ng/mL after 6 months.

Baseline Characteristics

Twenty participants (15 men, 5 women) with a mean age of 63 ± 1.6 years completed the study and data was analyzed for this interim analysis. The participants were overweight to obese, with a mean body mass index of 29 ± 0.4 kg/m² (Table 1). At baseline, no differences were observed between the two intervention groups for subject characteristics or any of the outcome parameters. During the entire study, participants were encouraged to maintain their initial body weight. Overtime, therefore, no difference was observed in total body weight in either of the groups (change in resveratrol group: -0.41 ± 0.72 kg; change in the placebo group: 0.71 ± 0.43 kg). In contradiction to previous studies, no improvement upon resveratrol was seen for systolic or diastolic blood pressure (Figure 2A-B).

Table 1. Subject characteristics per intervention group, upon start of the intervention period

Variable	Intervention	
	Resveratrol	Placebo
Gender, <i>n</i> (m/f)	8/2	7/3
Family history of T2D ^a , <i>n</i>	2	5
Age (y)	62 ± 2.1	63 ± 2.4
Body weight (kg)	87.2 ± 4.1	92.1 ± 4.2
Body mass index (kg/m ²)	29 ± 0.5	29 ± 0.7
Systolic blood pressure (mmHg)	134 ± 3.6	134 ± 2.0
Diastolic blood pressure (mmHg)	85 ± 1.9	85 ± 3.1
Medication use, <i>n</i>	4	3
Anti-hypertensives	2	2
Statins	1	1
Other	2	2

Data are presented as mean $\pm$ SEM; ^aFirst-degree relative of patient with T2D

Figure 2. Change in systolic and diastolic blood pressure upon 6 months of resveratrol or placebo. At baseline and month 6, blood pressure was measured in triplicate, using an automated electronic oscillometric device. Data are presented as minimum, first quartile, median, third quartile and maximum. Data from three participants was excluded because of the use of anti-hypertensive medication; $n = 8$ in the resveratrol group and $n = 9$ in the placebo group. Δ : Month 6 – baseline; RSV, resveratrol

Plasma Biochemistry

Plasma markers were analysed related to glucose homeostasis, dyslipidaemia and safety. Concerning glucose homeostasis, no changes were measured regarding glucose ($\Delta -0.05 \pm 0.12$ and $\Delta -0.05 \pm 0.08$ mmol/L, upon resveratrol and placebo respectively) or insulin upon 6 months of resveratrol supplementation compared to 6 months of placebo (Table 2). However, the change in HbA1c (%) differed significantly between resveratrol and placebo ($p=0.016$, Table 2). HbA1c tended to decrease slightly in the resveratrol group ($p=0.084$, within-group difference), while a tendency for a slight increase in the placebo group was observed ($p=0.096$, within-group difference). Plasma markers related to dyslipidaemia remained unaffected by resveratrol treatment (Table 2). Finally, in plasma values related to safety no changes were observed: bilirubin, creatinine, urea, AST and ALT did not change over 6 months within and between groups. Serum creatinine levels tended to decrease by -3.6 ± 6.19 $\mu\text{mol/L}$ upon resveratrol ($p=0.084$ for within-group differences), while they tended to increase by 6.0 ± 2.72 $\mu\text{mol/L}$ upon placebo ($p=0.096$, within-group). The differences between the groups were not statistically significant ($p=0.190$, Table 2).

Table 2. Plasma biochemistry

	resveratrol (n=10)				placebo (n=10)				p-values	
	baseline	month 6	delta	baseline	month 6	delta	baseline ^a	delta ^b		
Glucose, mmol/L	5.4 ± 0.12	5.4 ± 0.12	-0.05 ± 0.12	5.3 ± 0.12	5.2 ± 0.12	-0.05 ± 0.08	0.454	0.998		
Insulin, µU/mL	10.6 ± 1.36	10.5 ± 1.21	-0.08 ± 0.70	10.6 ± 1.40	10.0 ± 0.96	-0.67 ± 0.81	0.732	0.607		
HbA1c, %	5.6 ± 0.09	5.5 ± 0.12	-0.11 ± 0.06	5.6 ± 0.12	5.7 ± 0.10	0.13 ± 0.06	0.949	0.016		
Triglycerides, mmol/L	1.4 ± 0.16	1.6 ± 0.23	0.25 ± 0.15	1.5 ± 0.20	1.7 ± 0.23	0.19 ± 0.11	0.633	0.829		
Free fatty acids, µmol/L	469 ± 36.2	461 ± 38.2	-7.6 ± 38.9	446 ± 39.6	469 ± 49.6	23.2 ± 43.8	0.677	0.608		
Total cholesterol, mmol/L	5.2 ± 0.20	5.5 ± 0.21	0.28 ± 0.15	5.1 ± 0.22	5.4 ± 0.26	0.32 ± 0.22	0.826	0.870		
HDL cholesterol, mmol/L	1.3 ± 0.12	1.3 ± 0.15	-0.01 ± 0.06	1.3 ± 0.12	1.4 ± 0.08	0.08 ± 0.08	0.950	0.361		
LDL cholesterol, mmol/L	3.2 ± 0.21	3.4 ± 0.20	0.20 ± 0.16	3.1 ± 0.18	3.3 ± 0.22	0.13 ± 0.12	0.781	0.741		
Bilirubin, µmol/L	9.8 ± 1.19	9.4 ± 0.94	-0.39 ± 0.74	8.7 ± 1.13	9.7 ± 1.38	0.91 ± 1.22	0.514	0.374		
Creatinine, µmol/L	90 ± 4.9	87 ± 5.8	-3.6 ± 6.19	81 ± 4.1	87 ± 4.3	6.0 ± 2.72	0.153	0.190		
Urea, mmol/L	5.4 ± 0.41	5.1 ± 0.34	-0.25 ± 0.14	5.2 ± 0.22	5.2 ± 0.41	-0.01 ± 0.29	0.670	0.499		
ALT, U/L	31 ± 4.5	34 ± 5.9	2.5 ± 2.37	23 ± 1.9	24 ± 2.8	0.4 ± 2.06	0.125	0.486		
AST, U/L	24 ± 1.6	25 ± 2.5	1.0 ± 1.72	21 ± 1.1	23 ± 2.1	1.2 ± 2.03	0.184	0.938		

Data are presented as mean ± SEM. HDL, high-density lipoprotein; LDL, low-density lipoprotein; ALT, alanine transaminase; AST, aspartate transaminase; ^a baseline resveratrol group compared to baseline placebo group values; ^b delta: month 6 – baseline

Figure 3. Change in intrahepatic lipid content upon 6 months of resveratrol or placebo

Intrahepatic lipid content was determined at baseline and after 6 months of treatment by ^1H -MRS. Values are given as T2 corrected ratios of the CH_2 peak relative to unsuppressed water resonance, expressed as percentage. Changes over time were compared using independent samples t-test. Data are presented as minimum, first quartile, median, third quartile and maximum. Data from two participants was missing due to claustrophobia; $n=10$ in the resveratrol group and $n=8$ in the placebo group. Δ : Month 6 – baseline; RSV, resveratrol

Intrahepatic Lipid Content

IHL content appeared to be higher at baseline in the resveratrol group compared to those enrolled for receiving placebo (5.50 vs 2.60% respectively). However, this did not reach statistical significance ($p=0.126$). The change in IHL content over 6 months tended to be different between the groups ($p=0.066$, Figure 3), which could mainly be contributed to an increase within the placebo group ($\Delta 1.00 \pm 0.49\%$, $p=0.081$) and a slight decrease in IHL content within the resveratrol group ($\Delta -0.55 \pm 0.58\%$, $p=0.325$).

Body Composition

As described, no difference was observed in changes in total body weight between groups. There was also no significant change in total fat mass between the groups ($p=0.101$, Figure 4A). However, within the placebo group total body fat increased ($p=0.010$) while upon resveratrol total body fat was unchanged ($p=0.436$). The change in lean mass over the 6 months was not significantly different between the groups ($p=0.427$; Figure 4B). Nevertheless, lean body mass decreased significantly within the placebo group ($p=0.043$), while it remained stable upon resveratrol ($p=0.229$). When specifically looking at fat percentage of the trunk, which is an important risk factor for metabolic abnormalities, the same tendencies could be observed. An increase in trunk fat percentage upon placebo was observed ($p=0.010$), with no change upon resveratrol ($p=0.315$) and no significant difference in the change in trunk fat mass between groups ($p=0.189$, Figure 4C).

Figure 4. Change in body composition upon 6 months of resveratrol or placebo

Body composition was determined at baseline and after 6 months of treatment using dual-emission X-ray absorptiometry. Changes over time were compared using independent samples t-test. Data are presented as minimum, first quartile, median, third quartile and maximum; $n=10$ per group. Δ : Month 6 – baseline; RSV, resveratrol

Resting Energy Metabolism

Previously, it has been shown that resveratrol can lower sleeping metabolic rate and the respiratory exchange ratio. Nevertheless, in this study no differences were found between the two groups regarding changes in resting energy expenditure, respiratory exchange ratio, carbohydrate oxidation and fat oxidation (Table 3). Also within the groups no changes were detected in energy utilisation.

Physical Performance

Walking distance during the 6-minute walk-test increased significantly within both groups (resveratrol: $\Delta 21.1 \pm 7.76$ m; placebo: $\Delta 15.3 \pm 5.69$ m), with no difference between the groups ($p=0.550$, Figure 5A). However, there was a tendency towards a greater improvement in the stand-sit time of the Timed-Chair Stand Test after 6 months of resveratrol compared to placebo ($p=0.098$ between groups; Figure 5B). This close-to significant improvement in stand-sit time was initiated by a significant reduction in stand-sit time within the resveratrol group ($p=0.001$), with no change within the placebo group ($p=0.401$).

Table 3. Resting energy metabolism

	resveratrol (n=10)				placebo (n=10)				p-values	
	baseline	month 6	delta	baseline	month 6	delta	baseline	month 6	baseline ^a	delta ^b
Energy expenditure, kJ/min	4.90 ± 0.21	4.96 ± 0.20	0.07 ± 0.11	5.14 ± 0.15	5.09 ± 0.16	-0.05 ± 0.09	0.352			0.400
Carbohydrate oxidation, g/min g/min	0.10 ± 0.02	0.12 ± 0.01	0.02 ± 0.01	0.12 ± 0.01	0.11 ± 0.02	-0.01 ± 0.02	0.397			0.183
Fat oxidation, g/min	0.08 ± 0.01	0.08 ± 0.00	-0.01 ± 0.01	0.08 ± 0.00	0.09 ± 0.01	0.00 ± 0.01	0.876			0.346
Respiratory exchange ratio	0.79 ± 0.02	0.81 ± 0.01	0.01 ± 0.02	0.80 ± 0.01	0.80 ± 0.01	-0.01 ± 0.02	0.538			0.249

Differences in baseline values and deltas were analysed by independent samples t-test. Data are presented as mean ± SEM; ^a baseline resveratrol compared to baseline placebo group values; ^b delta: month 6 - baseline

Figure 5. Effect of resveratrol supplementation on functional parameters of physical performance. Between-group differences in change from baseline to month 6 were tested using independent samples t-test. A: Distance covered in the 6-minute walk test at baseline and follow-up per group. In both groups, data from one subject was omitted due to hip pain complaints ($n = 9$ in resveratrol, $n = 9$ in placebo). B: Time to complete the Timed Chair-Stand Test at baseline and follow-up. Data from one subject was omitted due to hip pain complaints; $n = 10$ in the resveratrol group, and $n = 9$ in the placebo group. Data is presented as individual data points. RSV, resveratrol

DISCUSSION

In this randomized, placebo-controlled, parallel group clinical trial, we investigated the effects of long-term treatment with 150mg resveratrol per day in an overweight population of men and women on metabolic health.

We investigated if long-term resveratrol treatment exerted beneficial effects on blood markers related to dyslipidemia and glucose homeostasis. We found that none of the plasma markers related to dyslipidemia was affected by resveratrol treatment: total cholesterol, HDL and LDL cholesterol, free fatty acids and triglycerides remained unaffected over the 6-month intervention period. So far, a few human clinical trials found reductions in cholesterol, while other studies reported inconclusive results or did not find effects [26]. Recently, a study has been published where treatment with a relatively high-dose of resveratrol lead to an increase in total and LDL cholesterol [27]. The combination of our interim-analysis and previous clinical trials indicates that resveratrol seems less suitable for targeting dyslipidemia. This interim-analysis does indicate that supplying resveratrol for 6 months leads to a significant reduction in HbA1c, when compared to placebo. HbA1c is an important marker of long-term glycemic control, and reflects average plasma glucose values of the preceding three months [28]. This beneficial effect of resveratrol has been found previously, but only in patients with T2D as described in a meta-analysis by Liu from 2014 [29]. Conversely, the decrease in HbA1c in the current study was not accompanied by

lower fasting plasma glucose or insulin values. Importantly, the American Diabetes Association states that HbA1c has greater pre-analytical stability and less day-to-day perturbations during stress and illness, compared to fasting plasma glucose values [30]. Thereby, HbA1c might be more suitable for detecting differences in a small sample size. Furthermore, in this preliminary analysis of our ongoing long-term resveratrol study, the power might not have been sufficient to detect effects on fasting plasma glucose and insulin values. We also measured plasma markers related to safety: bilirubin, creatinine, urea, AST and ALT. None of the plasma markers changed over the 6 months, neither within or between the groups, which implies that supplying 150mg resveratrol for 6 months can be regarded as safe as also previously established [27].

In a short-term human clinical trial, we found beneficial effects of resveratrol supplementation on intrahepatic lipid content in healthy obese men [7]. In this 6-month intervention trial we found a tendency towards a decrease in IHL in the resveratrol group, while we found a small increase upon placebo, resulting in a near significant effect of resveratrol on IHL content. Other studies in which participants were enrolled with disturbed glucose homeostasis, such as patients with T2D and men with the metabolic syndrome, did not find a reduction in IHL [13, 22, 27], despite using the same dose of resveratrol was used as well as the same measurement technique. This may suggest that resveratrol can only considerably lower IHL in relatively healthy obese individuals or that a longer duration of treatment with resveratrol is needed to affect IHL. Including more subjects in the current study will give more insight in whether 150mg of resveratrol is indeed capable of lowering IHL when supplied for 6 months.

Animal studies suggest that resveratrol could protect against high-fat diet induced weight gain and stimulate energy expenditure. So far, in humans no effects of resveratrol on body weight or body composition have been established [7, 8, 10, 11, 14, 16]. In this interim-analysis we found no significant effect of resveratrol on fat mass compared to placebo. However, upon placebo there was a significant increase in whole body fat mass and trunk fat mass compared to baseline. In the resveratrol group, whole body and trunk fat mass stayed stable without changes in body weight in either of the two groups. The absence of these changes in the resveratrol group could imply a blunting of a fat mass increase in time, and thereby resemble findings from animal studies [31-33]. Regarding whole body energy metabolism, we did not see any changes in resting metabolic rate in either of the groups. Resting energy expenditure, fat and carbohydrate utilization stayed stable over the 6-month intervention period, which is in contradiction to animal studies [32-34] but agrees with most human clinical trials in which also no effects of resveratrol treatment were observed on resting energy metabolism [6, 13].

Skeletal muscle plays an important role in glucose metabolism, and impaired skeletal muscle functioning contributes to the development of T2D [35]. Since the mitochondria produce over 90% of the ATP needed for movement, improvements in mitochondrial function can lead to

improvement in muscle functioning. It is known that resveratrol can induce the expression of PGC-1 α , a key regulator of mitochondrial biogenesis [4]. Multiple human clinical trials revealed that a relatively low-dose of resveratrol improves *ex vivo* muscle mitochondrial oxidative capacity, already after 30 days [7, 13, 21, 22]. Therefore, in this clinical trial we focused on the potential effects of these improvements on muscle function. To this end, the participants performed several physical performance tests, of whom two are included in this interim-analysis: a 6-Minute Walk Test and a Timed Chair-Rise-Test. Both the placebo and resveratrol group significantly improved walking performance, as represented by distance covered in 6 minutes of walking. In addition, all participants but one in the resveratrol group, - but not in the placebo group - improved time to complete the TCST. As a result, time to complete the TCST tended to be shorter within the resveratrol group after the intervention, compared to the placebo group. Considering the 6MWT and TCST measure different components of physical performance, it is not unusual that the outcomes are not in line. The 6MWT is a marker of aerobic capacity and endurance [36], while the TCST is representative of muscle strength [37]. In accordance, a recent clinical trial did not find effects of resveratrol on walking performance [38], while animal studies do show effects on both aerobic capacity and muscle strength [32, 39]. It is possible that in humans, resveratrol primarily targets muscle strength and does not affect aerobic capacity. However, a more complete analysis of these and other parameters upon completion of the entire study is needed before definitive conclusions can be drawn.

In conclusion, this interim-analysis suggests that 6-months of 150mg resveratrol per day lowers HbA1c, tends to decrease IHL, tends to blunt increases in fat mass and increases leg muscle strength. So far, these beneficial effects are not accompanied by improvements in outcome parameters related to dyslipidemia and energy metabolism. Considering this is an interim-analysis, and the primary study outcome parameter is not taken into account in this analysis, no preliminary conclusions can be drawn regarding insulin sensitivity. Completion of this study will give more robust answers to the question whether resveratrol can be beneficial in the prevention of metabolic disturbances and promotion of overall well-being.

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CHAPTER 7

Carnitine supplementation improves metabolic flexibility and skeletal muscle acetylcarnitine formation

Yvonne M.H. Bruls, Marlies de Ligt, Lucas Lindeboom, Esther Phielix, Bas Havekes, Joachim E. Wildberger, Matthijs K. Hesselink, Patrick Schrauwen and Vera B. Schrauwen-Hinderling

In preparation

ABSTRACT

Objective

Insulin resistant subjects and patients with type 2 diabetic are characterized by disturbed glucose homeostasis and decreased metabolic flexibility. Recent evidence indicates that low carnitine availability may be underlying the reduced metabolic flexibility and impaired glucose tolerance. Here, we investigated whether carnitine supplementation improves metabolic flexibility and insulin sensitivity in volunteers with impaired glucose tolerance (IGT).

Methods

Eleven IGT volunteers followed a 36-day placebo- and L-carnitine treatment (2 g/day) in a randomized, placebo-controlled, double blind crossover design. A hyperinsulinemic-euglycemic clamp (40 mU/m²/min), combined with indirect calorimetry (ventilated hood) was performed to determine insulin sensitivity and metabolic flexibility. Furthermore, metabolic flexibility was assessed in response to a high-energy meal. Skeletal muscle acetylcarnitine concentrations were measured *in vivo* using long echo time proton magnetic resonance spectroscopy (¹H-MRS, TE=500ms) in the resting state (7:00 AM and 5:00 PM). To stimulate acetylcarnitine formation as parameter for free carnitine availability, a 30-minute cycling exercise was performed at 70% of the volunteers' predetermined maximal output (W_{max}) followed by acetylcarnitine determination by ¹H-MRS. Twelve normal glucose tolerant (NGT) volunteers were included without any intervention as control group.

Results

Metabolic flexibility was lower in IGT compared to NGT (0.07 ± 0.01 and 0.10 ± 0.01 AU, $p=0.22$), but was completely restored upon carnitine supplementation (0.10 ± 0.1 , $p<0.05$) as measured during a hyperinsulinemic-euglycemic clamp. Similar results on metabolic flexibility were found in response to a high-energy meal test ($p<0.05$). Carnitine supplementation also enhanced the increase in resting skeletal muscle acetylcarnitine concentrations over the day (delta of 7:00 AM versus 5:00 PM: 1.57 ± 0.33 and 0.67 ± 0.18 mmol/kgww upon carnitine and placebo respectively, $p<0.05$). Besides, post-exercise acetylcarnitine concentrations increased compared to placebo (1.62 ± 0.27 and 1.08 ± 0.20 mmol/kgww respectively ($p<0.05$). Whole-body insulin sensitivity was not affected by carnitine supplementation ($p>0.05$).

Conclusion

Carnitine supplementation completely restored metabolic flexibility in impaired glucose tolerant subjects. A larger increase over the day in resting skeletal muscle acetylcarnitine concentration as well as the improved capacity to form acetylcarnitine with exercise may be underlying the beneficial effects on metabolic flexibility.

INTRODUCTION

Metabolic flexibility is defined as the capacity to switch from predominantly fat oxidation while fasting, to carbohydrate oxidation in the insulin-stimulated state and vice versa [1]. Decreased metabolic flexibility is an early hallmark in the development of type 2 diabetes mellitus. Impairments in metabolic flexibility are not only present in patients with type 2 diabetes but also in a pre-diabetic state, in individuals with so-called impaired glucose tolerance (IGT) [2]. Decreased metabolic flexibility in these individuals results in delayed postprandial glucose clearance and thereby eventually leads to disturbances in glucose homeostasis. Therefore, improving metabolic flexibility in people with IGT may be a good strategy to delay and/or prevent disturbed glucose homeostasis.

It has recently been suggested that the formation of acetylcarnitine is crucial in maintaining metabolic flexibility, resulting in improved glucose homeostasis [3-6]. Acetylcarnitine is synthesized by the conjugation of acetyl-CoA and free carnitine, mediated via the enzyme carnitine acyltransferase (CrAT) [3-6]. This formation of acetylcarnitine acts as a buffering system to regulate the acetyl-CoA pool, and thus to prevent intra-mitochondrial accumulation of acetyl-CoA. The buffering of acetyl-CoA is especially important under conditions of excessive substrate supply to the TCA cycle, such as during (over)feeding and exercise. Acetyl-CoA is an allosteric regulator of mitochondrial enzymes and high levels of acetyl-CoA have been linked to states of mitochondrial 'indecision'. This indecision refers to the fact that the regulation as to which substrate prevails for oxidation is overruled by signals of substrate overabundance. Thus, the inflow of acetyl-CoA from fatty acid oxidation can result in unresponsiveness to the regulatory signal of insulin to switch from fatty acid oxidation to glucose oxidation. Therefore, metabolic inflexibility at the mitochondrial level occurs [6]. In accordance, knocking-out the CrAT enzyme in mice results in lower acetylcarnitine formation and blunted metabolic flexibility. Furthermore, CrAT gain-of function studies in human primary myotubes showed elevated mitochondrial acetylcarnitine efflux, indicating enhanced acetylcarnitine formation with higher CrAT enzyme activity [3], resulting in higher PDH activity and metabolic flexibility [3]. These results suggest an important role for acetylcarnitine formation in maintaining metabolic flexibility.

When there is excessive substrate supply to the mitochondria, a mismatch between β -oxidation and TCA cycle occurs. Typically, this mismatch results in accumulation of β -oxidation intermediates, such as long chain acyl-CoA and long chain acylcarnitine species [6, 7]. Indeed, elevated long-chain acylcarnitines were reported in insulin resistance and in conditions of blunted metabolic flexibility [8]. The 'trapping' of free carnitine due to the accumulation of long chain acylcarnitines may diminish the capacity to form acetylcarnitine further, as free carnitine availability is crucial in maintaining the proposed mitochondrial rescue mechanism of acetylcarnitine formation [3, 4, 9]. Therefore, next to low CrAT activity, also a decreased

carnitine availability may be underlying the development of metabolic inflexibility. For example, it was found that diabetic mice (BAP-agouti transgenic mice) were characterized by reduced acetylcarnitine concentrations in muscle, which could be restored to levels of non-diabetic mice by carnitine supplementation [9]. This was accompanied by improved metabolic flexibility, insulin sensitivity and restoration of blood glucose levels in these diabetic mice [9]. Furthermore, Noland et al. [4] found that feeding rats a high-fat diet decreased the availability of free carnitine and hampered metabolic flexibility. Again, supplementation of carnitine in these rats restored metabolic flexibility and insulin sensitivity [4].

Whether carnitine supplementation can also improve metabolic flexibility in humans has so far not been studied. Moreover, it still remains elusive whether the capacity to form acetylcarnitine is underlying the beneficial effects of carnitine supplementation on metabolic flexibility and concomitantly improved glucose tolerance in humans. Intervention trials in humans have shown that oral carnitine supplementation can improve glucose tolerance in insulin resistant individuals with low carnitine status [10-12] and can have beneficial effects on glucose levels [13, 14], insulin levels [12] and markers of insulin resistance, such as the HOMA-IR index [12, 13] or glucose area under the curve after an oral glucose tolerance test [15]. These results seem to suggest that also in humans, carnitine supplementation may enhance the capacity to form acetylcarnitine and improve metabolic flexibility. To facilitate examination of this hypothesis, we recently set up a novel, proton magnetic resonance spectroscopy (¹H-MRS) protocol, using long echo times, to repeatedly determine acetylcarnitine concentrations in skeletal muscle *in vivo* [16]. This technique provides the unique opportunity to non-invasively and dynamically investigate the role of acetylcarnitine formation in the development of metabolic inflexibility and type 2 diabetes. Using this non-invasive approach, we here aimed to investigate in humans if carnitine supplementation leads to increased acetylcarnitine formation and improves metabolic flexibility and insulin sensitivity in individuals with impaired glucose tolerance.

METHODS

Participants

Twelve normal glucose tolerant (NGT) and eleven impaired glucose tolerant (IGT) individuals participated in this study. Impaired glucose tolerance was defined as a plasma glucose level between 7.8-11.1 mmol/L 2-hours after a 75-gram glucose bolus provided during an oral glucose tolerance test (OGTT). NGT was defined as plasma glucose levels <7.8 mmol/L and >11.1 mmol/L two hours after OGTT [4, 17]. Exclusion criteria were unstable body weight (weight gain or loss >3 kg in the previous 3 months), engagement in exercise >3 hours a week, impaired renal and/or kidney function, uncontrolled hypertension, history of cardiovascular disease, MRI contra-indications, use of medication known to interfere with glucose homeostasis and being

vegetarian, which can affect the whole-body carnitine status. Before written informed consent was obtained, the protocol was explained in full, including potential risks. The study protocol was approved by the institutional Medical Ethical Committee and conducted in accordance with the declaration of Helsinki. Monitoring was performed by the Clinical Trial Center of Maastricht. The study was registered at clinicaltrials.gov with identifier NTC02072759.

Experimental Design

The study was set up in a double blind, randomized cross-over design in volunteers with IGT. Normal glucose tolerant (NGT) volunteers served as control group for baseline comparison. IGT participants were randomly assigned to one of the two periods of the intervention trial: placebo or carnitine (2000 mg/day of L-carnitine). After a wash-out period of at least 4 weeks volunteers followed the other treatment. At the beginning of each period, a fasted blood sample was obtained after which supplementation was started and continued for 36 days (Figure 1). Participants reported to the laboratory on a weekly basis to obtain a fasting blood sample and to supply supplements for the next week. Participants were asked to maintain their normal diet and physical activity pattern during participation in the study.

On day 30, participants reported to the laboratory at 6:00 AM after an overnight fast. A meal test was performed to investigate metabolic flexibility by indirect calorimetry upon a high-energy breakfast provided to the volunteers at 8:00 AM at the laboratory. *In vivo* skeletal muscle acetylcarnitine concentrations were measured via proton magnetic resonance spectroscopy (¹H-MRS) [16] before and several time points after this breakfast. On a separate day (day 33), a biopsy was taken and a hyperinsulinemic-euglycemic clamp was performed to measure peripheral insulin sensitivity. Finally, on a third test day (day 36), participants came to the laboratory at 4:30 PM. After consumption of a light lunch at 12:00 AM, participants remained fasted for the following 5 hours. At 5:00 PM, participants were positioned in the MR scanner and skeletal muscle acetylcarnitine concentrations were measured using long-TE ¹H-MRS at rest and directly after a 30-minute cycling exercise at 70% of the subjects predetermined maximal output (W_{max}). NGT

Figure 1. Outline of the double blind, randomized cross-over design.

participants were subjected to the same measurements but without any intervention trial (no placebo and carnitine).

Carnitine Supplementation

L-Carnitine tartrate (NOW foods, Bloomingdale, IL, USA) was taken orally for 36 days (2000 mg carnitine/d); one 500-mg capsule in the morning during breakfast, one 500-mg capsule at noon during lunch and two 500-mg capsules in the evening during dinner. In the morning of test-day 30 and 33, subjects did not take any supplements. Compliance was checked by measuring acylcarnitines levels in the plasma before the start of the intervention at day 1 and on the morning of the clamp (day 33). Placebo capsules (Pharmacy Radboud UMC, Nijmegen, The Netherlands) containing 500-mg micro cellulose were provided in an identical regimen.

VO₂max and Body Composition

On a separate day, before the start of the intervention trials, participants underwent an incremental cycling test on a stationary bike to determine maximal oxygen uptake (VO₂max) and maximal power output (W_{max}) for characterization of the participants. On the same day, body composition was determined by DXA (DXA, discovery A; Hologic).

Meal Test

On day 30, participants reported to the laboratory at 6:00 AM after an overnight fast. To standardize the diet, all participants were provided with a standardized chicken-rice dinner for consumption at home on day 29. Furthermore, participants were asked to refrain from strenuous physical activity three days prior to the meal test. After arrival at the laboratory, subjects were bed-rested in supine position and a catheter was inserted in the median cubital vein for repetitive blood sampling throughout the test day. A first 10-ml blood sample was taken in the fasted condition before the meal. While resting, indirect calorimetry (Ventilated hood, Omnicol, Maastricht Instruments, Maastricht University) was performed to determine substrate oxidation at baseline ($t=-90$). Subsequently, a baseline proton magnetic resonance spectroscopy (¹H-MRS) scan was performed, using long echo times (TE) to quantify in vivo skeletal muscle acetylcarnitine ($t=-30$ min) as previously reported [16]. After these measurements, subjects consumed a high-energy breakfast. The high-energy breakfast consisted of sausage rolls (65.5% energy from fat, 31.6% energy of carbohydrates, and 6.9% energy from proteins). The total energy content of the breakfast was equal to 50% of the participants required daily energy intake. Daily energy intake was calculated on the basis of lean body mass as measured during the screening visit according to the Cunningham equation [18]. A correction factor of 1.3 was applied for physical activity. ¹H-MRS measurements were repeated directly after the breakfast ($t=30$ min), 2.5 hours ($t=150$ min) and 5.5 hours ($t=330$ min) after breakfast. Blood samples were drawn before each MR spectroscopy measurement to measure plasma glucose, insulin, free fatty acids and triglycerides (at $t=-45$, $t=15$, $t=150$, $t=330$). In between scans, volunteers bed-rested and indirect calorimetry

Figure 2. Flowchart of the meal test (day 30). Participants were asked to consume a standardized evening meal on the evening before. Participants reported to the laboratory at 06:00 AM in the fasted state. First, baseline indirect calorimetry and ¹H-MRS measurements were conducted. Between 07:45 AM and 08:15 AM, subjects consumed a high-energy breakfast. Directly after breakfast (t=30) as well as 2.5 (t=150) and 5.5 (t=330) hours after the meal, MRS measurements were repeated. Indirect calorimetry was measured three times using a ventilated hood system (t=-90, t=90, t=270). A blood samples was taken prior to each MRS measurement. IC, indirect calorimetry (ventilated hood), ¹H-MRS, proton magnetic resonance spectroscopy.

(ventilated hood) was used to determine metabolic flexibility ($\Delta\text{RER}_{t=90-\text{fasted}}$ and $\Delta\text{RER}_{t=270-t=90}$) and substrate oxidation 1.5 hours (t=90 min) and 4.5 hours (t=270 min) after consumption of the breakfast. A schematic overview of the test day is illustrated in Figure 2.

Hyperinsulinemic-euglycemic Clamp

On day 33, a one-step hyperinsulinemic-euglycemic clamp was performed to assess peripheral insulin sensitivity [19]. Participants were asked to refrain from physical activity three days prior to the clamp and to consume a standardized carbohydrate rich meal the evening before the clamp. At the day of the clamp, subjects reported to the laboratory at 7:30 AM after an overnight fast. A fasted blood sample was taken and subsequently a primed-continuous infusion of D-[6,6-²H₂]-glucose (0.04 ml/kg/min) was started to determine rates of glucose appearance (Ra), glucose disappearance (Rd) and endogenous glucose production (EGP) [20]. After 2 hours (t=120), infusion of insulin (40 mU/m²/min) was started for 2.5 hours combined with simultaneous infusion of glucose (glucose 20%) to maintain euglycemia (5.0 mmol/L). During the basal period (t=90-120 min) and the last 30 minutes of insulin infusion (t=240-270 min) blood samples were collected and indirect calorimetry (ventilated hood) was performed to assess metabolic flexibility and substrate utilization. Metabolic flexibility is expressed as the insulin stimulated RER – the basal RER. Carbohydrate and lipid oxidation was calculated according to Peronnet et al. [21].

Cycling Test

Finally, on a third test day (day 36), participants reported to the laboratory at 4:30 PM. After consumption of a light lunch at 12:00 AM, participants remained fasted for the following 5 hours and refrained from physical activity. After arrival at the laboratory, subjects rested for 30 minutes until the start of the baseline proton magnetic resonance spectroscopy (¹H-MRS), using long echo times (TE) as previously reported [16], to quantify in vivo skeletal muscle acetylcarnitine. At 5:00 PM, participants were positioned in the MR scanner and baseline skeletal muscle acetylcarnitine

concentrations were measured. Subsequently, a 30-minute cycling exercise at 70% of the participants predetermined maximal power output (W_{max}) was performed on an ergometer in a room next to the MR scanner. Directly after the exercise, a second ^1H -MRS measurement was performed to quantify skeletal muscle acetylcarnitine concentrations.

^1H -MRS (acetylcarnitine)

In vivo skeletal muscle acetylcarnitine concentration was measured using a long-TE ^1H -MRS protocol as described previously [16]. All experiments were performed on a 3T clinical MR scanner (Achieva 3T-X, Philips Healthcare, Best, The Netherlands). Participants were positioned in supine position feet first in the MR scanner and a two-element surface coil was placed around the left upper leg. The voxel of interest (40mm x 20mm x 60mm) was positioned in the *m. vastus lateralis*. The spectra were acquired with the following acquisition parameters: TR=6000, spectral bandwidth 2 kHz and number of acquired data points 2048. A series of spectra were acquired with variable TE and NSA (300-12, 325-16, 350-20, 400-32, 450-52, 500-76 respectively). Due to considerable lipid contamination, acetylcarnitine concentration was analyzed in spectra with TE=500 ms as the shorter echo times showed considerable lipid contamination in overweight participants.

Baseline correction was performed for all acquired spectra with a custom-made MATLAB script (The Mathworks Inc.). Spectra were analyzed using the AMARES algorithm in jMRUI software [22]. The creatine resonance (t-Cr) was used as internal reference and acetylcarnitine concentration was calculated assuming a creatine concentration of 30 mmol/kg ww. T2 corrections were performed for creatine (T2=166ms) and acetylcarnitine (T2=262ms) and a correction for the dipolar coupling of creatine was applied and set at 30% of the signal as reported earlier [16].

Blood Biochemistry

Arterialized blood samples (hotbox heated) were collected from a hand vein during the hyperinsulinemic-euglycemic clamp and were immediately centrifuged. Venous blood samples obtained during the meal test were collected identically. Plasma was frozen in liquid nitrogen and stored at -80°C until analyzed. Plasma concentrations of insulin, glucose, free fatty acids (FFAs), and total and free glycerol were determined in duplicate. Plasma FFAs, glucose and free and total glycerol were measured using enzymatic assays automated on a Cobas Fara/Mira analyzer [glucose: hexokinase method (Roche); FFAs: Wako Nefa C test kit (Wako Chemicals); glycerol: Enzytec glycerol kit (R-biopharm)]. Free glycerol and total glycerol were used to calculate triacylglyceride concentrations. Plasma insulin concentrations were determined using an RIA (Millipore). Plasma acylcarnitine concentrations were measured by tandem mass spectrometry as previously reported [23]. Acylcarnitine concentrations were determined at the start end of the intervention (day 0 and 33).

Muscle Biopsy

On the morning of the hyperinsulinemic euglycemic clamp, a muscle biopsy was taken from the vastus lateralis muscle according to the Bergström method [24] under local anesthesia (2% Lidocaine, Accord Healthcare Limited, Harrow, United Kingdom). Muscle tissue was directly frozen in melting isopentane and stored at 80°C until further processing. Skeletal muscle acylcarnitines were analyzed as previously described using mass spectrometry [25]. Glycogen concentration was determined by using a commercial glycogen assay kit (ab65620, Abcam, Cambridge, United Kingdom) according to instructions of the manufacturer.

Tracer Calculations

Isotopic enrichment of plasma glucose was determined by electron ionization gas chromatography-mass spectrometry as described previously [26]. Steele's single pool non-steady state equations were used to calculate glucose Ra and Rd [20]. Volume of distribution was assumed to be 0.160 L/kg for glucose. Insulin-stimulated glucose disposal (Δ Rd) was computed as the difference between Rd under insulin-stimulated conditions and Rd under basal non-insulin stimulated conditions. EGP was calculated as Ra minus exogenous glucose infusion rate. Non-oxidative glucose disposal (NOGD) was calculated as Rd minus carbohydrate oxidation, derived from the indirect calorimetry measurements.

Statistics

Data are presented as means $\pm$ SEM. Statistical analysis were performed using SPSS 24.0 software (SPSS, Chicago, IL.). Results were considered to be significantly different when p value <0.05 (two-sided testing). To evaluate if the data were normally distributed a Shapiro-Wilk normality test was performed. Statistical comparisons between the two intervention trials (carnitine and placebo) were performed using a Student's paired sample t-test. Student's independent sample t-tests were performed to compare NGT participants to IGT participants. Potential carry-over effect between treatment and period as well as period effect were examined by unpaired t-test analyses according to Pocock et al. [27]. No carry-over or period effects were found.

RESULTS

Subject Characteristics

Body composition and maximal oxygen uptake ($\text{VO}_{2\text{max}}$) were comparable between groups. Fasting glucose levels were slightly higher in IGT participants ($p=0.034$) and, by design, glucose levels 120 minutes after an oral glucose tolerance test (OGTT_{120}) were higher in IGT ($p<0.01$). Also, insulin levels at $t=120$ minutes of the OGTT were significantly different between groups ($p=0.01$). Fasting insulin, cholesterol and triglyceride levels were similar across groups at the onset of the study. Baseline characteristics are reported in Table 1. Carnitine supplementation did not alter fasting plasma glucose, insulin, cholesterol, triglycerides and liver function (Supplementary Table 1).

Table 1. Baseline participant characteristics

	NGT (n=12)	IGT (n=11)
Sex (m/f)	10 / 2	10 / 1
Age (y)	61 ± 2	62 ± 2
BMI (kg/m ²)	28.9 ± 0.7	29.7 ± 0.5
Body composition		
Fat mass (kg)	26.8 ± 1.8	27.0 ± 1.1
Fat free mass (kg)	64.0 ± 2.5	64.2 ± 2.9
Fat percentage (%)	29.6 ± 1.9	29.8 ± 1.0
Visceral adipose tissue (kg)	0.6 ± 0.1	0.8 ± 0.1
Physical fitness		
VO ₂ max (ml*min ⁻¹ *kg ⁻¹)	29.7 ± 1.5	28.3 ± 1.4
Wmax (W*kg ⁻¹)	2.2 ± 0.1	2.3 ± 0.1
Oral glucose tolerance test (OGTT)		
Fasting glucose (mmol/L)	5.3 ± 0.1	5.7 ± 0.1*
Glucose OGTT ₁₂₀ (mmol/L)	4.6 ± 0.3	8.2 ± 0.3*
Fasting insulin (pmol/L)	56.6 ± 9.1	80.1 ± 14.2
Insulin OGTT ₁₂₀ (pmol/L)	223.5 ± 36.1	669.3 ± 149.3*
Blood lipid profile		
Total cholesterol (mmol/L)	5.6 ± 0.2	5.3 ± 0.1
HDL cholesterol (mmol/L)	1.5 ± 0.2	1.3 ± 0.1
LDL cholesterol (mmol/L)	3.4 ± 0.2	3.2 ± 0.2
Triglycerides (mmol/L)	1.64 ± 0.36	1.76 ± 0.28

Data are represented as mean ± SEM. * p<0.05, IGT significantly different from NGT. Wmax, maximal workload.

Plasma Free Carnitine and Acetylcarnitine Concentrations

No difference in plasma free carnitine (C0) concentrations were found between the placebo and carnitine group at the start of the intervention (p=0.312). In line, plasma acetylcarnitine concentration did not differ between the groups. Upon carnitine supplementation, plasma free carnitine as well as acetylcarnitine concentrations increased in all individuals (C0 from 40.8±1.6 to 50.5±1.7 µmol/L, p<0.01 and C2 from 6.6±0.4 to 8.3±0.6 µmol/L, p=0.035), indicating compliance to the study protocol (Figure 3). C3 acylcarnitine species also increased upon carnitine supplementation. No differences in other acylcarnitine species were detected upon carnitine supplementation (Supplementary Table 2). Carnitine levels did not change after placebo treatment (C0 from 39.4±1.4 to 39.8±1.3 µmol/L, p=0.785 and C2 from 6.0±0.4 to 6.2±0.3 µmol/L, p=0.489).

Metabolic Flexibility and Insulin Sensitivity

Metabolic flexibility, expressed as the change in respiratory exchange ratio upon insulin stimulation (ΔRER), was significantly lower in IGT compared to NGT participants (Figure 4A,

Figure 3. Plasma free carnitine concentrations (A) and plasma acetylcarnitine concentration (B). In A and B, black bars represent day 0 and white bars represent day 33 in NGT, IGT P (IGT placebo), and IGT C (IGT carnitine). Data are expressed as means $\pm$ SEM. * significantly different ($p < 0.05$).

Figure 4. Metabolic flexibility measured as the change from the fasted state to the insulin-stimulated state (ΔRER) (A), CHO oxidation (B), and lipid oxidation (C). In B and C, black bars represent the fasted state and white bars represent the insulin-stimulated state in NGT, IGT P (IGT placebo), and IGT C (IGT carnitine). Data are expressed as means $\pm$ SEM. * insulin stimulated state significantly different from fasted state, # different from insulin stimulated state NGT, ^ different from fasted state NGT and IGT on placebo ($p < 0.05$).

$p = 0.022$). Interestingly, carnitine supplementation completely restored metabolic flexibility to NGT values (Figure 4A, $p < 0.05$). Overnight fasted lipid and carbohydrate oxidation rates were not different between NGT and IGT. However, carnitine supplementation increased overnight fasted basal whole-body lipid oxidation compared to placebo ($p = 0.034$), resulting in fat oxidation levels that were higher than in normal glucose tolerant participants (Figure 4C, $p = 0.007$). In line with the notion that fat- and carbohydrate oxidation rates generally exhibit reciprocal trends, basal carbohydrate oxidation was reduced on carnitine supplementation compared to the placebo group ($p = 0.013$) and tended to be lower in comparison with NGT participants ($p = 0.055$). Despite the reduction in carbohydrate oxidation in the fasted state in IGT participants upon carnitine supplementation, non-oxidative glucose disposal in the fasted state was increased ($p < 0.05$) indicating that a larger fraction of the glucose taken up was incorporated into glycogen.

We also examined if carnitine supplementation had beneficial effects on peripheral insulin sensitivity. To this end, we performed a hyperinsulinemic euglycemic clamp combined with

deuterated glucose tracers to allow the determination of the rate of disappearance of glucose, which mainly reflects skeletal muscle glucose uptake. Basal EGP was lower in IGT participants compared to NGT ($p<0.01$) but was not affected by carnitine supplementation ($p=0.962$). Also, insulin-stimulated suppression of EGP was not significantly different between groups ($p>0.05$). As expected, the insulin-stimulated rate of disappearance of glucose (ΔR_g) was significantly higher in NGT compared to IGT participants ($p<0.01$, Table 2). Carnitine supplementation did not significantly affect insulin stimulated glucose disposal (ΔR_g : 11.74 ± 1.99 vs 13.32 ± 3.08 after placebo and carnitine respectively, $p=0.512$). Interestingly, however, upon carnitine supplementation, a larger fraction of this insulin stimulated glucose disposal was directed towards glucose oxidation, thereby completely restoring the insulin-stimulated glucose oxidation in IGT towards levels observed in NGT (Table 2). Conversely, carnitine supplementation reduced insulin-stimulated non-oxidative glucose disposal. These data suggest that, although carnitine supplementation did not affect peripheral insulin sensitivity, it did result in a redistribution of the glucose taken up after insulin stimulation towards oxidative disposal and less to glycogen storage.

Skeletal Muscle Glycogen Concentrations

To directly test if carnitine supplementation affected baseline muscle glycogen concentrations, we measured glycogen concentration in muscle biopsies taken before the clamp. Muscle glycogen was not different between NGT and IGT participants ($p>0.05$). Although not significant, skeletal muscle glycogen concentrations after an overnight fast tended to increase upon carnitine supplementation ($p=0.140$, Figure 5). These data are in line with a higher non-oxidative glucose disposal (NOGD) in the overnight fasted state upon carnitine supplementation ($p=0.006$), suggesting that under basal conditions more glucose is shuttled towards glycogen storage. It also may explain the reduction in insulin-stimulated non-oxidative glucose disposal as basal glycogen levels are known to determine further rates of glycogen storage.

Metabolic Flexibility Assessed by A High-Energy Meal Test

Next to the assessment of metabolic flexibility during a clamp, metabolic flexibility was also determined in a more physiological setting, in this case before and after a meal. In line with the metabolic flexibility results from the clamps, metabolic flexibility in response to a high-energy meal was also decreased in IGT and could be completely restored with carnitine supplementation ($p<0.05$ Figure 6A). IGT participants on carnitine supplementation had a lower RER in the fasted state compared to placebo ($p=0.024$) as well as when compared to NGT controls ($p=0.027$) (Table 3). The lower basal RER upon carnitine supplement reflected a decreased carbohydrate oxidation ($p=0.031$, Figure 4B) and a trend towards increased whole-body lipid oxidation ($p=0.056$, Figure 6C) compared to placebo. Although metabolic flexibility improved meal-induced changes in plasma glucose, insulin, triacylglycerides and free fatty acids were unaffected by carnitine supplementation (Figure 7).

Table 2. Substrate kinetics and insulin sensitivity

	NGT (n=12)	IGT Placebo (n=11)	IGT Carnitine (n=11)
RER (arbitrary units AU)			
Basal	0.798 ± 0.008	0.791 ± 0.012	0.762 ± 0.010 ^{a,b}
Insulin-stimulated	0.899 ± 0.009	0.857 ± 0.011 ^a	0.863 ± 0.010 ^a
Δ	0.101 ± 0.012	0.066 ± 0.007 ^a	0.100 ± 0.010 ^b
CHO oxidation (μmol*kg ⁻¹ *min ⁻¹)			
Basal	6.71 ± 0.61	5.93 ± 0.71	4.01 ± 0.66 ^{a,b}
Insulin-stimulated	14.22 ± 0.78	10.77 ± 0.75	11.09 ± 0.79 ^a
Δ	7.51 ± 1.01	4.84 ± 0.48	7.08 ± 0.74 ^b
Lipid oxidation (μmol*kg ⁻¹ *min ⁻¹)			
Basal	1.10 ± 0.05	1.12 ± 0.08	1.27 ± 0.07 ^{a,b}
Insulin-stimulated	0.55 ± 0.05	0.77 ± 0.07	0.73 ± 0.06 ^a
Δ	-0.54 ± 0.07	-0.35 ± 0.04	-0.54 ± 0.05 ^b
Rd glucose (μmol*kg ⁻¹ *min ⁻¹)			
Basal	10.80 ± 0.70	7.64 ± 0.49 ^a	7.97 ± 0.80 ^a
Insulin-stimulated	36.51 ± 3.48	19.38 ± 2.02 ^a	21.29 ± 2.98 ^a
Δ	25.71 ± 3.39	11.74 ± 1.99 ^a	13.32 ± 3.08 ^a
NOGD (μmol*kg ⁻¹ *min ⁻¹)			
Basal	5.41 ± 0.70	3.09 ± 0.49 ^a	5.94 ± 0.80 ^b
Insulin-stimulated	23.16 ± 3.48	10.98 ± 2.02 ^a	9.08 ± 2.98 ^a
Δ	17.76 ± 3.39	7.89 ± 1.99 ^a	3.15 ± 3.08 ^a
Glucose (mmol/L)			
Basal	5.5 ± 0.1	5.8 ± 0.1 ^a	5.9 ± 0.1 ^a
Insulin-stimulated	5.5 ± 0.2	5.3 ± 0.1	5.2 ± 0.1
Plasma insulin (pmol/L)			
Basal	63.85 ± 11.48	84.31 ± 9.16	84.24 ± 11.48
Insulin-stimulated	945.07 ± 52.39	1022.01 ± 43.04	981.08 ± 5.56
Plasma FFA (μmol/L)			
Basal	522.86 ± 30.96	612.00 ± 56.68	597.4 ± 34.75
Insulin-stimulated	72.31 ± 6.61	119.90 ± 14.02 ^a	110.30 ± 11.28 ^a

Data are expressed as mean ± SEM. ^a IGT placebo or IGT carnitine significantly different from NGT. ^b IGT carnitine significantly different from IGT placebo.

Figure 5. Skeletal muscle glycogen concentrations. Data are expressed as means ± SEM.

Figure 6. Respiratory exchange ratio (A), black bars represent $\Delta RER_{t=90-\text{basal}}$ and white bars represent $\Delta RER_{t=270-t=90}$. * significantly different from IGT on placebo, # different from NGT. CHO oxidation (B), and lipid oxidation (C). In B and C, black bars represent the fasted state, white bars represent $t=90$ min and grey bars represent $t=270$ min after the meal in NGT, IGT P (IGT placebo), and IGT C (IGT carnitine). Data are expressed as means $\pm$ SEM. * significantly different from fasted state, # different from NGT and IGT, ^ different from $t=90$ min, & different from NGT, % different from $t=90$ ($P<0.05$). Values refer to twelve participants in the NGT group and nine participants in the IGT P and IGT C groups. Two IGT participants were not able to perform the meal test due to technical problems.

Figure 7. Plasma metabolites during the meal test, glucose (A), free fatty acids FFA (B), triglycerides (C), insulin (D). The black line represents the NGT group, the striped line the IGT on placebo and the dotted line IGT on carnitine. Data are expressed as means $\pm$ SEM. * significantly different ($p<0.05$). Values refer to twelve participants in the NGT group and nine participants in the IGT P and IGT C groups. In two IGT participants, the meal test could not be performed due to technical problems.

Table 3. Substrate kinetics during the high-energy meal test

	NGT	IGT Placebo	IGT Carnitine
RER			
T1=-90 (baseline)	0.836 ± 0.009	0.829 ± 0.012	0.795 ± 0.015 ^{a,b}
T2=90	0.860 ± 0.006	0.830 ± 0.012 ^a	0.833 ± 0.009 ^a
T3=270	0.816 ± 0.010	0.806 ± 0.016	0.796 ± 0.007
Δ T2-T1	0.022 ± 0.007	0.001 ± 0.013	0.037 ± 0.008 ^b
Δ T3-T2	-0.044 ± 0.009	-0.024 ± 0.012	-0.037 ± 0.008
CHO oxidation (μmol*kg ⁻¹ *min ⁻¹)			
T1=-90 (baseline)	9.56 ± 0.82	8.66 ± 0.86	6.86 ± 1.04 ^{#,b}
T2=90	12.50 ± 0.58	9.57 ± 0.82 ^a	9.88 ± 0.61 ^a
T3=270	8.83 ± 0.74	8.08 ± 1.21	7.25 ± 0.61
Δ T2-T1	3.74 ± 1.06	0.91 ± 0.98 [#]	3.02 ± 0.80 ^b
Δ T3-T2	-3.67 ± 0.70	-1.49 ± 0.89 [#]	-2.63 ± 0.58
Lipid oxidation (μmol*kg ⁻¹ *min ⁻¹)			
T1=-90 (baseline)	0.83 ± 0.06	0.91 ± 0.07	1.04 ± 0.10 ^{%,b}
T2=90	0.85 ± 0.05	1.01 ± 0.10	0.99 ± 0.07
T3=270	1.12 ± 0.08	1.17 ± 0.09	1.23 ± 0.05
Δ T2-T1	0.09 ± 0.08	0.10 ± 0.08	-0.05 ± 0.06 [%]
Δ T3-T2	0.26 ± 0.06	0.16 ± 0.08	0.25 ± 0.05

Data are expressed as mean ± SEM. ^a IGT placebo or IGT carnitine significantly different from NGT. ^b IGT carnitine significantly different from IGT placebo. [#] IGT placebo or IGT carnitine tending to be different from NGT. [%] IGT carnitine tending to be different from IGT placebo.

Carnitine Supplementation Improves Capacity to Form Acetylcarnitine in Muscle

To investigate if the improved metabolic flexibility upon carnitine supplementation was due to improved skeletal muscle acetylcarnitine metabolism, we used ¹H-MRS to determine acetylcarnitine levels in the vastus lateralis muscle. This non-invasive technique allows the repeated measurement of acetylcarnitine in the same muscle group. We first determined if carnitine supplementation would elevate overnight fasted acetyl-carnitine levels. Contrary to our expectations, skeletal muscle acetylcarnitine concentrations in the morning at 7:00 AM were not significantly different between NGT and IGT (0.37±0.09 vs 0.65±0.15 μmol/kgww respectively, p=0.111), and if anything tended to be elevated in IGT. Also, carnitine supplementation did not alter muscle acetylcarnitine concentrations (0.44±0.12 mmol/kgww for carnitine, p=0.674) as measured by ¹H-MRS. These data match with the acetylcarnitine concentrations as measured in biopsies taken after an overnight fast. Likewise, in the muscle biopsies no difference between the groups or between the placebo and carnitine treatment were detected (see data below). Given the hypothesized role of acetyl-carnitine in metabolic flexibility, it can be expected that these levels are low in the overnight fasted state when the body mainly relies on fat as a substrate source. We therefore also measured acetylcarnitine levels later during the day. Interestingly, skeletal muscle acetylcarnitine levels were higher when measured at 5:00 PM when compared to

7:00 AM in NGT (Figure 8), suggesting that acetylcarnitine levels in skeletal muscle rise during the day. Intriguingly, this increase in skeletal muscle acetylcarnitine levels during the day (assessed in a subset of 8 participants) was markedly blunted in the IGT group (delta acetylcarnitine concentration: 0.67 ± 0.18 vs 1.51 ± 0.33 $\mu\text{mol/kgww}$ in IGT compared to NGT, $p=0.037$, Figure 8), but was completely restored upon carnitine supplementation (1.57 ± 0.33 mmol/kgww). As a result, acetylcarnitine levels measured at 5:00 PM tended to be lower in IGT participants on placebo compared to NGT controls (1.08 ± 0.20 , 1.64 ± 0.28 mmol/kgww for IGT and NGT respectively, $p=0.064$) and in fact, carnitine supplementation restored acetylcarnitine levels towards values observed in NGT (1.62 ± 0.27 mmol/kgww).

Finally, we also determined the maximal capacity to form acetylcarnitine. Exercise is known to lead to an increase in skeletal muscle acetylcarnitine, possibly because substrate load into the mitochondria is rapidly increased upon exercise thereby elevating acetyl-CoA levels. At high exercise intensity, this increase in acetylcarnitine reflects the capacity individuals have to produce acetylcarnitine and can be seen as a parameter for free carnitine availability. Therefore, acetylcarnitine was measured in skeletal muscle before exercise (5:00 PM measurement) and after 30 minutes of exercise at $70\% W_{\text{max}}$. Acetylcarnitine levels in skeletal muscle increased in all three groups ($p<0.05$), but post-exercise acetylcarnitine concentrations were markedly higher after carnitine supplementation compared to placebo (4.23 ± 0.53 vs 3.60 ± 0.49 mmol/kgww for carnitine and placebo respectively, $p=0.017$) and reached NGT-levels (4.15 ± 0.28 mmol/kgww , Figure 9), suggesting that carnitine supplementation boosted the maximal capacity to form acetylcarnitine.

Skeletal Muscle Acylcarnitine Profiles

A limitation of MRS is that only acetylcarnitine levels can be measured and no information is provided about other acylcarnitine species. Therefore, we examined the complete acylcarnitine profile in the muscle biopsies taken after an overnight fast after placebo or carnitine supplementation. As described above, no difference in free carnitine (C0), acetylcarnitine (C2) or other short-chain acylcarnitine concentrations were detected in muscle biopsies between NGT and IGT ($p>0.05$) and carnitine supplementation did not affect this ($p>0.05$). Long-chain acylcarnitines C16:2 and C20:2, however, tended to be elevated in IGT on placebo compared to NGT ($p<0.05$), suggesting less complete β -oxidation in IGT volunteers. Interestingly, carnitine supplementation lowered long-chain C18:2, C18:1, C18 and C20:1 ($p<0.05$) and tended to lower C16 and C20 ($p<0.1$) acylcarnitine species (Figure 10, Supplementary Table 3), suggesting carnitine supplementation contributed to more complete β -oxidation.

Figure 8. Skeletal muscle acetylcarnitine concentrations over the day (A) and delta acetylcarnitine concentrations (B). In A, the black line represents the NGT group, the striped line the IGT on placebo and the dotted line IGT on carnitine. Data are expressed as means $\pm$ SEM. * IGT placebo significantly different from IGT placebo and NGT, # IGT placebo significantly different from IGT on carnitine ($p < 0.05$). Values refer to twelve participants in the NGT group and eight participants in the IGT P and IGT C groups. Two IGT participants were not able to perform the 07:00 AM measurement due to technical problems and one subject was excluded due to protocol violations.

Figure 9. Skeletal muscle acetylcarnitine concentrations in the resting state and the capacity to form acetylcarnitine with exercise in NGT, IGT P (IGT placebo), and IGT C (IGT carnitine). Data are expressed as means $\pm$ SEM. * significantly different from IGT on carnitine, # tending to be different from NGT ($p = 0.064$). Values refer to twelve participants in the NGT group and eleven participants in the IGT P and IGT C groups.

Figure 10. Skeletal muscle long-chain acylcarnitine concentrations measured in biopsies. Black bars represent the NGT group, white bars the IGT on placebo and grey bars IGT on carnitine. Data are expressed as means $\pm$ SEM. * IGT on carnitine significantly from IGT placebo ($p < 0.05$), # IGT on carnitine tending to be different from IGT on placebo ($p < 0.10$), ^ IGT placebo tending to be different from NGT ($p < 0.10$).

DISCUSSION

Animal studies have indicated that free carnitine availability in skeletal muscle may be crucial in the formation of acetylcarnitine and subsequently maintenance of metabolic flexibility as indicated by Noland et al. [4]. We here aimed to investigate in humans if carnitine supplementation leads to increased acetylcarnitine formation and improves metabolic flexibility and insulin sensitivity in individuals with impaired glucose tolerance. In the current study, we show that metabolic flexibility was lower in IGT compared to NGT participants and could be completely restored upon carnitine supplementation. Concomitantly, free carnitine availability in plasma increased. Likewise, the increase in *in vivo* resting skeletal muscle acetylcarnitine concentrations during the day was enhanced upon carnitine supplementation compared to placebo in impaired glucose tolerant volunteers, as well as the maximal capacity to form acetylcarnitine during exercise. These findings are in line with the notion that the capacity to form acetylcarnitine is important in mitochondrial substrate switching and may be limited by availability of free carnitine [3, 4]. Interestingly, improved metabolic flexibility was not only measured during the hyperinsulinemic euglycemic clamp, but also in a more physiological setting upon consumption of an energy-rich meal. Together these data suggest that also in humans, carnitine supplementation may help to improve metabolic flexibility via increasing skeletal muscle acetylcarnitine levels.

In the current study, we determined acetylcarnitine concentrations *in vivo* using ¹H-MRS both in the morning after an overnight fast as well as in the afternoon. Interestingly, acetylcarnitine concentrations were consistently higher in the afternoon compared to the morning, suggesting that acetylcarnitine levels rise during the day. This increase in acetylcarnitine levels during the day fits with a role of muscle acetylcarnitine in facilitating metabolic flexibility, as it is known that after an overnight fast the body mainly relies on fatty acid oxidation, whereas substrate oxidation is more mixed later during the day when the body is in a fed state [1]. Strikingly, this increase in acetylcarnitine over the day was substantially blunted in IGT, leading to significant differences in acetylcarnitine concentrations between the groups in the afternoon, which were not detectable in the morning. Interestingly, upon carnitine supplementation the increase of acetylcarnitine levels during the day was completely restored to NGT levels, which makes it tempting to speculate that the variation in acetylcarnitine levels over the day may be related to flexibility in substrate use.

Underlying mechanisms of improved metabolic flexibility upon carnitine supplementation are yet unknown, but indirect calorimetry showed that especially basal lipid oxidation was increased, thereby sparing glycogen stores. In line with the latter, muscle glycogen concentrations and non-oxidative glucose disposal rate in the basal, fasted state were enhanced upon carnitine supplementation. With respect to the elevated fat oxidation, accumulation of long-chain acylcarnitines in skeletal muscle has previously been reported in situations when high fat

availability drives fat oxidation. Under such circumstances, fatty acid availability surpasses fat oxidative capacity, resulting in incomplete fat oxidation [4, 6, 7]. Contrary to such availability-driven increases in fat oxidation, the increased fat oxidation in the current study was accompanied by decreased levels of skeletal muscle long-chain acylcarnitines. Thus, this suggests that in the current study, carnitine supplementation resulted in pronounced improvements in fat oxidative capacity, stimulating complete fat oxidation. In accordance, findings of higher fat oxidative capacity and lower skeletal muscle long-chain acylcarnitines were found when wild type mice were compared with SIRT3 knock-out mice [28-31]. SIRT3 is a deacetylase located in the mitochondrial matrix and is known for its function in regulating acetylation of multiple metabolic proteins and enzymes. The downregulation of SIRT3 results in increased lysine acetylation of mitochondrial proteins/enzymes, which may give rise to incomplete fat oxidation and hence the accumulation of long-chain acylcarnitines [28-32]. Lysine acetylation of mitochondrial proteins is thought to be the resultant of the mass action of excessive carbon load, such as during exercise or over-feeding, as accumulating acetyl-CoA levels are expanding the acetyl donor pool [33-35]. Davies et al. showed that high acetyl-CoA concentrations can indeed induce lysine acetylation in mice. Interestingly with respect to the current study, this effect can be blocked by supplementing the mice with carnitine [33]. Although we did not investigate protein acetylation, it is well known that important regulatory enzymes of the β -oxidation and glycogen synthesis pathway can be acetylated [33]. Therefore, it is tempting to speculate that the low metabolic flexibility in pre-diabetic volunteers is due to increased lysine acetylation and that carnitine supplementation may have reduced lysine acetylation by stimulating the formation of acetylcarnitine and thereby keeping intramitochondrial acetyl-CoA levels low. Such changes in acetyl-donor pool and consequently reduced acetylation could explain the beneficial effects observed on metabolic flexibility and fat oxidation capacity in pre-diabetic patients. Future studies are necessary to investigate if acetylation might underlie the beneficial effects on metabolic flexibility in pre-diabetic patients.

Despite the beneficial effects of carnitine supplementation on skeletal muscle acetylcarnitine concentration and metabolic flexibility, no changes in insulin sensitivity were observed in the current study. Although the current study cannot reveal the reason for this lack of effect on insulin sensitivity, previous results suggest that the duration of the carnitine supplementation may have been too short to improve insulin sensitivity. Thus, Gonzalez-Ortiz et al. [36] and Galloway et al. [37] did not report changes in glucose homeostasis after four and two weeks of 3g L-Carnitine supplementation respectively. Indeed, changes in fasting plasma glucose and insulin, as well as improved HOMA-IR indices were observed upon 12, 24 and 48 weeks of oral L-carnitine supplementation suggesting that the duration of 36 days as in our study might be too short to improve insulin sensitivity. Future studies are therefore needed to reveal if longer duration of carnitine supplementation would result in improved insulin sensitivity.

In conclusion, we here show that carnitine supplementation has very pronounced effects of on metabolic flexibility in impaired glucose tolerant volunteers, and in fact can completely restore metabolic flexibility. Carnitine supplementation enhanced the increase in acetylcarnitine concentration in resting muscle over the day as well as the capacity to form acetylcarnitine with exercise. These changes in acetylcarnitine formation may be underlying the beneficial effects on metabolic flexibility. Longer studies are needed to investigate if carnitine supplementation can also improve insulin sensitivity. Taken together, carnitine supplementation may be an interesting aid in improving disturbed metabolism in subjects prone to develop type 2 diabetes mellitus.

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SUPPLEMENTARY DATA

Supplementary Table 1. Plasma acylcarnitine before and after 30 days of carnitine and placebo

	IGT placebo	IGT carnitine	P-value
Glucose (mmol/L)			
Day 0	5.7 ± 0.2	5.6 ± 0.2	p=0.315
Day 7	5.5 ± 0.1	5.6 ± 0.2	
Day 14	5.4 ± 0.1	5.5 ± 0.2	
Day 21	5.4 ± 0.5	5.9 ± 0.2	
Day 28	5.6 ± 0.2	5.6 ± 0.2	
Day 33	5.4 ± 0.1	5.6 ± 0.1	
Insulin (pmol/L)			
Day 0	68.8 ± 9.0	78.6 ± 11.7	p=0.501
Day 7	75.0 ± 13.7	71.8 ± 13.2	
Day 14	72.8 ± 10.6	88.0 ± 17.0	
Day 21	89.5 ± 17.5	114.9 ± 21.1	
Day 28	46.2 ± 6.4	58.2 ± 9.8	
Day 33	56.2 ± 9.3	59.6 ± 10.5	
HbA1C (%)			
Day 0	5.5 ± 0.1	5.5 ± 0.1	p=0.921
Day 33	5.5 ± 0.1	5.6 ± 0.1	
Blood lipid profile			
Total cholesterol (mmol/L)			
Day 0	5.7 ± 0.3	5.9 ± 0.2	p=0.521
Day 7	5.7 ± 0.3	5.8 ± 0.2	
Day 14	5.6 ± 0.3	6.0 ± 0.3	
Day 21	5.5 ± 0.2	5.7 ± 0.2	
Day 28	5.6 ± 0.3	5.1 ± 0.3	
Day 33	5.4 ± 0.2	5.5 ± 0.3	
HDL cholesterol (mmol/L)			
Day 0	1.4 ± 0.1	1.4 ± 0.1	p=0.391
Day 7	1.4 ± 0.1	1.4 ± 0.2	
Day 14	1.2 ± 0.1	1.4 ± 0.2	
Day 21	1.4 ± 0.1	1.2 ± 0.1	
Day 28	1.6 ± 0.2	1.1 ± 0.1	
Day 33	1.3 ± 0.1	1.3 ± 0.1	
LDL cholesterol (mmol/L)			
Day 0	3.4 ± 0.3	3.6 ± 0.2	p=0.620
Day 7	3.5 ± 0.3	3.6 ± 0.3	
Day 14	3.5 ± 0.3	3.5 ± 0.2	
Day 21	3.3 ± 0.3	3.3 ± 0.2	
Day 28	3.3 ± 0.1	2.9 ± 0.2	
Day 33	3.2 ± 0.2	3.2 ± 0.2	
Triglycerides (mmol/L)			
Day 0	2.1 ± 0.3	2.6 ± 0.5	p=0.512
Day 7	2.1 ± 0.3	2.6 ± 0.5	
Day 14	2.5 ± 0.5	3.6 ± 1.5	
Day 21	2.9 ± 0.5	3.0 ± 0.4	
Day 28	2.6 ± 0.3	2.2 ± 0.4	
Day 33	2.0 ± 0.2	2.1 ± 0.3	
Liver function			
ASAT (U/L)			
Day 0	27.1 ± 2.3	25.2 ± 2.0	p=0.678
Day 33	23.7 ± 1.5	23.6 ± 3.4	
ALAT (U/L)			
Day 0	28.1 ± 3.6	29.8 ± 3.4	p=0.758
Day 33	25.6 ± 2.7	32.3 ± 4.5	
Gamma-GT (U/L)			
Day 0	39.2 ± 8.2	39.4 ± 6.3	p=0.546
Day 33	34.6 ± 6.0	32.3 ± 6.3	

Data are expressed as mean ± SEM. P-value reflect time*treatment effect by two-way repeated measures ANOVA. Plasma values are obtained after an overnight fast.

Supplementary Table 2. Plasma acylcarnitine before and after 30 days of carnitine and placebo

	NGT	IGT placebo	IGT carnitine	IGT placebo	IGT carnitine
	Day 0	Day 0	Day 0	Day 33	Day 33
C0	42.48 ± 1.19	39.43 ± 1.44	40.77 ± 1.57	39.75 ± 1.31	50.52 ± 1.66 ^{a,b,c}
C2	5.52 ± 0.35	5.95 ± 0.36	6.63 ± 0.44	6.24 ± 0.35	8.28 ± 0.61 ^{a,b,c}
C3	0.44 ± 0.02	0.42 ± 0.04	0.45 ± 0.05	0.47 ± 0.03	0.55 ± 0.04 ^{a,b,c}
C4	0.35 ± 0.01	0.31 ± 0.01	0.33 ± 0.03	0.32 ± 0.02	0.34 ± 0.02 ^c
C5:1	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00 ^a
C5	0.11 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.10 ± 0.01
C4-3OH	0.04 ± 0.01	0.04 ± 0.01	0.06 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
C6	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.08 ± 0.01
C5OH	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C8	0.21 ± 0.03	0.23 ± 0.02	0.25 ± 0.03	0.20 ± 0.02	0.23 ± 0.03
C3 DC	0.06 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.07 ± 0.01
C10:1	0.16 ± 0.02	0.20 ± 0.02	0.22 ± 0.03	0.17 ± 0.02	0.18 ± 0.02
C10	0.25 ± 0.03	0.28 ± 0.03	0.31 ± 0.04	0.24 ± 0.03	0.27 ± 0.03
C4 DC	0.02 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.06 ± 0.01 ^c
C5 DC	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.00	0.06 ± 0.01
C12:1	0.09 ± 0.01	0.10 ± 0.01	0.12 ± 0.01	0.09 ± 0.01	0.11 ± 0.01
C12	0.09 ± 0.01	0.12 ± 0.01	0.13 ± 0.02 ^d	0.10 ± 0.01	0.11 ± 0.01
C6 DC	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
C12:1OH	0.03 ± 0.01	0.07 ± 0.04	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
C12OH	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
C14:2	0.04 ± 0.00	0.05 ± 0.00	0.05 ± 0.01	0.04 ± 0.00	0.04 ± 0.01
C14:1	0.09 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
C14	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.01	0.03 ± 0.00	0.03 ± 0.00
C8 DC	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00 ^b
C14:1OH	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
C14OH	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C16:1	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
C16	0.10 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
C10 DC	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C16:1OH	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C16OH	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C18:2	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
C18:1	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
C18	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
C18:2OH	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C18:1OH	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C18OH	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Data are expressed as mean ± SEM. ^a significantly different from day 0, ^b different from IGT on placebo at day 33, ^c significantly different from NGT day 33, ^d different from NGT day 0.

Supplementary Table 3. Skeletal muscle acylcarnitine concentration (in biopsies) before and after 30 days of carnitine and placebo

	NGT	IGT placebo	IGT carnitine
	Day 33	Day 33	Day 33
C0	6820.44 ± 838.28	7647.93 ± 495.58	8490.26 ± 933.94
C2	644.88 ± 80.30	731.41 ± 113.50	885.53 ± 175.97
C3	10.96 ± 1.39	12.73 ± 1.18	12.91 ± 1.34
C4	9.41 ± 1.37	10.59 ± 2.65	7.46 ± 1.31
C5:1	0.41 ± 0.09	0.73 ± 0.13	0.80 ± 0.22
C5	3.22 ± 0.62	4.95 ± 1.35	4.94 ± 1.15
C4-3OH	2.59 ± 0.41	4.55 ± 1.35	4.62 ± 1.25
C6	5.75 ± 1.55	7.54 ± 2.54	3.79 ± 1.36
C5-OH	1.03 ± 0.24	1.51 ± 0.29	1.48 ± 0.32
C8:1	1.03 ± 0.17	1.26 ± 0.19	0.78 ± 0.10 ^a
C8	3.24 ± 1.08	3.59 ± 1.22	1.82 ± 0.51
C3DC	0.71 ± 0.45	0.42 ± 0.15	0.72 ± 0.18
C4DC	1.77 ± 0.60	1.42 ± 0.20	1.48 ± 0.20
C10	1.78 ± 0.65	2.18 ± 0.74	1.30 ± 0.49
C5DC	1.18 ± 0.78	0.43 ± 0.12	0.52 ± 0.15
C12:1	0.42 ± 0.11	0.78 ± 0.27	0.50 ± 0.21
C12	1.62 ± 0.46	3.38 ± 1.22	2.22 ± 1.11
C8:1DC or C12:1-OH	0.12 ± 0.02	0.19 ± 0.05	0.21 ± 0.05
C8DC or C12-OH	0.13 ± 0.02	0.17 ± 0.04	0.17 ± 0.03
C14:2	0.88 ± 0.21	1.90 ± 0.66	1.33 ± 0.77
C14:1	3.17 ± 0.88	8.62 ± 3.65	5.73 ± 3.93
C14	3.20 ± 0.91	8.87 ± 3.40	5.38 ± 3.03
C10:1DC or C14:1-OH	0.32 ± 0.05	0.62 ± 0.14 ^c	0.48 ± 0.13
C16:2	0.61 ± 0.17	1.65 ± 0.57 ^d	0.98 ± 0.62
C16:1	3.21 ± 0.90	9.16 ± 3.59	5.29 ± 3.31
C16	9.15 ± 2.24	18.58 ± 5.36	12.84 ± 3.63 ^b
C12:1DC or C16:1-OH	0.31 ± 0.04	0.59 ± 0.16 ^d	0.43 ± 0.11
C18:2	3.22 ± 0.85	5.29 ± 1.35	3.50 ± 0.84 ^a
C18:1	10.72 ± 2.66	20.42 ± 6.24	12.69 ± 3.95 ^a
C18	4.02 ± 0.88	6.95 ± 1.97	5.31 ± 1.49 ^a
C20:2	0.06 ± 0.01	0.11 ± 0.02 ^d	0.08 ± 0.02
C20:1	0.19 ± 0.04	0.34 ± 0.10	0.22 ± 0.06 ^a
C20	0.07 ± 0.02	0.12 ± 0.04	0.11 ± 0.03 ^b

Data are expressed as mean ± SEM. ^asignificantly different from IGT placebo, ^btending towards a difference from IGT on placebo, ^csignificantly different from NGT, ^dtending towards a difference from NGT.

CHAPTER 8

General Discussion

GENERAL DISCUSSION

Type 2 Diabetes Mellitus (T2D) is a complex multifactorial disease with severe complications. The incidence of type 2 diabetes is rising dramatically all over the world, and it is predicted that 600 million people will suffer from T2D by 2030 [1]. Reduced insulin sensitivity and mitochondrial function are key characteristics of metabolic diseases. Lifestyle intervention programmes, like caloric restriction and exercise training, have proven to be most effective for protecting against metabolic diseases but adherence is poor. In addition, although great progress has been made in the development of drugs for glycaemic control there is a demand for non-pharmacological treatment options. The combination of these factors stresses the need for novel effective therapies to prevent and treat T2D.

Mitochondrial dysfunction has been related to the development of insulin resistance and T2DM. For example, T2D is associated with reduced skeletal muscle mitochondrial function and reduced metabolic flexibility [2]. Even though it is still unclear whether a direct relationship exists between mitochondrial dysfunction and insulin resistance [2], targeting mitochondrial function is regarded a promising strategy for improving insulin sensitivity and thereby prevent T2DM.

Caloric restriction is the most robust non-pharmacological intervention for preserving metabolic health. Caloric restriction is usually defined as a modest reduction in caloric intake of around 20-40%, as compared to ad libitum intake [3]. Much research has focussed on unravelling how exactly caloric restriction promotes metabolic health, and there is an ongoing search for compounds that can mimic beneficial effects of caloric restriction without extensive changes in lifestyle. Among the different mechanism by which caloric restriction might act, silent information regulator 2 (SIRT1) is regarded as specifically interesting, considering it incorporates the sensing of the metabolic energy status with adaptive transitional outputs [4]. Based on the idea that sirtuins are essential for the beneficial effects of caloric restriction, a large *in vitro* screening was performed to identify small molecule activators of SIRT1 [5]. Upon this screening resveratrol was identified as the most potent activator of SIRT1 [5]. In this thesis, we evaluated if resveratrol can be used in humans to target mitochondrial function - and thereby have beneficial metabolic health effects - including improvements in insulin sensitivity.

Can resveratrol improve mitochondrial function and metabolic health?

Animal studies confirmed that resveratrol can indeed have substantial effects on metabolic health, for example by improving glucose homeostasis. The beneficial health effects of resveratrol in mice have been principally attributed to the potential of resveratrol to enhance energy utilisation by boosting mitochondrial oxidative capacity and lipid oxidation [6]. The underlying mechanism is based on the potential of resveratrol to activate SIRT1. Activation of SIRT1 leads to de-acetylating PGC-1 α , which can translocate to the nucleus and act as a master

regulator of mitochondrial biogenesis [7]. Indeed, animal studies revealed that treatment with resveratrol increased mitochondrial content in among others skeletal muscle [6].

In **chapter 3 and 4** of this thesis, two studies are described where 150mg resveratrol was supplied for 30 days to a group of male patients with T2D and a group of males at increased risk of developing T2D. After 30 days, a significant improvement could be measured in both groups in *ex vivo* muscle mitochondrial oxidative capacity compared to placebo. These data are consistent with a previous study in healthy obese men [8], where the same dose and treatment duration of resveratrol supplementation were used and also led to improvements of mitochondrial function.

How can resveratrol improve metabolic health?

While we found that resveratrol can improve muscle mitochondrial function, in the two studies presented in **chapter 3 and 4**, yet no effects on metabolic health were observed. Likewise, when the data of these two studies was combined with the data of a previously performed clinical trial in healthy obese men – as described in **chapter 5** - a robust improvement of *ex vivo* muscle mitochondrial function was established upon resveratrol supplementation. In addition, although not found in each study separately, a significant decrease in both systolic blood pressure and sleeping metabolic rate was found when resveratrol treatment was compared to placebo. Still, even in this combined data-analyses described in **chapter 5**, no effects were found of resveratrol on glucose homeostasis.

Intriguingly, there are discrepancies between results from *in vivo* experiments with resveratrol supplementation and SIRT1-overexpression models. For example, most rodent studies show that resveratrol treatment improves glucose homeostasis [6, 9, 10], while muscle-specific SIRT1 overexpression does not affect insulin sensitivity [11]. In addition, rodent studies have shown that resveratrol treatment can blunt high-fat diet induced weight-gain, while SIRT1 transgenic mice have similar weight gain as control wild-type mice [12-14]. More importantly, resveratrol has been shown to be able to improve glucose homeostasis in SIRT1 deficient mice [15]. These results indicate that additional mechanisms are required - beside SIRT1 activation or improvement of mitochondrial biogenesis - for resveratrol to improve whole body metabolic health. Although indirect, our findings that resveratrol did improve mitochondrial function in human skeletal muscle, but did not affect insulin sensitivity would fit with this suggestion.

Activation of SIRT1 and AMP-activated protein kinase (AMPK) have largely overlapping outcomes. AMPK plays a central role in cellular energy homeostasis, and is activated upon energy stress triggered by for example exercise and fasting [16]. Activation of AMPK results in among other stimulation of hepatic and skeletal muscle fatty acid oxidation, increased glucose uptake in skeletal muscle, and inhibition of adipocyte lipolysis and lipogenesis [7]. Several animal studies have revealed that resveratrol can activate AMPK, and metabolic effects of resveratrol largely

depend on AMPK activation [17-20]. Interestingly, a study with AMPK-deficient mice revealed that in the absence of AMPK α_1 or AMPK α_2 , resveratrol failed to increase insulin sensitivity, glucose tolerance or expression of PGC-1 α [21]. In our clinical trial with patients with T2D (**chapter 3**) protein expression of pAMPK, AMPK and PGC-1 α was measured in skeletal muscle. However, consistent with the lack of effect of resveratrol on insulin sensitivity, resveratrol also did not activate AMPK - as represented by the pAMPK/AMPK ratio - nor did it change the expression of PGC-1 α . In a previous clinical trial in healthy obese men with the same dose of resveratrol, activation of AMPK and increased expression of PGC-1 α has been observed which was paralleled by improvements in glucose homeostasis [8]. It can therefore be speculated that AMPK is a crucial regulator of the metabolic actions of resveratrol, targeting alternative pathways beyond those affecting muscle mitochondrial function. This may also suggest that merely improving mitochondrial function is not sufficient to improve whole body metabolic health. This theory is supported by data from Hoehn et al. [22], who revealed that acute or chronic up-regulation of mitochondrial fatty acid oxidation has no net effect on whole-body energy expenditure or adiposity. In addition, a mouse study by Wong et al. [23] showed that skeletal muscle PGC-1 α overexpression improved mitochondrial function, but did not prevent high-fat diet induced insulin resistance. The PGC-1 α induced increase in mitochondrial function was only accompanied by improved insulin sensitivity when the mice were stimulated to become physically active. It could therefore be speculated that merely increasing mitochondrial function is insufficient to boost metabolic health, but that using the higher mitochondrial capacity - for example during physical activity - is important for the efficacy of resveratrol. Future studies could test the concept that resveratrol, in addition to a physical activity program, may have beneficial effects in volunteers with disturbed glucose homeostasis.

For which population is resveratrol beneficial?

Although the results from pre-clinical studies were promising, the results from human studies are less straight forward. The number of clinical trials failing to show beneficial effects of resveratrol, almost outbalance the number of studies that do show positive effects. Major underlying problems are that it is still unknown what the optimal dose and treatment duration are of resveratrol, what the exact mechanisms of action are, and for which subject populations specifically resveratrol could be beneficial. Thus, it can be questioned if the dose of 150mg/day resveratrol used in the studies described in **chapter 3 and 4** might have been too low, or the treatment duration of a month too short to elicit effects on glucose metabolism in humans with disrupted glucose homeostasis. A recent clinical trial in obese men with moderate insulin resistance used the same relatively low dose of resveratrol (150 mg/day) and compared it to a higher dose of 1000 mg/day [24]. In this study resveratrol was supplemented for a period of six months, but no effects on glucose or lipid metabolism were found upon neither of the doses. These findings agree with our findings from **chapter 3 and 4**, namely that resveratrol supplementation might not be beneficial for improving glucose homeostasis in men with

decreased insulin sensitivity. In addition, we observed a potential interaction between resveratrol and metformin (**chapter 3**), a commonly used oral glucose-lowering drug. This suggests that medication use can be an important determinant for efficaciousness of resveratrol. Thus, in **chapter 3** we observed a strong correlation between plasma dihydro-resveratrol (DHR) levels at day 30 of the resveratrol treatment period, and the dose of metformin used by the T2D patients. Post-hoc analysis revealed that plasma DHR levels were higher during 30 days of resveratrol supplementation in T2D patients taking a relatively high dose of metformin (> 1000 mg/day metformin) compared to patients treated with a significant lower dose (≤ 1000 mg/day metformin). These results suggest that there could be an interaction between metformin and the metabolism of resveratrol. In addition, it has been suggested that metformin treatment can lead to phosphorylation of the threonine residue 172 (thr172) in the α -subunit of AMPK [25]. Potentially, a daily dose of 150 mg of resveratrol may not have been able to further activate AMPK in T2D patients receiving metformin, thereby explaining the lack of effect of resveratrol on insulin sensitivity in our study where resveratrol was supplemented on-top of metformin treatment (**chapter 3**). The combination of these findings suggest that resveratrol might not be suitable for patients on metformin, but resveratrol could still be beneficial for patients on other oral glucose-lowering medication. Movahed et al. [26] performed a sub-analysis on their data to check the effectiveness of resveratrol on clinical and biochemical parameters for the different oral glucose-lowering medications included in their study. Interestingly, the patients treated with metformin only, showed no significant improvement of resveratrol on circulating insulin and HbA1c levels compared to the control group, whereas the computed HOMA-IR index did improve upon resveratrol. In contrast, patients receiving sulphonylurea derivatives (SUDs) or metformin in combination with SUDs demonstrated a resveratrol-induced improvement in both plasma insulin and HbA1c levels. Together with our study results, these findings reinforce the importance of investigating the relationship between resveratrol and metformin in future research and underline that resveratrol still might be beneficial for patients on other oral-glucose lowering medication beside metformin. In addition, our data from **chapter 3** and **4** indicate that resveratrol might not be suitable for individuals with disrupted glucose homeostasis. In **chapter 5**, we tried to identify if participant characteristics, such as plasma glucose and HOMA-IR, can influence effectiveness of resveratrol. However, no relationship was found between baseline HOMA-IR, used as marker of insulin sensitivity, and resveratrol-induced changes in outcome parameters. To be more specific, baseline HOMA-IR did not significantly predict resveratrol-induced change in plasma glucose, insulin, intrahepatic lipid (IHL) content and blood pressure. These results suggest that having a decrease in insulin sensitivity does not influence effectiveness of resveratrol, and individuals with decreased insulin sensitivity do not specifically benefit from resveratrol treatment.

Surprisingly, baseline total serum bilirubin significantly predicted resveratrol-induced change in diastolic blood pressure, alanine aminotransferase (ALT), intrahepatic lipid content and plasma glucose. Previous studies reported that serum bilirubin itself can significantly contribute to total antioxidant capacity [27, 28]. For example, at the molecular level bilirubin can scavenge reactive oxygen species [29] and thereby prevent oxidation of intracellular lipids [30]. Potentially there is a synergetic effect of bilirubin and resveratrol on metabolic health. Furthermore, bilirubin is conjugated with glucuronic acid by the same enzyme as *trans*-resveratrol, namely UDP-glucuronosyltransferase (UGT1A1) [33]. It has been proposed that bilirubin can be seen as a surrogate for UGT1A1 activity [34, 35]. Thus, higher serum bilirubin levels could indirectly imply higher UGT1A1 activity. Although speculative, this could mean that glucuronidation - by UGT1A1 - of *trans*-resveratrol is important for effectiveness of resveratrol. To conclude, the results from **chapter 3, 4 and 5** combined give strong indications that resveratrol supplementation cannot improve metabolic health in everyone, and subject characteristics - such as serum bilirubin levels - could play an important role in effectiveness of resveratrol.

In the clinical trial described in **chapter 6**, a varied population of overweight men and women was enrolled. Men and women with overweight or obesity used 150mg resveratrol or a placebo for six months. The interim-analyses reported in chapter 6 gave indications that resveratrol might be beneficial when consumed for a longer period. Thus, this interim-analysis suggests that 6-months resveratrol lowers HbA1c, tends to decrease IHL content, tends to blunt increases in fat mass and tends to increase leg muscle strength, as represented by time to complete a Timed Chair-Stand Test. Other studies in which participants were enrolled with disturbed glucose homeostasis, such as patients with T2D and men with the metabolic syndrome, did not find a reduction in IHL [8, 24, 36]. This again may suggest that low-dose resveratrol can only have beneficial metabolic effects in relatively healthy obese individuals or that a longer duration of treatment with resveratrol is needed to observe whole body improvements. Considering that this was an interim-analysis, and the primary study outcome parameter was not taken into account in this analysis, no conclusions can be drawn regarding insulin sensitivity. Completion of this study will give more robust answers to the question whether resveratrol can be beneficial in the prevention of metabolic disturbances in a varied population of overweight men and women.

Other strategies to combat metabolic diseases

Next to resveratrol, other promising food supplements that may affect mitochondrial energy and substrate metabolism and whole-body metabolic health have been identified in the last decades. In **chapter 7** we investigated one of those new promising supplements, namely L-carnitine, and its effects on metabolic health. Carnitines are involved in mitochondrial transport of fatty acids and are important for maintaining normal mitochondrial function [37, 38]. Carnitine availability can be limiting step for β -oxidation, and carnitine supplementation can potentially enhance fatty acid metabolism in the mitochondria [38]. Recently, carnitine has been suggested to play an even

more important role in metabolic health by buffering acyl-CoA concentrations by conversion to acyl-carnitines. Especially the buffering of acetyl-CoA by conversion to acetylcarnitine has received a lot of attention. By these mechanisms, the maintenance of glucose homeostasis and metabolic flexibility may depend on availability of carnitine. In the clinical trial described in **chapter 7**, overweight men and women with disrupted glucose homeostasis received carnitine supplementation and placebo both for a period of 36 days. A group of individuals with normal glucose homeostasis was added as a control group. Metabolic flexibility - expressed as the change in respiratory exchange ratio as measured during a hyperinsulinemic-euglycemic clamp - was completely restored upon carnitine supplementation to the level of the healthy controls. Similar results on metabolic flexibility were found in response to a high-energy meal. Despite beneficial effects of carnitine on metabolic flexibility, no improvement in insulin sensitivity was measured. Based on previous studies, it can be suggested that the duration of the carnitine supplementation may have been too short to improve insulin sensitivity [39, 40]. Thus, Galloway et al.[39] and Gonzalez-Ortiz et al.[40] did not report changes in glucose homeostasis after four and two weeks of 3g L-Carnitine supplementation respectively. Therefore, future research is needed to investigate the mechanism behind the improvement in metabolic flexibility, and if the improvements in metabolic flexibility will result in improvements in insulin sensitivity when carnitine is given for a longer period. Especially studies in individuals with severely compromised metabolic health such as patients with T2D are warranted. What we can conclude so far is that carnitine supplementation is an interesting strategy for instigating a healthy metabolism in subjects prone to develop T2D.

Future perspectives

After reading the first four chapters of this thesis one can wonder: is resveratrol really the answer in the fight against metabolic diseases? Should we shift our focus from resveratrol to new strategies to improve metabolic health, or is there still a window of opportunity for resveratrol? What we can conclude from the research presented in this thesis is that resveratrol can boost muscle mitochondrial function significantly. It however remains unclear whether such improvements in mitochondrial function are sufficient to improve whole body metabolic health, and if certain participant characteristics play a role herein. So before definitive conclusions can be made on the potential of resveratrol as a food supplement to improve metabolic health in humans, more research is needed. Based on the results presented in this thesis and available literature, the following suggestions can be made for future research:

- It would be interesting to explore the concept of combining resveratrol treatment with a physical activity program in individuals with disrupted glucose homeostasis. It can be hypothesized that merely increasing mitochondrial function is insufficient to boost metabolic health, and actually using the higher mitochondrial capacity - for example during physical activity - could be important for the efficacy of resveratrol.

- Completion of the long-term resveratrol treatment study, described in chapter 6, will give more robust answers to the question whether a longer treatment duration with resveratrol is needed to elicit beneficial effects and metabolic health in a population of overweight men and women.
- Measuring plasma concentrations of free-, glucuronidated and sulfated resveratrol separately could shed light on which form of resveratrol actually induces beneficial effects on metabolic health. In turn, this could provide additional information on why resveratrol is more effective in certain individuals than others, and which subject characteristics play a role herein. For example, *in vitro* studies should be performed to explore the potential synergetic effect of serum bilirubin and resveratrol on glucose homeostasis. In addition, the importance of glucuronidation and de-conjugation of *trans*-resveratrol can be investigated into in more depth. Specifically, the gene UGT1A1 and the enzyme β -glucuronidase could play a role in bioavailability of resveratrol, and the conversion of resveratrol into its bioactive form.
- From chapter 3, it became apparent that medication use can be an important determinant for efficaciousness of resveratrol. Thus, we observed a correlation between plasma dihydro-resveratrol values and dose of metformin. This reinforces the importance of investigating the relationship between resveratrol and metformin in future research and underlines that resveratrol still might be beneficial for patients on other oral-glucose lowering medication such as sulphonylurea derivatives.
- Finally, even though more research on resveratrol is needed, alternative treatments should be explored for improving metabolic health. An interesting new approach is the food supplement L-carnitine. From chapter 7 can be concluded that carnitine supplementation has pronounced effects on metabolic flexibility in humans with impaired glucose tolerance. Clinical trials are needed with a longer period of supplementation, to investigate if carnitine supplementation can also improve insulin sensitivity. In addition, it would be interesting to investigate the molecular mechanism behind the improvement in metabolic flexibility, and if carnitine supplementation can also be beneficial for individuals with more severely compromised metabolic health such as patients with T2D.

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APPENDICES

Summary

Nederlandse Samenvatting

Valorization

Dankwoord

Curriculum Vitae

List of Publications

SUMMARY

Over the past decades, the average life expectancy of humans has gradually increased. Better nutrition and health care are fundamental factors for this increase in longevity. Although life expectancy is increasing, the prevalence of non-communicable diseases is also rapidly increasing. A common non-communicable disease is Type 2 Diabetes Mellitus (T2D). Insulin resistance is the most important factor in the development of T2D. In healthy humans the hormone insulin is responsible for the uptake of glucose (sugar) from the blood into the cells, this is dysregulated in patients with T2D (they are insulin resistant). Over the years it has become evident that the mitochondria (who are present in all cells of the human body and break down nutrients to produce energy) play an important role in the preservation of muscle insulin sensitivity. Thus, therapies that target mitochondrial function could be interesting strategies to improve insulin sensitivity and thereby prevent and treat T2D. For such therapies we do not directly have to turn into the direction of medication, dietary supplements might also offer a solution. Two promising nutritional supplements that could influence insulin sensitivity and mitochondrial function are resveratrol and carnitine. To explore the potentials of these supplements, in this thesis a review was written, four human clinical trials were performed, and a combined data analysis was conducted. The data from these studies is presented and discussed in this thesis.

Resveratrol is a polyphenol that is naturally present in several plants. Small amounts of resveratrol can be found in peanuts, grapes and red wine. In 2003, researchers discovered that resveratrol can activate the molecule sirtuin 1 (SIRT1). In the human body, SIRT1 plays an important role in the regulation of mitochondrial function. This aspect makes SIRT1 an interesting molecular target for the treatment of metabolic disorders such as T2D. Given that resveratrol can activate SIRT1, in turn this makes resveratrol an interesting strategy to prevent and treat T2D. In **chapter 2**, we evaluated if resveratrol can relieve metabolic disturbances by reviewing existing literature of animal studies and human clinical trials. Main findings described in **chapter 2** were that animal studies show beneficial effects of resveratrol supplementation on mitochondrial function, glucose metabolism, body composition and accumulation of fat in the liver. However, it was also concluded that these findings cannot yet be confirmed in humans: too few well-controlled clinical studies have been performed, and it is still unclear what the optimal dose of resveratrol is. In addition, in **chapter 2** an interesting new option for future research was introduced, namely to focus on brown adipose tissue. Since a couple of years we know that humans have so-called brown adipose tissue, beside the regular white adipose tissue. In contrast to white adipose tissue (which mainly stores fat), brown adipose tissue stores little fat and instead burns fat to produce heat and thus regulate body temperature. This characteristic of brown adipose tissue led to the idea that stimulating brown adipose tissue could lead to weight loss. A number of animal studies have been conducted that have investigated whether resveratrol can stimulate brown adipose tissue and these studies were generally encouraging. Whether resveratrol can also have

an effect on brown adipose tissue in humans has not yet been investigated. The main conclusions of **chapter 2** are that more placebo-controlled clinical trials are needed, including studies that investigate whether resveratrol can stimulate brown adipose tissue in humans.

In **chapter 3** and **4**, two human clinical trials are described. In both studies 150mg/day resveratrol was given as a supplement to either a group of male patients with T2D (**chapter 3**) or a group of men at increased risk of developing T2D (**chapter 4**). For both studies, the main outcome parameter was insulin sensitivity, which was assessed by the hyperinsulinemic-euglycemic two-step clamp technique. In addition, in both studies muscle mitochondrial function, liver fat content, muscle fat content, energy expenditure, blood pressure and several plasma metabolites were measured. In **chapter 4** some additional measurements were performed to examine effects of resveratrol on brown adipose tissue.

In **chapter 3**, seventeen patients with T2D were treated with placebo and resveratrol on top of standard medication for diabetes. After 30 days, resveratrol significantly improved mitochondrial function. However, insulin sensitivity was not affected by resveratrol treatment and also the amount of fat in the liver did not change. On the other hand, fat accumulation in the muscle increased, and systolic blood pressure tended to decrease upon resveratrol treatment. Intriguingly, there appeared to be an interaction between the dose of medication used by the patients and the concentration of resveratrol found in the blood upon supplementation. It could therefore be speculated that the lack of effect of resveratrol on insulin sensitivity is caused by this interaction, suggesting that the medication use of the T2S patients interfered with the effects of resveratrol. In **chapter 4**, thirteen men at increased risk of T2D received resveratrol for 30-34 days. Again, resveratrol treatment significantly improved muscle mitochondrial function. However, resveratrol did not improve insulin sensitivity nor affected accumulation of fat in the liver or muscle, substrate utilization, energy metabolism or brown-adipose tissue.

The main conclusions of **chapter 3** and **4** are that resveratrol can stimulate *ex vivo* muscle mitochondrial function, which is in concordance with previous results. However, resveratrol supplementation does not improve insulin sensitivity in either patients with T2D or in men at increased risk of T2D. Our results question the generalized value of resveratrol as therapy in the prevention and treatment of T2D and emphasize the need to explore whether the dose might have been too low or the treatment duration too short to elicit effects on insulin sensitivity. In addition, in **chapter 4** it was suggested that differences in subject characteristics, such as age, body weight and gut microbiota composition could be important influential factors for effectiveness of resveratrol. It would therefore be interesting to investigate whether, and if so which, participant characteristics play a role in the effectiveness of resveratrol. To investigate this, we conducted a combined data-analysis (**chapter 5**), in which we looked at which participant characteristics could predict the effectiveness of resveratrol.

In **chapter 5**, data from three human clinical trials was combined. In all three clinical trials the same protocol was applied, and only the populations varied from healthy obese men, patients with T2D and men at increased risk of T2D. What among others emerged from this data analysis, was that bilirubin (which is a breakdown product of red blood cells measured in the blood) predicted whether diastolic blood pressure, plasma ALT, fat accumulation in the liver and glucose changed upon resveratrol supplementation. What can be concluded from **chapter 5** is that higher bilirubin levels in the blood are associated with stronger improvements in metabolic health upon supplementation with resveratrol. However, the exact mechanistic link between bilirubin and the efficacy of resveratrol needs to be investigated further.

Considering the treatment duration - used in the studies described in chapter 3, 4 and 5 - may have been too short for the translation of improvements in mitochondrial function to whole body effects, we performed a long-term resveratrol supplementation study in a mixed population of overweight men and women. In **chapter 6**, the results are presented of an interim-analyses (the study is not finalised yet). In this study, overweight men and woman received 150 mg/day resveratrol or placebo for six months. Twenty participants completed the study and their data was used for the interim-analyses. The primary outcome was insulin sensitivity, but this was not taken into account in the interim-analyses to preserve integrity of the study until completion. Secondary outcome measures were liver fat content, body composition, resting energy metabolism, blood pressure and several plasma markers. After six months, no significant differences were found in the plasma markers except in HbA1c (%). HbA1c is a measure of the concentration of glucose in the blood over the past months. HbA1c decreased in the participants who had received resveratrol, while it increased in the people who had received the placebo. Furthermore, the amount of fat in the liver seemed to decrease in the people upon resveratrol while it increased slightly in the placebo group. However, no differences were found between the groups regarding body composition, blood pressure or energy utilisation. Since it was an interim analysis, no definitive conclusions can be drawn yet. Completion of this study will provide more robust answers.

Next to resveratrol, other promising dietary supplements - that can have positive effects on insulin sensitivity - have been identified over the last decades. In **chapter 7** we examined one of these supplements, namely carnitine. Carnitine occurs naturally in meat, fish and milk. Carnitine supplementation could be beneficial, because recently it has been suggested that availability of carnitine is important for maintaining metabolic flexibility. Metabolic flexibility, like insulin resistance, is an important feature in the development of T2D. In our study we gave carnitine (2 g/day) as a supplement to 11 participants at an increased risk of developing T2D, for a period of 36 days. What can be concluded from **chapter 7** is that supplementation with carnitine can fully restore the metabolic flexibility in people at increased risk of T2D. In addition, we observed that carnitine caused an increase in acetylcarnitine concentrations (which is formed by means

of carnitine) in skeletal muscle, and improved the ability to form acetylcarnitine upon exercise. However, no effects were seen on insulin sensitivity.

In conclusion, the research presented in this thesis demonstrates that resveratrol can boost muscle mitochondrial function significantly. However, it remains unclear whether such improvements in mitochondrial function are sufficient to improve insulin sensitivity. Furthermore, it remains unclear how exactly characteristics of the participants play a role in the effectiveness of resveratrol. Before definitive conclusions can be drawn regarding the potential of resveratrol to improve metabolic health in humans more research is needed. In addition, alternative treatment options for T2D, such as carnitine supplementation, should be further investigated.

SAMENVATTING

De afgelopen decennia is de gemiddelde levensverwachting geleidelijk toegenomen. Betere voeding en gezondheidszorg zijn belangrijke factoren voor deze toename. Hoewel de levensverwachting stijgt, neemt het aantal patiënten met zogenaamde welvaartsziekten snel toe. Een veelvoorkomende welvaartsziekte is Type 2 Diabetes Mellitus (T2D), ook wel ouderdomssuikerziekte genoemd. Insulineresistentie is de belangrijkste factor in de ontwikkeling van T2D. Het hormoon insuline zorgt bij gezonde mensen voor de opname van glucose (suiker) uit het bloed in de cellen, deze opname is verstoord bij mensen met T2D (ze hebben insulineresistentie). De afgelopen jaren is het duidelijk geworden dat de mitochondriën (deze zijn aanwezig in elke cel van het lichaam en verbranden voedingsstoffen om energie te produceren) een belangrijke rol spelen bij het behouden van de insulinegevoeligheid van de spieren. Therapieën die gericht zijn op het verbeteren van het functioneren van de mitochondriën kunnen daarom interessante strategieën zijn om de insulinegevoeligheid te verbeteren en daarmee T2D te voorkomen of te behandelen. Voor zulke therapieën hoeven we niet direct te denken aan medicatie; voedingssupplementen zouden mogelijk ook een uitkomst kunnen bieden. Twee veelbelovende voedingssupplementen die de insulinegevoeligheid en mitochondriële functie zouden kunnen beïnvloeden zijn resveratrol en carnitine. Om de potentie van deze supplementen te onderzoeken werd in dit proefschrift een review geschreven, vier humane klinische studies uitgevoerd en een gecombineerde data-analyse uitgevoerd. De resultaten van deze onderzoeken staan in dit proefschrift beschreven en worden hierin bediscussieerd.

Resveratrol is een polyfenol dat van nature voorkomt in verschillende planten. Kleine hoeveelheden resveratrol kunnen worden gevonden in pinda's, druiven en rode wijn. In 2003 hebben onderzoekers ontdekt dat resveratrol het molecuul sirtuin 1 (SIRT1) kan activeren. In het menselijk lichaam speelt SIRT1 een belangrijke rol bij het reguleren van de mitochondriële functie. Dit maakt SIRT1 een interessant molecuulair doelwit voor de behandeling van metabole stoornissen zoals T2D. Gezien het feit dat resveratrol SIRT1 kan activeren, maakt dit resveratrol suppletie op haar beurt een interessante strategie om T2D te voorkomen en te behandelen. In **hoofdstuk 2** is geëvalueerd of resveratrol metabole verstoringen kan verhelpen, door bestaande literatuur van dierstudies en humane klinische studies te evalueren. De belangrijkste bevindingen beschreven in **hoofdstuk 2** zijn dat dierstudies gunstige effecten laten zien van resveratrol suppletie op de mitochondriële functie, de regulatie van glucose, lichaamssamenstelling en opslag van vet in de lever. Er werd echter ook geconcludeerd dat deze bevindingen nog niet bevestigd kunnen worden in de mens. Er zijn nog te weinig goed gecontroleerde klinische studies uitgevoerd en ook is nog onduidelijk wat de optimale dosis is van resveratrol. Daarnaast wordt in **hoofdstuk 2** een interessante nieuwe optie voor toekomstig onderzoek geïntroduceerd, namelijk om te focussen op bruin-vetweefsel. We weten sinds enkele jaren dat mensen naast het gewone witte vetweefsel (dat voornamelijk vet opslaat) ook zogenaamd bruin vetweefsel

hebben. In tegenstelling tot wit vetweefsel, slaat bruin vetweefsel weinig vet op en verbrandt in plaats daarvan juist veel vet om warmte te produceren en daarmee de lichaamstemperatuur te regelen. Deze eigenschap van bruin vetweefsel leidde tot het idee dat het stimuleren van bruin vetweefsel tot gewichtsverlies zou kunnen leiden. Er zijn een aantal dierstudies uitgevoerd die hebben onderzocht of resveratrol bruin vetweefsel kan stimuleren en deze onderzoeken waren over het algemeen bemoedigend. Of resveratrol ook effect kan hebben op bruin vetweefsel in mensen is echter nog niet onderzocht. De belangrijkste conclusies van **hoofdstuk 2** zijn dat er meer placebo-gecontroleerde klinische onderzoeken nodig zijn en dat er studies nodig zijn die onderzoeken of resveratrol bruin-vetweefsel kan stimuleren in mensen.

In **hoofdstuk 3 en 4** worden twee humane placebo-gecontroleerde, klinische studies beschreven. In beide onderzoeken werd 150 mg/dag resveratrol als supplement gegeven aan een groep mannelijke patiënten met T2D (**hoofdstuk 3**) of aan een groep mannen met een verhoogd risico op het ontwikkelen van T2D (**hoofdstuk 4**). Voor beide studies was de belangrijkste uitkomstparameter insulinegevoeligheid, die werd bepaald doormiddel van de hyperinsulinemische-euglycemische tweestaps-clamptechniek. Daarnaast werden in beide studies mitochondriële functie, vetstapeling in de lever, vetstapeling in de spier, energiegebruik, bloeddruk en verschillende plasma metabolieten gemeten. In **hoofdstuk 4** zijn enkele aanvullende metingen uitgevoerd om de effecten van resveratrol op bruin vetweefsel te onderzoeken.

In **hoofdstuk 3** werden zeventien patiënten met T2D behandeld met placebo en resveratrol in aanvulling op de standaard medicatie voor hun diabetes. Na 30 dagen zagen we dat de insulinegevoeligheid niet verbeterde door de behandeling met resveratrol en ook de hoeveelheid vet in de lever bleef hetzelfde. De hoeveelheid vet in de spieren nam echter toe en de systolische bloeddruk daalde na resveratrol behandeling en bovendien verbeterde resveratrol significant de mitochondriële functie. Tot onze verbazing leek er een interactie tussen de hoeveelheid resveratrol in het bloed, en de medicatie die de patiënten gebruikten. Het zou daarom kunnen dat deze interactie tussen de gebruikelijke medicatie en het ingenomen resveratrol ervoor zorgt dat we geen effecten zagen van resveratrol op de insulinegevoeligheid. In **hoofdstuk 4** ontvingen dertien mannen met een verhoogd risico op T2D gedurende 30-34 dagen resveratrol. De behandeling met resveratrol verbeterde weer significant de mitochondriële functie. Echter, resveratrol had weer geen effect op de insulinegevoeligheid, de hoeveelheid vet in de lever en spier, het energiegebruik en ook niet op het bruin vetweefsel.

De belangrijkste conclusies van **hoofdstuk 3 en 4** zijn dat resveratrol mitochondriële functie kan stimuleren, wat in overeenstemming is met eerdere resultaten. Suppletie met resveratrol verbetert de insulinegevoeligheid echter niet bij patiënten met T2D of bij mannen met een verhoogd risico op T2D. Onze resultaten trekken de algemene waarde van resveratrol als

therapie bij de preventie en behandeling van T2D in twijfel en benadrukken de noodzaak om te onderzoeken of de dosis mogelijk te laag was of de behandelingsduur te kort om effecten op insulinegevoeligheid te zien. Bovendien werd in **hoofdstuk 4** gesuggereerd dat verschillen tussen de proefpersonen, zoals leeftijd, lichaamsgewicht en samenstelling van de darmflora, invloed kunnen hebben op de effectiviteit van resveratrol. Het zou daarom interessant zijn om te onderzoeken of en welke proefpersoon karakteristieken een rol spelen in de effectiviteit van resveratrol. Om dit te onderzoeken voerden we een gecombineerde data-analyse uit (**hoofdstuk 5**), waarin we bekeken welke proefpersoon karakteristieken de effectiviteit van resveratrol kunnen beïnvloeden.

In **hoofdstuk 5** werden de gegevens uit hoofdstuk 3 en 4 gecombineerd met de gegevens van een eerder uitgevoerde klinische studie waarin resveratrol werd gegeven aan gezonde mannen met obesitas. In alle drie de klinische onderzoeken werd hetzelfde studieprotocol toegepast, alleen de populaties varieerden van gezonde mannen met obesitas, patiënten met T2D en tot mannen met een verhoogd risico op T2D. Wat onder andere uit deze data-analyse naar voren kwam was dat bilirubine (een afbraakproduct van rode bloedcellen, gemeten in het bloed) voorspelde of de diastolische bloeddruk, plasma-ALT, vetstapeling in de lever en glucose veranderde door resveratrol suppletie. Wat uit **hoofdstuk 5** geconcludeerd kan worden, is dat hogere bilirubinespiegels in het bloed worden geassocieerd met sterkere verbeteringen in de metabole gezondheid bij suppletie met resveratrol. De exacte link tussen bilirubine en de werkzaamheid van resveratrol moet echter verder worden onderzocht.

Gezien dat de behandelingsduur - gebruikt in de studies beschreven in hoofdstuk 3 en 4 - mogelijk te kort is geweest om de verbeteringen in de mitochondriële functie ook te laten resulteren in algehele gezondheidseffecten, hebben we een lange-termijnstudie uitgevoerd met resveratrol. In **hoofdstuk 6** zijn de resultaten gepresenteerd van een tussentijdse-analyse (de studie is nog niet afgerond) waarbij mannen en vrouwen gedurende zes maanden 150 mg/dag resveratrol of placebo innamen. Twintig deelnemers met overgewicht voltooiden het onderzoek en de gegevens werden geanalyseerd voor de tussentijdse-analyse. De belangrijkste uitkomstparameter was insulinegevoeligheid, maar deze meting is niet meegenomen in de tussentijdse-analyse om de integriteit van het onderzoek te bewaren tot voltooiing van de gehele studie. Andere uitkomstparameters waren hoeveelheid vet in de lever, lichaamssamenstelling, energiegebruik in rust, bloeddruk en verschillende plasmamarkers. Na zes maanden werden geen significante verschillen gevonden in de plasmamarkers, behalve in HbA1c (%). HbA1c, zegt iets over de concentratie glucose in het bloed van de afgelopen maanden. Bij de deelnemers die resveratrol in hadden genomen nam HbA1c af, terwijl het toenam bij de mensen die de placebo hadden geslikt. Verder leek de hoeveelheid vet in de lever af te nemen bij de mensen die resveratrol hadden kregen en nam dit juist licht toe in de placebogroep. Er werden echter geen verschillen gevonden tussen de groepen in verandering in lichaamssamenstelling, bloeddruk

of energieverbruik. Aangezien het een tussentijdse-analyse was, kunnen er nog geen definitieve conclusies worden getrokken. Afronding van deze studie zal meer robuuste antwoorden geven.

Naast resveratrol zijn er in de laatste decennia ook andere veelbelovende voedingssupplementen geïdentificeerd die positieve effecten kunnen hebben op de insulinegevoeligheid. In **hoofdstuk 7** onderzochten we een van deze supplementen, namelijk carnitine. Carnitine komt van nature voor in vlees, vis en melk. Carnitine suppletie zou goed kunnen zijn omdat recent is gesuggereerd dat beschikbaarheid van carnitine belangrijk is voor het behouden van metabole flexibiliteit. Metabole flexibiliteit is, net als insulineresistentie, een kenmerk van de ontwikkeling van T2D. Voor ons onderzoek gaven we carnitine (2 g/dag) als supplement aan 11 deelnemers met een verhoogd risico op T2D voor een periode van 36 dagen. Wat uit **hoofdstuk 7** kan worden geconcludeerd, is dat suppletie met carnitine de metabole flexibiliteit in mensen met een verhoogd risico op T2D volledig kan herstellen. Daarnaast zagen we dat carnitine zorgde voor een toename van acetylcarnitine-concentraties (wat wordt gevormd doormiddel van carnitine) gedurende de dag in de skeletspier en het verbeterde het vermogen om acetylcarnitine te vormen bij inspanning. Er werden echter geen effecten gezien op de insulinegevoeligheid.

Wat we kunnen concluderen uit de onderzoeken gepresenteerd in dit proefschrift is dat resveratrol de mitochondriële functie aanzienlijk kan stimuleren in de spieren. Het blijft echter onduidelijk of dergelijke verbeteringen in de mitochondriële functie voldoende zijn om de insulinegevoeligheid te verbeteren. Ook blijft het onduidelijk hoe bepaalde karakteristieken van de deelnemers een rol spelen in de effectiviteit van resveratrol. Voordat definitieve conclusies kunnen worden getrokken over de potentie van resveratrol om de metabole gezondheid van mensen te verbeteren, is meer onderzoek nodig. Daarnaast moeten ook alternatieve behandelingsopties voor T2D, zoals carnitine suppletie, verder worden onderzocht.

VALORISATION

Societal Relevance

Obesity, cardiovascular diseases and Type 2 Diabetes (T2D) are tightly linked to our modern lifestyle, which is characterised by overconsumption of food and a lack of physical activity [1]. Currently, these so called non-communicable diseases are the leading cause of death worldwide. It is therefore of utmost importance that effective preventive and curative treatments are being developed for this class of diseases. T2D is characterised by a decreased sensitivity to the hormone insulin, leading to a dysregulation of glucose metabolism [2]. On the long-term high blood glucose levels can give complications such as a stroke, heart disease, kidney failure and blindness [3]. Existing strategies that promote a healthy diet and stimulate physical activity are effective but have been shown to be challenging to adhere to for most people. Therefore, it is important to explore other treatment options for cardiovascular diseases and T2D that are easier to adhere to.

Over the years, the interest in red wine has steadily increased. There is much debate about whether red wine has beneficial health effects or not. This debate originates from the late 1980s when an apparently paradoxical epidemiological observation was made in French people: they had a relatively low incidence of coronary heart disease while having a diet rich in saturated fats [4]. Since the publication of this epidemiological observation, research aimed at identifying which compound was responsible for these so-claimed beneficial health effects. In 2003, it was discovered that the polyphenol resveratrol – present in red wine – could activate sirtuin 1 (SIRT1) [5]. SIRT1 is an important regulator in cellular energy homeostasis and its activity is linked to longevity [6], thereby making resveratrol an interesting candidate for further research and was marked as a potential player in the French paradox. Even though soon it became clear that the amounts of resveratrol in red wine are too low to explain health effects of red wine – and the French paradox has been suggested to be an illusion – animal studies with resveratrol presented promising results.

The aim of this dissertation was to investigate if the food supplement *trans*-resveratrol can be used in prevention of T2D or can be used as a supportive tool in the management of T2D in humans. Three human clinical trials have been performed, that were specifically designed to provide evidence that resveratrol can help in prevention and treatment of T2D, by improving insulin sensitivity, mitochondrial function and liver fat accumulation. Unfortunately, despite robust improvements in mitochondrial function, resveratrol supplementation for a period of 30 days did not improve insulin sensitivity and liver fat accumulation. Nevertheless, when resveratrol is supplied for a longer period this might be beneficial for prevention of T2D, since for example a decrease in HbA1c was seen when resveratrol was supplied for six months. HbA1c is a marker of long-term glucose homeostasis, and thereby an important biomarker for the onset of T2D.

Therefore, these results are promising and indicate that resveratrol supplementation might still be beneficial in the prevention and treatment of metabolic disturbances - such as T2D - when supplied for a longer period.

Economic Relevance

Novel, easy to adhere to, interventions that can prevent or treat T2D can have an enormous impact on the costs of health care. T2D embodies a major economic burden on health care systems since it is one of the most costly health problems [7]. According to the Dutch Government, the costs for diabetes care were 1.7 billion euro in 2011 in just The Netherlands [8]. Ever since, the number of people suffering from diabetes has only increased and thereby the health costs as well. Thus, food supplements can be an inexpensive and easy supportive tool to prevent and treat T2D, which could scale down the medical costs significantly.

Translation into Practice

Even though in the 30-day clinical trials described in this dissertation we did not see effects on insulin sensitivity, this does not mean we did not make important progress in this field of research. A very important finding is that we saw a robust improvement in mitochondrial function in both patients with T2D and men at increased risk of T2D. Since mitochondrial dysfunction is linked to insulin resistance and metabolic diseases, achieving an improvement in mitochondrial function by just using a food supplement is astonishingly and promising. It should be explored further if the treatment duration of 30-days might have been too short, or if resveratrol supplementation should be combined with a physical activity program to achieve beneficial effects on whole body energy metabolism.

Furthermore, the results presented in this dissertation are or will be used for original scientific articles. These articles have been published in or will be submitted to international well-known peer-reviewed journals. Thereby, the knowledge is shared that can be acquired from these studies with scientists worldwide. In addition, the data from the different studies has also been presented at several national and international conferences and symposia which also helps to increase visibility of the results and thereby could contribute to new insights and ideas for future research.

Planning and Realisation

Supplementation for 30-days with resveratrol in patients with T2D and men at increases risk significantly increased mitochondrial function but did not improve insulin sensitivity or other markers related to metabolic health. An interim-analyses of a long-term study with resveratrol indicated that the treatment duration of 30-days might have been too short to elicit beneficial effects on whole body energy metabolism. Completion of this long-term study will help to answer the question if resveratrol is a promising candidate to improve metabolic health in

humans or not. Furthermore, it became apparent that resveratrol seems to be beneficial in certain individuals but not in others. Differences in participant characteristics could play a role herein, for example the serum bilirubin levels seem to be predictive for response to resveratrol. This relationship has not been observed before and should be explored further, to be able to identify for who resveratrol might still be useful for promoting health. The latter also matches the increasing call for so-called personalised medicine, which is based on the principle of dividing patients into different groups, with medical decisions, practices, interventions and/or products being tailored to the patient based on their predicted response or risk of disease [9]. The focus on individualisation, by personalised medicine, can have economic benefits as well as limit the expenditure on treatments to just the population who are most likely to benefit, thus saving health care costs [9]. Underlining the importance to explore the role of bilirubin - and potentially also other participants characteristics - in the effectiveness of resveratrol.

To achieve these goals, the long-term resveratrol study will be finalised- to see if resveratrol supplementation does have beneficial effects beyond improving mitochondrial function when supplied for a longer period - and extra analysis will be performed related to bilirubin metabolism to shed light on the relationship between serum bilirubin levels and effectiveness of resveratrol.

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Emmaniiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiii**Emmani**iiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiii
iiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiii**Emmani**iiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiiii

Eigenlijk wilde ik een hele pagina zo vullen, maar misschien gaat dat toch wat ver :P Dan had jij natuurlijk wel mooi kunnen zeggen ‘Lekker bezig!’ en eigenlijk was het wel terecht geweest voor de vader van mijn kinderen. Waar zijn ze nu trouwens?! Ik heb geen zin dat ze bij de promotie aanwezig zijn hoor, breng ze maar naar de crèche ofzo (of zijn ze daar te oud voor?). Sorry, nu maak ik het wel een beetje oreo-awkward-turtle.. En nu serieus: wat heb ik toch een leuke tijd met jou hier in het verre zuiden Maastricht! Alle cocktails die we samen hebben gedronken/gaan drinken/nu drinken hoop ik, shop tripjes, bios bezoeken, bak avonturen (zouden we inmiddels de KitKat cake al gemaakt hebben?), alle (90s) party's, salsa/merengue/bachata dansen die we hebben opgevoerd (ik ben blij dat je zo goed kan leiden en niemand weet hoe dramatisch ik eigenlijk ben ;)), fancy etentjes, Tea Zone bezoeken en zelfs de Intratuin is een happening met jou. Naast alle leuke dingen sta je ook altijd voor me klaar zowel op werkgebied als privé. Ik kan me geen betere vriend wensen als jij en hoop dat je ook nog een paar jaartjes in Maastricht blijft. Especially now **Axel** has joined our little pact, making our adventures even more fabulous.

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altijd zo 'sereen' door mij en Dirk werd gedeeld. De koffie consumptie is inmiddels echt buiten proportioneel (I'm tasting colours begint een dagelijke uitspraak te worden). Ik vraag me soms echt af of de concentratie cafeïne in ons bloed niet hoger is dan in die cupjes zelf (Envivo is Life!). Ondanks onze verschillende muzieksmaak hebben we gelukkig wel dezelfde interesse qua kunst, wat dan ook heeft geresulteerd in meerdere prachtige decoraties in ons kantoor (benieuwt wie ooit de post-it Pickachu vindt). Dank ook voor de prachtige aankleding van mijn bureaustoel en alle nuttige briefjes om argeloze andere PhD'ers op afstand te houden als ik thesisstress had. Ik denk dat als jij geen thesis dagen in mijn agenda had gezet dit moment nooit was aangebroken! Ik ben blij dat we ook lekker op een lijn zitten qua lay-autisme en creativiteit. Ik weet alleen niet goed hoe ik de man cave ooit nog kan overtreffen... Gelukkig nog ruim een jaar om over na te denken en we hebben altijd project big flush nog! Patrick gaat spijt krijgen dat hij ons op een kamer heeft gezet.

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Yvo, alias L.P. we draaien een pittig project maar wij kunnen dit want wij een sterk team; we houden elkaar scherp. We gaan prachtige resultaten behalen (NEJM here we come) en anders kunnen we altijd nog ons bier-bonbon zaakje beginnen en nieuwe afleveringen schrijven van ExpeditieRotizooi (heb al lang niks meer van De Rat/The Wizzard gehoord, zou de S. Daarmee te maken hebben?). **Bas D**, alias BD, jij maakt sinds kort ook deel uit van team DAPA. Ik vind het heel leuk dat je besloten hebt je bij ons te voegen. Je bent een fijne collega en vriend! Ik hoop dat we naast nog vele succesvolle testdagen mogen draaien en ook nog vele BBQs enzo samen organiseren (en dan weer per ongeluk te veel ijsjes kopen). Ik zou het wel prettig vinden als je wat kan doen aan de geest van het MRUM...

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The Nerds: Alex (Alexxxxx, how are you!?!), **Dennis** (Denniiiiis, Dennis Dubois, Do you want a cookie?), **Emmani** (Can I touch your planets?), **Yvo** (onze selectieve participant), **Charlotte T** (enfante terrible), **Niels** (Dirk 2.0), **Max** (Vengefull Spirit of the Day), **Dirk** (THE Dirk), **Cyril** (Laurent-Voulzy) and (Dries) **Roel Vink**. What more can I say then 'tatatatataaaataa tadadaaa, Charlie do you want to go to Candy Mountain, You're not joining for lunch because of aqua pony club?!' and 'To Violece!'. Now serious: thanks for all the fun guys! And not thanks for showing me the dark side of the internet and Funky Forest (big NOT thanks :P). With an extra note to **Roel**: dank dat ik met je mag samen werken op het DAPA project. Dankzij jou ben ik denk ik de enige persoon op de hele uni (hele universum?!) die het leuk vindt als de monitor langskomt. Ow and before I forget **Nerds**, I've got something very special for you guys:

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CURRICULUM VITAE

Marlies de Ligt was born on February the 22nd of 1989 in Boskoop, The Netherlands. She completed her high school at Het Rhedens in Rozendaal in 2007. Consecutively, she studied Nutrition and Health at Wageningen University in Wageningen The Netherlands. Within the study Nutrition and Health she specialised in the directions of Nutrition and Physiology and Molecular Nutrition and Toxicology. As part of her master she performed an internship at the department of Human Nutrition of Otago University located in New Zealand. The two research topics of this internship were: added sugar consumption and obesity in New Zealand adults (an epidemiological study), and sugar sweetened beverage consumption and the metabolic syndrome (a human clinical trial). Her final master thesis was performed at the department of Nutrition and Pharmacology of Wageningen University. The goal of her master thesis research was to identify changes in muscle protein synthesis, muscle protein breakdown and energy metabolism in a murine cancer cachexia model, on gene-expression and protein level. In August 2013, she started her PhD research at the Department of Nutrition and Movement Sciences at Maastricht University (NUTRIM, School of Nutrition and Translational Research in Metabolism), under the supervision of Prof. Dr. P. Schrauwen, Dr. V. Schrauwen-Hinderling and Dr. E. Phielix. Her research, as described in this thesis, was primarily focused on the food supplement resveratrol as a tool to prevent and treat Type 2 Diabetes Mellitus in humans. Main outcome parameters were insulin sensitivity and mitochondrial function. Upon finalization of her PhD, M. de Ligt will pursue her scientific career at the department of Nutrition and Movement Sciences of Maastricht University as a postdoctoral researcher.

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M.d.L and S.T. contributed equally to this work

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