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master in de biomedische wetenschappen: klinische moleculaire wetenschappen

Masterproef

Dietary polyphenols as modulators of lipid oxidation and mitochondrial function

Promotor :
prof. dr. ELLEN BLAAK
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Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting klinische moleculaire wetenschappen

Maastricht University

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UNIVERSITEIT VAN DE TOEKOMST

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List of abbreviations

ACC: acetyl-CoA carboxylase	HOMA-IR: homeostatic model assessment of insulin resistance
ALAT: alanine aminotransferase	HSL: hormone-sensitive lipase
AMPK: 5' adenosine monophosphate-activated protein kinase	iAUC: incremental area under the curve
ANOVA: analysis of variance	IGF-1: insulin-like growth factor-1
ATGL: adipose triglyceride lipase	IMCLs: *intramyocellular* lipids
CGI-58: comparative gene interaction-58	IMTGs: *intramyocellular* triglycerides
COMT: catechol O-methyltransferase	IRS-1: insulin receptor substrate-1
COX: cytochrome c oxidase	LPL: lipoprotein lipase
CPT1: carnitine palmitoyltransferase I	PDK1: 3-phosphoinositide-dependent kinase 1
CS: citrate synthase	PGC-1α: peroxisome proliferator-activated receptor gamma coactivator-1 alpha
CVD: cardiovascular disease	PI3K: phosphatidylinositol 3-kinase
DAG: diacylglycerol	PKB: protein kinase B
DIOGENES: diet, obesity and genes	PKC: protein kinase C
DPS: diabetes prevention study	PPAR: peroxisome proliferator-activated receptor
E%: energy percentage	ROS: reactive oxygen species
EE: energy expenditure	Rpm: revolutions per minute
EGCG: epigallocatechin gallate	RQ: respiratory quotient
FABPc: cytosolic fatty acid binding protein	RSV: resveratrol
FABPpm: plasma membrane fatty acid binding protein	RT-PCR: real-time polymerase chain reaction
FAT: fatty acid translocase	SEM: standard error of mean
FFA: free fatty acid	SIRT1: silent mating type information regulation 2 homolog 1
G0S2: G_0/G_1 *switch* gene 2	SLIM: study on lifestyle intervention and impaired glucose tolerance Maastricht
GEN: genistein	T2DM: type 2 diabetes mellitus
HAD: 3-hydroxy fatty-acyl CoA dehydrogenase	UCP2: uncoupling protein 2
HbA1c: glycated haemoglobin	VLDL: very low-density lipoprotein

Preface

This report is my master thesis for concluding my education Biomedical Sciences at the University of Hasselt. Furthermore, it is also a conclusion of my internship at the department of Human Biology from the university of Maastricht, the Netherlands. For eight months I worked at this department and it would be impossible to complete this project without the help of some people. Therefore, I would like to take this opportunity to express my gratitude to them.

First, I would like to thank my principal and second supervisor, Prof. Dr. Ellen Blaak and Dr. Johan Jocken, for their guidance, support and constructive feedback on this report during this internship. Thanks also to Drs. Jasper Most for his advice and encouragement during my senior practical training. I also want to thank him for letting me work as independent as possible and for the support during the clinical investigation days. Furthermore, I want to express my gratitude to my second examiner, Dr. Marielle Thewissen, for giving me constructive feedback on research presentations and for reading this report.

Moreover, I want to thank the subjects, which participated in this study. Writing this report was impossible without their help. In addition, I would like to show appreciation to all the members of the department of Human Biology and my fellow students for yielding a very pleasant working environment. Finally, I want to thank my family, my boyfriend and friends for their support throughout my education.

Abstract (in English)

Background: There is an urgent need of preventive strategies to slow down the progression rate of obesity and type 2 diabetes mellitus (T2DM). Recently, there has been increased interest in the use of polyphenols to combat these diseases. Combining the polyphenols epigallocatechin gallate (EGCG), resveratrol (RSV) and genistein (GEN) may increase lipid oxidation and lipolysis and enhance mitochondrial function.

Objective: To examine the short-term (3 days) effects of combining 2 (EGCG and RSV) or 3 polyphenols (EGCG, RSV and GEN) on adipose tissue lipolysis, lipid oxidation and mitochondrial function in overweight volunteers.

Study design: A double-blinded, randomized, placebo-controlled *cross-over* design was used. A total of 18 overweight subjects (9M/9F) will receive the combination of EGCG and RSV with or without the addition of GEN and a placebo treatment for 3 days, in randomized order (6 subjects were analyzed in this thesis). Substrate oxidation and energy expenditure (EE) were determined during baseline fasting conditions and for 6 hours after intake of a high-fat meal. In addition, blood samples were taken to assess circulating metabolites. Finally, at the end of the postprandial period, an adipose tissue biopsy was taken to determine gene and protein expression of factors involved in lipolysis and fatty acid handling.

Results: Combining the polyphenols had no effect on substrate oxidation and EE compared to placebo treatment. Likewise, treatment with these polyphenols had no effect on the rate of lipolysis or glycemic control. However, lactate concentrations tended to be reduced after treatment with a combination of 3 polyphenols compared to treatment with a combination of 2 polyphenols. This effect on lactate concentrations was significantly different in the late postprandial period.

Conclusion: Our findings may suggest that supplementation with a combination of EGCG, RSV and GEN reduces the glycolytic pathways, shifting towards more lipid oxidation. Furthermore, glucose and free glycerol concentrations tended to be reduced after treatment with a combination of EGCG, RSV and GEN. Assuming that insulin concentrations are comparable, this may indicate a tendency towards an improved insulin sensitivity. These data need to be confirmed when analyzing the complete data set of 18 subjects.

Abstract (in Dutch)

Inleiding: Er zijn dringend preventieve strategieën nodig om de progressie van obesitas en type 2 diabetes te vertragen. Momenteel is er veel interesse in het gebruik van polyphenolen om deze ziekten te bestrijden. Men veronderstelt dat de combinatie van de polyphenolen epigallocatechin gallate (EGCG), resveratrol (RSV) en genistein (GEN) vetoxidatie, lipolyse en mitochondriele functie kan verbeteren.

Doel: Het onderzoeken van de korte termijn effecten van de combinatie van 2 (EGCG en RSV) en 3 (EGCG, RSV en GEN) polyphenolen op vetoxidatie, lipolyse en mitochondriele functie in personen met overgewicht.

Studieopzet: Voor deze studie werd er een dubbel geblindeerd, *random*, placebo gecontroleerd *cross-over* opzet gebruikt. In totaal zullen 18 mensen met overgewicht (9 mannen en 9 vrouwen) in willekeurige volgorde de combinatie van EGCG en RSV met of zonder de toevoeging van GEN en een placebo behandeling krijgen voor 3 dagen. In deze thesis werden de resultaten van 6 proefpersonen geanalyseerd. Substraatoxidatie en energie-uitgaven werden bepaald tijdens vastende condities en voor 6 uur na inname van een hoge vet maaltijd. Daarenboven werd er bloed afgenomen om circulerende metabolieten te bepalen. Uiteindelijk werd er op het einde van de postprandiale periode ook nog een vetbiopt afgenomen. Dit was nodig om de gen- en eiwitexpressie van factoren, betrokken in lipolyse en vetzuurmetabolisme, te bepalen.

Resultaten: Het combineren van de polyphenolen had geen effect op substraatoxidatie en energie-uitgaven, vergeleken met de placebo behandeling. Bovendien hadden de polyphenolen ook geen effect op lipolyse en glucosewaarden. Daarentegen leken de lactaat concentraties verminderd na een behandeling met een combinatie van 3 polyphenolen, vergeleken met een combinatie van 2 polyphenolen. Dit effect op lactaat concentraties was significant verschillend in de late postprandiale periode.

Conclusie: Deze bevindingen wijzen erop dat supplementatie met een combinatie van EGCG, RSV en GEN bijdraagt tot een vermindering van de glycolyse en bijgevolg tot een verschuiving naar vetoxidatie. Deze data moeten echter bevestigd worden wanneer de complete data set van 18 proefpersonen wordt geanalyseerd.

1 Introduction

Diabetes is a growing public health care problem in both developing and developed countries (1, 2): there is an explosive rise in the number of people having diabetes worldwide (3). Currently, approximately 32 million people in Europe need treatment for diabetes (4). The global figure of people diagnosed with diabetes is expected to rise from the current estimate of 220 million people to 334 million people in 2025 (3, 5). This finding supports previous predictions of the epidemic character of diabetes worldwide in the first quarter of the 21st century (6). The diabetes epidemic especially refers to type 2 diabetes mellitus (T2DM), which is strongly associated with the increasing prevalence of physical inactivity and obesity (1). More than half of the adult population across Europe is considered to be either overweight ($25 \leq BMI \leq 29.9 kg/m^2$) or clinically obese ($BMI > 30 kg/m^2$). Approximately 143 million adults are overweight and 68 million adults are obese (7). As suggested above, obesity is one of the most important risk factors for the development of T2DM (8). Obesity is associated with disturbances in lipid metabolism (**Figure 1**). These disturbances are characterized by an impaired function of adipose tissue, as well as by impairments in skeletal muscle fatty acid handling (8, 9). One aspect of adipose tissue dysfunction is an impaired buffering capacity, resulting in lipid *overflow* in the circulation (10). This increased supply of lipids, together with an impaired ability of skeletal muscle to adjust lipid oxidation to fatty acid availability, may enhance the storage of lipids and lipid metabolites within skeletal muscle (11). This accumulation of lipid metabolites interferes with insulin signalling, thereby promoting the development of insulin resistance and T2DM (12, 13). The prevalence of both obesity and T2DM is increasing at an alarming rate (1, 2). Therefore, there is an urgent need of successful preventive strategies to slow down the progression rate of these diseases and to reduce dependence on costly medical treatment (6).

Long-term trials have illustrated that lifestyle interventions may reduce the cumulative incidence of T2DM by 58%. However, more than 30% of the high-risk population does not respond to lifestyle interventions (14-16). This ineffectiveness of chronic studies underscores the need of more preventive strategies to combat T2DM. Recently, there has been increased interest in the use of natural ingredients, such as polyphenols, as functional foods for the prevention of obesity and T2DM (17). These natural substances are present in foods and drinks of plant origin (18). Recent evidence has revealed that polyphenols have beneficial effects on human health, more particularly on both lipid and energy metabolism. It has been suggested that they increase adipose tissue lipolysis, thereby increasing fatty acid availability

and that they improve mitochondrial function, leading to an elevation of lipid oxidation in skeletal muscle (19-24) (**Figure 1**). This may reduce the accumulation of lipid metabolites within skeletal muscle, thereby improving insulin sensitivity. In recent years, there has been considerable interest in using a combination of various polyphenols to combat obesity and T2DM (25, 26). This increased interest may be explained by the fact that the synergistic activity of a combination of polyphenols offers advantages over the effect of single polyphenols. For this reason, this study examined the acute effects of combining specific polyphenols, with distinct mechanisms of action, on adipose tissue lipolysis, lipid oxidation and mitochondrial function in overweight individuals after an overnight fast and after intake of a high-fat meal (postprandial).

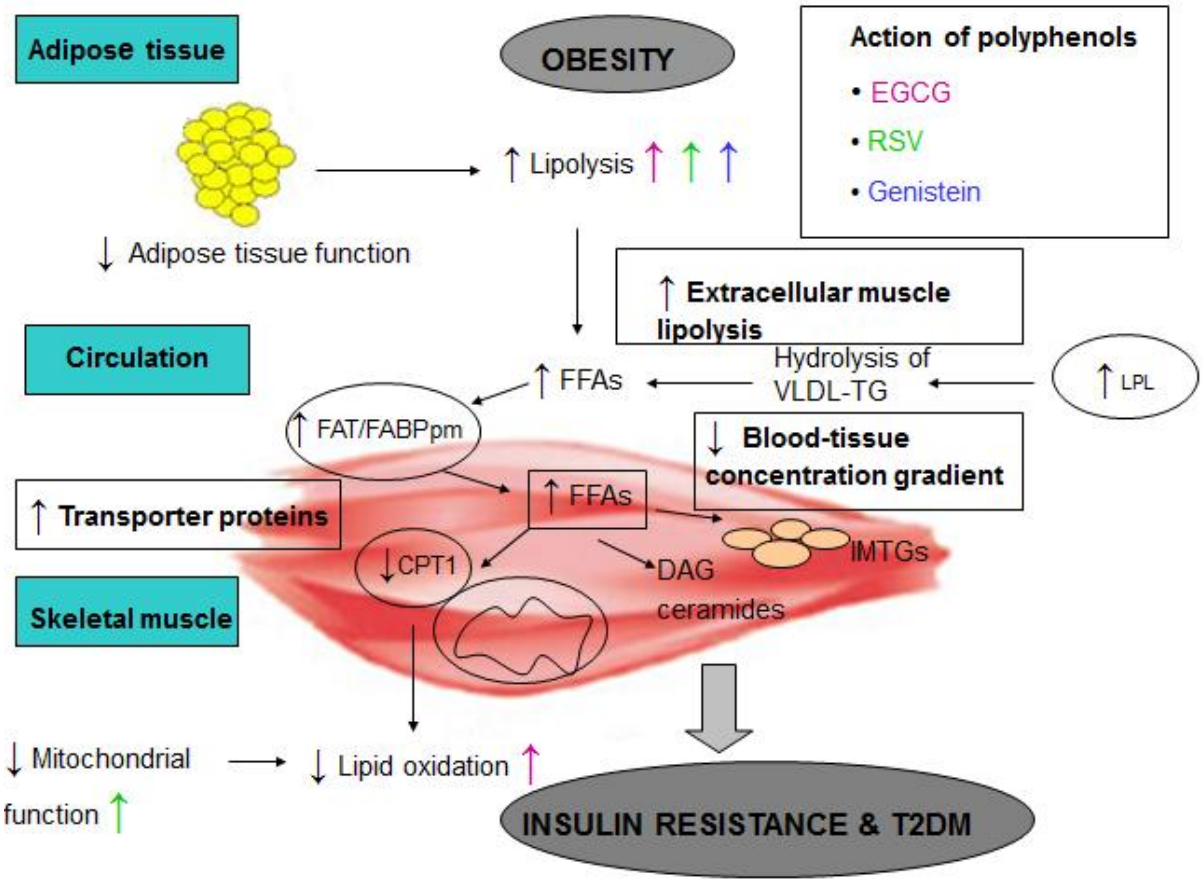

Figure 1. Disturbances in lipid metabolism in obese individuals and the distinct mechanisms of polyphenols to prevent insulin resistance and T2DM. Obesity is characterized by an impaired adipose tissue buffering capacity and an increased adipose tissue lipolytic rate, which cause lipid *overflow* in the circulation. Next to this increased FFA availability, obese skeletal muscle exhibits both impairments in the regulation of fatty acid uptake and a disturbed lipid oxidation. A possible explanation for this hampered lipid oxidation is mitochondrial dysfunction. The imbalance between adipose tissue lipolysis and lipid oxidation causes excessive accumulation of lipids in skeletal muscle (IMCLs). Subsequently, lipid metabolites, like DAG and ceramides, interfere with insulin signalling and action, encouraging the

development of insulin resistance and T2DM. Dietary polyphenols are considered to contribute to the prevention of these diseases. In particular, EGCG, RSV and GEN give rise to an increase in FFA availability. Furthermore, EGCG has a positive effect on lipid oxidation and RSV improves mitochondrial function. In this way, the combined effects of these polyphenols (an increased supply of fatty acids accompanied by the ability of skeletal muscle to adjust lipid oxidation to this increased FFA availability) may prevent lipid accumulation in skeletal muscle. Consequently, these polyphenols might combat the development of insulin resistance and T2DM. Abbreviations: CPT1: carnitine palmitoyltransferase I, DAG: diacylglycerol, EGCG: epigallocatechin gallate, FABPpm: plasma membrane fatty acid binding protein, FAT: fatty acid translocase, FFAs: free fatty acids, GEN: genistein, IMTGs: *intramyocellular* triglycerides, LPL: lipoprotein lipase, RSV: resveratrol, T2DM: type 2 diabetes mellitus, TG: triglycerides, VLDL: very low-density lipoprotein

1.1 Adipose tissue dysfunction

Obesity results from an imbalance between energy intake and energy expenditure (EE). The excessive energy is mainly stored in adipose tissue, an organ important for buffering the daily *influx* of dietary fat (9). Adipose tissue exerts its buffering capacity by responding quickly to ingestion of meals: it sequesters free fatty acids (FFAs) in the postprandial period and releases them later when appropriate (9, 27). Thus, adipose tissue stores FFAs, originating from dietary intake, as triglycerides in lipid droplets. In the meantime, insulin suppresses the rate of adipose tissue lipolysis (10, 28). In lean people, adipose tissue fatty acid metabolism is well regulated: FFAs are released relative to the energy needs of the corresponding tissues (29). In contrast, adipocytes of obese individuals are characterized by an impaired capacity to store dietary lipids in the postprandial period. Furthermore, insulin-mediated inhibition of adipose tissue lipolysis is blunted in obese individuals after intake of a meal (10).

Obese persons exhibit enlarged (hypertrophic) adipocytes, in which adipose tissue lipolysis is reduced (9). *Jocken* et al. have reported a decreased systemic FFA or glycerol *efflux* per unit fat mass in obese individuals after an overnight fast (30). This decreased fasting lipolysis may be attributed to the *hyperinsulinemia* that is often present in insulin-resistant conditions, like obesity and T2DM (10, 28). On one hand, this *hyperinsulinemia* prevents a massive increase in circulating FFA levels. However, on the other hand, this decreased fasting lipolysis contributes to a continuous increase in adipocyte size and subsequently to an increase in adipose tissue mass (9, 30). This increase in adipose tissue mass impairs the dynamic function of adipose tissue to store lipids in order to accommodate to a rise in FFA availability (9). The metabolic consequences of excessive FFA release in the postprandial period are detrimental. *Coppack* et al. have revealed that the differences between adipose tissue metabolism of lean and obese subjects are more obvious in the postprandial period. Thus, they have proposed that

a disturbed postprandial buffering capacity of adipose tissue and the subsequent prolonged elevation of circulating FFA levels, rather than a rise in fasting lipolysis, contribute most to an increased adipocyte size, lipid *overflow* and *ectopic* lipid accumulation (31). In conclusion, the imbalance between energy intake and EE provokes an increased number of adipocytes, resulting in an increased adipose tissue mass and an increased adipocyte size (due to a disturbed adipocyte differentiation) (26). The impaired adipose tissue function in obese individuals leads to insulin resistance and subsequently to a reduced suppression of insulin-mediated lipolysis during fasting conditions and after a meal (27, 32, 33). The impaired capacity of adipocytes to store dietary lipids and to suppress endogenous lipolysis causes lipid *overflow* in the circulation and storage of triglycerides and lipid metabolites within *ectopic* sites, such as skeletal muscle, which is strongly associated with muscle insulin resistance (11, 34).

1.1.1 Impairments in skeletal muscle fatty acid handling

Next to the increased availability of lipids derived from adipose tissue, disturbances in skeletal muscle lipid metabolism contribute to *ectopic* accumulation of triglycerides (8, 35). Current evidence implies that both regulation of fatty acid uptake and lipid oxidation are impaired in skeletal muscle of obese individuals and type 2 diabetics (36). During fasting conditions, healthy skeletal muscle predominantly relies on lipid oxidation for energy production, whereas postprandially it quickly changes to glucose oxidation (35, 37). On the contrary, the obese and T2DM condition is characterized by a hampered *switch* from lipid to glucose oxidation postprandially or in response to insulin (8, 38). The ability of healthy skeletal muscle to increase lipid oxidation upon increased fatty acid availability and to change between fuels following a meal is called *metabolic flexibility* (38). Accordingly, the impaired capacity to increase lipid oxidation during fasting conditions and the lost ability of muscle to *switch* between fuels postprandially is called *metabolic inflexibility* (8, 37). Additionally, defects in the regulation of skeletal muscle lipolysis might contribute to the increased storage of lipids in skeletal muscle (39).

1.1.2 Disturbed skeletal muscle fatty acid uptake

Ravikumar and colleagues have shown a substantial increase in postprandial storage of triglycerides in skeletal muscle of T2DM subjects. They have suggested that this triglyceride accumulation is due to an increased postprandial uptake of triglyceride-derived fatty acids (40). This enlarged uptake may result from an increased muscle *lipoprotein lipase* (LPL) activity (41). LPL is responsible for the *hydrolysis* of triglycerides in *chylomicrons* and *very-*

low density lipoproteins (VLDLs) (42). Accordingly, the higher enzymatic activity of LPL overwhelms skeletal muscle with triglyceride-derived fatty acids and therefore causes a rise in fatty acid uptake in T2DM subjects (42, 43). In addition, researchers in our department have investigated the *handling* of FFAs in skeletal muscle of insulin-resistant men during fasting conditions and in the postprandial period. After distinguishing the contribution of circulating FFAs, endogenous triglycerides (VLDLs) and dietary triglycerides (*chylomicrons*) to skeletal muscle lipid metabolism, they detected a significant difference in the *handling* of VLDL triglycerides among insulin-resistant men and control subjects. In particular, the postprandial *extraction* of VLDL triglycerides was higher in muscle of insulin-resistant subjects. Moreover, the uptake of *chylomicron* triglycerides tended to increase in the insulin-resistant men following a meal. However, this result was not statistically significant (42). Furthermore, the study of *Ravikumar* and colleagues has revealed a steady rise in mean plasma *chylomicrons* and VLDLs in T2DM subjects in the postprandial period. Nevertheless, there was no significant difference in the level of plasma triglycerides or VLDLs between control subjects and T2DM patients at any time point during the study (40).

Next to an elevated extracellular muscle lipolysis, a higher concentration of transporter proteins on the cell membrane may result in an increased uptake of FFAs in skeletal muscle (44-46). Putative fatty acid transporters in skeletal muscle are *cytosolic fatty acid binding protein* (FABPc), *plasma membrane fatty acid binding protein* (FABPpm) and *fatty acid translocase* (FAT/CD36) (47). A study of *Bonen* et al. has reported that long-chain fatty acid transport is elevated in obese and T2DM subjects as a consequence of an increased skeletal muscle expression of FAT (44). Moreover, previous research has revealed a higher expression of the transporter protein FABPpm in skeletal muscle of obese (46) and T2DM subjects (45).

However, not all studies have been affirmative about an increased uptake of FFAs in obese and T2DM subjects (47, 48). *Blaak* et al. have reported a reduced skeletal muscle FFA uptake in obese subjects with T2DM. In addition, they have found that the FABPc content in skeletal muscle of subjects with T2DM is lower compared to the content of FABPc in skeletal muscle of lean control subjects (47). Nevertheless, it has recently been shown that FABPc is abundantly present in skeletal muscle and that it plays an essential role in muscular fatty acid utilization (49). This finding indicates that a reduced content of FABPc is probably not *rate-limiting* for fatty acid consumption by skeletal muscle. Hence, the reduced uptake of FFAs, detected in obese and T2DM subjects, might be secondary to other mechanisms that control the exchange of fatty acids across the skeletal muscle membrane. One of these mechanisms

may be the concentration gradient of FFAs between blood and skeletal muscle (50). *Blaak* et al. have shown a higher lipolysis rate and a reduced oxidation of FFAs in T2DM subjects during fasting conditions (47). This discovery encourages this hypothesis: a rise in lipolysis leads to an increase in FFA concentrations in skeletal muscle. As a consequence, the blood-tissue FFA concentration gradient is reduced and the uptake of FFAs is hampered. In conclusion, whether uptake of FFAs is reduced or increased in skeletal muscle of obese and T2DM subjects is still a topic of discussion. Anyway, in combination with reduced lipid oxidation the end result is the same: accumulation of lipids in skeletal muscle.

1.1.3 Impaired lipolysis in skeletal muscle

As mentioned above, obesity is characterized by defects in the regulation of skeletal muscle lipolysis. *Blaak* and colleagues have shown that the *β2-adrenergically-mediated* increase in glycerol concentration is blunted in obese subjects (51). In addition, they have demonstrated a disturbed fasting muscle lipolysis in obese men (39). These findings suggest that the regulation of intracellular muscle lipolysis is impaired in obese individuals. Furthermore, *Blaak* et al. have reported a normal *intramyocellular* lipid (IMCL) content in obese subjects (51). This result indicates that a restricted ability to regulate intracellular muscle lipolysis is already present before the accumulation of lipids within the skeletal muscle occurs. In conclusion, the inability to regulate intracellular muscle lipolysis may be an early event in the process that leads to increased lipid storage in obesity.

1.1.4 Impaired lipid oxidation in skeletal muscle

Next to defects in the regulation of FFA uptake and intracellular lipolysis, skeletal muscle lipid oxidation is hampered. Skeletal muscle of obese and T2DM subjects is characterized by a reduced capacity to oxidize FFAs during fasting (35, 52). The cellular mechanisms that are responsible for this disturbed lipid oxidation in obese human skeletal muscle are not completely understood (53). A possible explanation for this impaired lipid oxidation is a defect in the transport of fatty acids into the mitochondria by means of the enzyme *carnitine palmitoyltransferase I* (CPT1) (8, 35). This defective transport is attributable to an increased content of *malonyl coenzyme A* (malonyl-CoA), an inhibitor of CPT1 (8, 54). *Rasmussen* et al. have shown that *hyperglycemia* combined with *hyperinsulinemia* gives rise to an increase in the level of malonyl-CoA in human skeletal muscle. As a result, the activity of CPT1 is inhibited and long-chain fatty acids are not oxidized but instead stored in human skeletal muscle (54). Furthermore, a study carried out by *Schrauwen* and colleagues has revealed a rise in lipid oxidation after a 3-month low-intensity training program in healthy non-obese

men. This increase in lipid oxidation is accompanied by a reduction in skeletal muscle mRNA expression of *acetyl-CoA carboxylase-2* (ACC-2), an enzyme that induces the formation of malonyl-CoA (55). This finding implies that a diminished inhibition of mitochondrial fatty acid transport through CPT1 contributes to an improvement of lipid oxidation.

Besides a defect in the transport of fatty acids, a decreased ability of oxidative enzymes might play a role in disturbed lipid oxidation (56). *Blaak* et al. have examined the activity of key enzymes involved in β-oxidation and the Krebs cycle. They have shown that the oxidative capacity of muscle, reflected by the activity of *3-hydroxy fatty-acyl CoA dehydrogenase* (HAD) and *citrate synthase* (CS), is decreased in type 2 diabetic patients compared to control subjects (47). Finally, because skeletal muscle strongly depends on oxidative phosphorylation for energy production, defects in this mechanism might play a role in the hampered lipid oxidation in obese and T2DM subjects (57). *Simoneau* and colleagues have measured the enzymatic activity of a marker of oxidative phosphorylation, namely *cytochrome c oxidase* (COX) and the content of *uncoupling protein 2* (UCP2) in skeletal muscle of obese non-diabetic volunteers. They have found a lower muscle activity of COX and a higher UCP2 content in obese subjects (58). This result suggests that the increased content of UCP2 within skeletal muscle in obesity corresponds with a reduced skeletal muscle lipid oxidation during fasting conditions. Furthermore, *Kelley* et al. have shown that T2DM is characterized by smaller and seriously damaged mitochondria and that mitochondria of obese individuals and type 2 diabetics are characterized by diminished rates of oxidative phosphorylation (59). This reduced oxidative phosphorylation activity has also been exhibited in healthy subjects at risk of developing T2DM (60) and in elderly subjects with T2DM (61). These findings indicate that mitochondrial dysfunction may contribute to impairments in lipid oxidation and may eventually predispose individuals to obesity and T2DM.

As has been noted, many reports have revealed a correlation between mitochondrial dysfunction and insulin resistance in patients with T2DM. However, there are also data indicating that mitochondrial dysfunction is not a primary etiological event in T2DM. For instance, defective insulin signalling may promote mitochondrial dysfunction, which suggests that it is more a secondary effect. This has recently been shown in patients with genetic defects in the insulin receptor (62). Furthermore, studies with genetically manipulated mouse models have suggested that a reduction in mitochondrial oxidative capacity does not worsen but in fact improves insulin sensitivity (63, 64). In addition, *Schrauwen-Hinderling* et al. have shown no difference in the content of *intramyocellular* triglycerides (IMTGs) in spite of

reductions in mitochondrial function in T2DM patients (65). These findings denote that mitochondrial dysfunction in skeletal muscle is a secondary phenomenon that is not primarily implicated in the development of insulin resistance and T2DM. Thus, whether a reduced mitochondrial function is the cause or the consequence of T2DM, is still a topic of discussion.

1.2 Insulin resistance

As mentioned before, both adipose tissue dysfunction and impairments in skeletal muscle lipid metabolism contribute to excessive accumulation of triglycerides in skeletal muscle. There is a strong relation between increased storage of triglycerides in skeletal muscle and insulin resistance (66). This *inverse* relation between IMTGs content and insulin sensitivity has previously been demonstrated in normal weight non-diabetic (66-68), obese non-diabetic (69) and diabetic patients (69). On the contrary, this relation is not applicable to trained athletes: these individuals are really insulin-sensitive regardless of the presence of high levels of IMTGs. This phenomenon is known as the athlete's paradox and can be explained by a higher muscle oxidative capacity of individuals participating in regular exercise (34, 65).

Next to the *inverse* relation between the content of IMTGs and insulin sensitivity, it has become obvious that in particular other lipid metabolites cause insulin resistance. Accumulation of less abundant lipid metabolites, such as diacylglycerol (DAG), interferes with insulin signalling and action, promoting the development of insulin resistance and T2DM (13, 57). Likewise, a recent study, which measured lipolysis both *in vitro* and *in vivo*, has revealed that inhibition of lipolysis induces accumulation of DAG and insulin resistance (70). On the other hand, recent research in our department has not shown any DAG accumulation in obese, insulin-resistant men but rather a reduction in total DAG content. This surprising result does not support the suggested key role of DAG muscle content in the development of insulin resistance. However, it does not completely exclude the possibility that changes in DAG could contribute to insulin resistance (71). It indicates that specific DAG species and stereoisomers or other lipid intermediates, like ceramides, might be possibly of greater importance in obese, insulin-resistant men. In several studies with obese, insulin-resistant subjects, an increase in ceramide content has been observed (72, 73), but results were not consistent (74). These findings indicate that the mechanism by which elevated levels of FFAs produce insulin resistance is not completely understood. Therefore, the role of both DAG and ceramides in the development of insulin resistance has to be examined in more detail in the future.

As suggested above, the molecular mechanism by which insulin resistance develops is not entirely figured out. There is evidence for the involvement of both DAG and ceramides in the development of insulin resistance (13, 72, 73). Anyway, the general mechanism underlying insulin resistance in skeletal muscle is described as follows: an increase in lipid metabolites, owing to an increased delivery from plasma or a reduced lipid oxidation, activates a *serine/threonine* kinase cascade. In particular, lipid metabolites activate an *isoform* of *protein kinase C* (PKC) in skeletal muscle. As a result, this *isoform* phosphorylates the insulin receptor and *insulin receptor substrate-1* (IRS-1) on serine instead of tyrosine residues (which would normally be the case). Subsequently, this leads to a decrease in IRS-1-associated *phosphatidylinositol 3-kinase* (PI3K) activity, resulting in a reduced insulin-stimulated *protein kinase B* (PKB) or AKT activity (75). Normally, PKB activates glycogen synthesis and provokes translocation of *glucose transporter 4* (GLUT4) from inside the cell to the plasma membrane, where it permits glucose uptake into the cell. Accordingly, lowered AKT activity fails to activate GLUT4 translocation and consequently insulin-induced glucose uptake is reduced. Thus, impairments in the insulin signalling pathway result in hampered glucose uptake, utilization and storage (76).

1.3 Importance of preventive strategies

The prevalence of both obesity and T2DM is rising at a frightening rate (1, 2). This increasing prevalence results in a huge impact on health care needs and costs (2, 6). For this reason, preventive strategies are urgently needed (6). However, due to the complex character of obesity and related metabolic complications, prevention and treatment of obesity is a difficult issue (19). As has been noted, obesity is caused by an imbalance between energy intake and EE. This imbalance is the outcome of our modern lifestyle (77, 78). Hence, there are two ways to attain weight loss: decrease energy intake or increase EE (19, 21). Subjects at high risk for T2DM can undertake lifestyle interventions to obtain a negative energy balance. Such lifestyle interventions can be a healthy diet and more physical activity (79). Several dietary intervention studies have revealed that the nutrient composition of the diet represents an important factor in the success of preventing obesity and T2DM (80, 81).

1.4 Effect of lifestyle interventions

Long-term studies have shown that lifestyle interventions can prevent T2DM in high-risk populations (14-16). Nevertheless, the effect of these interventions varies among individuals (16). *Corpeleijn* et al. have revealed that weight loss (± 15 kg) leads to an enhanced stimulation of lipid oxidation during fasting conditions. Moreover, they have found an

improvement in the postprandial *switch* from lipid to carbohydrate oxidation (37). These findings suggest that *metabolic flexibility* can be restored by weight loss. However, not all results about weight loss have been affirmative (16, 82). The *lifestyle intervention and impaired glucose tolerance Maastricht* (SLIM) study performed in our department has shown that a lifestyle intervention, consisting of a healthy diet and increased physical activity, has an advantageous effect on insulin resistance and reduces the risk of developing T2DM. Besides these positive effects, this study has improved dietary composition**:** fat intake has been reduced in individuals at high risk for T2DM. Unfortunately, the SLIM study has shown relatively small weight changes. After 1 year, subjects had lost approximately 2,8 kg and in the following years they even (re)gained weight (82). These results are consistent with data from the Finnish *diabetes prevention study* (DPS) in which a weight reduction of 4,5 kg was observed during the first year of the intervention and a regain of approximately 1 kg after 3 years (16). These results indicate that lifestyle interventions are able to prevent T2DM in high-risk populations. However, the ability of these intervention programmes to induce weight loss exhibits high relapse rates: at first, the response is encouraging, but after 1 to 3 years the subjects regain weight.

In addition, the importance of the composition of a diet for prevention and management of obesity is discussed (80, 81). The *diet, obesity and genes* (DIOGENES) study is a randomized, 26-weeks dietary intervention study, which assessed the effectiveness of moderate-fat diets, varying in protein content and glycemic index, for preventing weight regain. This study has shown that a low-protein high-glycemic index diet is associated with weight regain. Therefore, the authors have concluded that an increase in protein content and a decrease in glycemic index give rise to maintenance of weight loss (80). Moreover, another dietary intervention study, called LIPGENE, has been performed to address the effect of lipid modification in a European population. This study compared the impact of different types and quantities of dietary fatty acids on insulin sensitivity (81). LIPGENE has determined the efficiency of reducing dietary saturated fatty acids by replacement with mono-unsaturated fatty acids or as part of an iso-energetic low-fat, high-complex carbohydrate diet (83). Unfortunately, it has been revealed that iso-energetic reduction of saturated fatty acids has no effect on insulin sensitivity in subjects with the metabolic syndrome (81). Because of the restricted long-term success of conventional weight management programs, there is an increasing interest in alternative strategies for weight management, such as the use of natural ingredients (17, 19).

1.5 Dietary polyphenols

In recent years, a lot of research on the action of polyphenols has been performed. These natural substances are considered to contribute to the prevention of chronic diseases, including insulin resistance and T2DM (84). They are present in foods and drinks of plant origin, such as tea, red wine and soy products. The beneficial effects of polyphenols on human health are predominantly due to their anti-oxidant properties: they modulate biological oxidative stress to protect cellular DNA, lipids and proteins against damage. This modulation of oxidative stress occurs by means of scavenging reactive oxygen species (ROS). Furthermore, there is increasing evidence that polyphenols act beyond their anti-oxidant capacity. Over the last few years, they have been studied for their role in cellular signalling (18). Research has shown that they can exert additional effects on signalling pathways for cellular energy and fatty acid catabolism, thereby ameliorating lipid oxidation and mitochondrial function (18, 19, 22).

Green tea is a rich source of polyphenols like catechins, such as epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate (EGCG) (85). Besides these catechins, green tea also contains caffeine (86). Because green tea comprises both caffeine and catechins, a lot of research has focused on potential interactions among these components (19, 21, 87). Green tea is considered to contribute to beneficial health properties, such as protection against cancer and CVD (88). Recently, there has been evidence that it contributes to the prevention of obesity and T2DM (89-91). Scientific research has shown an advantageous effect of green tea supplementation on insulin sensitivity and lipid oxidation in mice and rats (89, 90). Furthermore, *Tsuneki* et al. have reported a positive effect of green tea supplementation on glucose metabolism in healthy humans (91).

The most abundant (>50%), most pharmacologically active and most studied catechin is EGCG (19, 92). Several studies have shown that EGCG is responsible for most beneficial health properties of green tea (21, 92). Albeit the mechanism of action of EGCG is not completely clear yet, there is sufficient evidence that EGCG exerts its effect on lipid oxidation through its impact on the sympathetic nervous system (85). This part of the nervous system regulates diet-induced thermogenesis, a component of total daily EE. It performs this action through the interaction of noradrenalin with adrenergic receptors (93). This neurotransmitter is degraded by *catechol O-methyltransferase* (COMT) (94). At this point, EGCG intervenes: it inhibits COMT. Consequently, noradrenalin remains at the synaptic junction for a longer

time and confers a prolonged interaction with the adrenergic receptors. This leads to an increased thermogenesis and lipid oxidation (19, 21).

EGCG was the first polyphenol to demonstrate its beneficial actions under *in vivo* conditions in men: *Dulloo* and colleagues have shown that the acute effect of green tea, consisting of EGCG and caffeine, on energy metabolism (24h-EE) and lipid oxidation is greater than the effect of caffeine alone (21). This finding indicates that EGCG and caffeine work synergistically (21, 87). On the contrary, a study of *Kovacs* et al. has shown that the effectiveness of green tea extract depends on the magnitude of habitual caffeine intake. This study investigated whether green tea improves weight maintenance by preventing weight regain after weight loss in overweight and moderately obese subjects. They have concluded that weight maintenance is not affected by treatment with green tea. They questioned whether the magnitude of regular caffeine intake affected the effectiveness of green tea administration and they found that regular caffeine use influenced weight maintenance in the green tea treatment group. In particular, they have shown that low caffeine consumers (< 300 mg/day) exhibit stronger body weight maintenance after body weight loss compared to high caffeine consumers (> 300 mg/day). This larger weight regain in high caffeine consumers might be explained by saturation of the ability of green tea to further stimulate noradrenalin-related mechanisms as both caffeine and green tea work through mechanisms involving noradrenalin (17). Similarly, *Westerterp-Plantenga* and colleagues examined the effect of a green tea-caffeine mixture on weight maintenance after body weight loss in obese subjects in relation to habitual caffeine intake. They have shown that body weight maintenance after body weight loss is considerably better in regular low caffeine consumers (95). Thus, these findings indicate that supplementation with green tea is only effective when usual caffeine intake is low and that a higher dose is needed when regular caffeine intake is high. For this reason, one of the inclusion criteria to participate in our study is a low habitual caffeine intake (< 300 mg/day).

Another polyphenol with beneficial effects on human health is resveratrol (RSV). It belongs to the category of viniferins and is predominantly found in grape skin (96). For this reason, red wine is a rich source of RSV (22, 97). RSV is well known for its anti-oxidant properties (96). In this way, it contributes to the prevention of cancer. It is also believed that RSV contributes to the prevention of CVD (97). The diminished risk of CVD related to habitual use of red wine is known as the ‘French paradox’. This term explains the observation that French people display a low risk of CVD, notwithstanding a diet that is high in lipids (96).

Recently, there has been evidence that RSV contributes to the prevention of T2DM (22, 23, 96-99). *Baur* et al. have shown that long-term administration of RSV causes increased survival and insulin sensitivity in rodents (98). It has been proposed that this increased insulin sensitivity results from a decreased expression of *insulin-like growth factor-1* (IGF-1) and an increased activity of *5' adenosine monophosphate-activated protein kinase* (AMPK), an enzyme that is important in energy metabolism (96, 99). Furthermore, *Lagouge* and colleagues have stated that treatment with RSV improves mitochondrial function and protects mice against diet-induced obesity and insulin resistance (22). They have suggested that these outcomes are derived from activation of *silent mating type information regulation 2 homolog 1* (SIRT1). SIRT1 deacetylates *peroxisome proliferator-activated receptor gamma coactivator-1 alpha* (PGC-1α). This leads to an increased activity of PGC-1α and induction of genes for oxidative phosphorylation and mitochondrial biogenesis (22). Obviously, in spite of clear positive effects on insulin sensitivity and mitochondrial function, the molecular targets through which RSV acts have not yet been univocally defined (99). It is possible that RSV works simultaneously on several targets. In accordance with this hypothesis, *Lasa* and colleagues have shown that RSV regulates lipolytic activity in human and murine adipocytes, as well as in white adipose tissue from mice, acting mainly on ATGL at transcriptional and posttranscriptional levels. Enzyme activation seems to be induced via AMPK (23).

Furthermore, evidence is emerging that the phyto-oestrogen genistein (GEN) exhibits anti-obesity and anti-diabetic effects (20, 24, 100-103). GEN is a member of the isoflavones and appears principally in soy and soy products (103). Isoflavones bind to oestrogen receptors and display both oestrogenic and anti-oestrogenic effects (102). Besides oestrogen receptors, *peroxisome proliferator-activated receptors* (PPARs) represent a target of genistein (101). Dose-dependent effects of GEN are determined by the balance between oestrogen receptors and PPARs (102). GEN has a valuable role in the prevention of many disorders, such as CVD, cancer, osteoporosis and most importantly diabetes (103).

In vitro experiments have shown that GEN acts as an estrogen at low concentrations, namely it restricts adipogenesis and enhances osteogenesis. Nevertheless, at high concentrations it improves adipogenesis and restricts osteogenesis, acting as a *ligand* of PPAR (101). Thus, GEN influences adipogenesis and osteogenesis in a *biphasic* dose-dependent manner. In addition, another *in vitro* experiment has revealed that genistein affects lipid metabolism. It regulates both lipogenesis and lipolysis in adipocytes, namely it restricts lipogenesis and increases lipolysis (24). Analogously, an animal study has shown that GEN exhibits *anti-*

lipogenic effects in adipose tissue. It has been proposed that this is caused by a decrease in the concentration of LPL, an enzyme that regulates lipid uptake (100). Moreover, enhancement of lipid and glucose metabolism after ingestion of soy in mice has recently been associated with activation of AMPK in adipose tissue and skeletal muscle (20). Although these results signify that GEN modulates lipid deposition in cultured cells and mice, it is not sure whether genistein displays *anti-lipogenic* effects in humans. Some evidence indicates that isoflavones, such as genistein, only positively affect plasma lipid profiles when consumed along with the protein part of soybeans (104).

Recently, there has been increased interest in research that uses a combination of polyphenols (25, 26). Combining numerous natural products that have synergistic actions may offer benefits over treatments that only use one product. This synergistic activity can be explained by the fact that different polyphenols act on multiple molecular targets (105). *Rayalam* et al. have examined whether combining RSV and GEN provokes enhanced effects on lipolysis, apoptosis and adipogenesis in adipocytes. They have concluded that the effects of combining RSV and GEN were more pronounced than the effects of the individual substances (26). Likewise, *Park* et al. have conducted an *in vitro* study. They have studied the effect of combining RSV, GEN and quercetin on adipogenesis and apoptosis in both primary human and murine adipocytes. This study has revealed that the combination of these products resulted in an enhanced inhibition of lipid accumulation in both human and murine adipocytes. Furthermore, the combined treatment induced adipocyte apoptosis, thereby reducing the number of adipocytes in adipose tissue, whereas the individual compounds did not induce apoptosis (25). Furthermore, a recent *in vivo* study has revealed that the combination of RSV, GEN, quercetin and vitamin D reduces weight gain and adiposity in a postmenopausal rat model (unpublished results). Thus, these findings suggest that combining polyphenols is a useful strategy to combat obesity.

In summary, the findings mentioned above indicate that polyphenols have an impact on the signalling pathways for cellular energy and fatty acid metabolism, thereby ameliorating lipid oxidation and mitochondrial function. However, most of these findings have been acquired from *in vitro* and animal studies and remain to be examined in humans. Therefore, we performed a clinical study with overweight subjects. During the last years, multiple studies have investigated the actions of individual polyphenols on energy and lipid metabolism. However, until now, few studies have dealt with the additive or possibly synergistic effects of combining several polyphenols in humans, albeit they emerge naturally as combinations in

low concentrations. For this reason, we examined the acute effects of combining polyphenols on adipose tissue lipolysis, lipid oxidation and mitochondrial function in humans. The **research question** that we explored in this study is: "Do polyphenols have an effect on adipose tissue lipolysis, lipid oxidation and mitochondrial function in humans and what are the underlying mechanisms"? We **hypothesized** that the combination of specific polyphenols with partly different mechanisms of action has advantageous effects on lipid oxidation through additive or synergistic actions on lipolysis, thereby increasing fatty acid availability, and on mitochondrial function. **To test this hypothesis**, we investigated the short-term (3 days) effects of combining the polyphenols EGCG, RSV and GEN on adipose tissue lipolysis, lipid oxidation and mitochondrial function in overweight subjects after an overnight fast and after ingestion of a high-fat meal.

2 Subjects & Methods

2.1 Subjects

A total of 18 healthy, overweight ($25 \leq BMI \leq 29.9 kg/m^2$) males and females, aged between 20 and 50 years, volunteered to participate in this study (the 6 subjects, who completed the study, were included in the interim analysis for this thesis). Recruitment of subjects occurred by means of advertisements in local newspapers, posters at the university and from existing cohorts within our research group. Additional inclusion criteria were Caucasian and weight stable in the last 3 months (± 2 kg). Exclusion criteria included smoking, regular use of green tea (> 1 cup per day) or products comprising green tea extract, total caffeine use > 300mg/day, any dietary vitamins or dietary supplements, alcohol intake > 20g/day, (post)menopausal, pregnant or lactating women, intensive fitness training (≥ 3 per week ≥ 1 hour training), diabetes mellitus, blood donation 2 months prior to the study and during the study, use of drugs, serious cardiovascular, pulmonary, renal or hepatic disorders and history of CVD. Before entering the study, subjects were invited for a standard screening visit to evaluate eligibility and to assess dietary intake, physical activity and medical history of all subjects by using questionnaires. The screening included a physical investigation and determination of body weight, height and waist-hip ratio. In addition, a resting electrocardiogram was conducted to exclude subjects at cardiovascular risk. Seated blood pressure was measured to exclude individuals with a current state of CVD. Moreover, body composition of the subjects was measured with bioimpedance analysis and blood samples were taken to determine the concentrations of plasma glucose, insulin, creatinin, alanine aminotransferase (ALAT), haemoglobin, hematrocrit and glycated haemoglobin (HbA1c). In women, also a pregnancy test was done. If the results of the screening were in accordance with the inclusion criteria, subjects were allowed to participate in the study. The nature and possible risks of the experimental procedures were explained to the subjects before their written informed consent was obtained. The study protocol was approved by the medical ethical committee of the Maastricht University Medical Centre.

2.2 Study design

A double-blinded, randomized, placebo-controlled *cross-over* design with 3 arms was used for this short-term study. The duration of each treatment was 3 days with a *wash-out* period of at least one week in between. The volunteers received following treatments in randomized order:

1) The combination of EGCG (282 mg daily) and RSV (200 mg daily)
2) These 2 polyphenols in combination with GEN (48 mg daily)
3) Placebo

The capsules were taken orally: three capsules, twice a day, at breakfast and dinner.

2.3 Clinical investigation day

On the 3rd day of each treatment, a clinical investigation day took place (**Figure 2**). Subjects were instructed to come to the university at 8:30h after an overnight fast. After arrival, they were asked to ingest 3 capsules at once. The remaining 3 capsules were ingested after a high-fat meal. All tests were conducted in the supine position.

2.3.1 Indirect calorimetry

Oxygen uptake and carbon dioxide production were continuously measured throughout the clinical investigation day with an open circuit *ventilated hood* system to calculate changes in EE, RQ (VCO_2 produced/VO_2 consumed) and substrate (lipid/carbohydrate) oxidation rates. Once a week, validation of the *ventilated hood* system was assessed by means of an alcohol-burning test. Measurements occurred during overnight fasted conditions and for 6 hours after ingestion of a high-fat meal (2.6 megajoule; 61 E% fat, 33 E% carbohydrate and 6 E% protein). The equation of Weir (107) was used to calculate EE: EE (kg/cal) = 3.9 * mean VO_2 + 1.1 * mean VCO_2; EE (kJ) = EE (kg/cal) * 4.84. Moreover, the equations of Frayn (108) were employed to compute lipid and carbohydrate oxidation rates: lipid oxidation = 1,67 * VO_2 – 1,67 * VCO_2 – 1,92 * N; carbohydrate oxidation = 4,55 * VCO_2 – 3,21 * VO_2 – 2,87 * N. This N is calculated by means of 0,15 * EE (kJ)/6,25/17. Alterations in EE and lipid and carbohydrate oxidation were calculated as *incremental area under the curve* (iAUC) using the trapezium method.

2.3.2 Blood sampling

At the beginning of the clinical investigation day, a *cannula* was inserted in an *antecubital* forearm vein for blood sampling. Blood samples were taken at baseline, every 30 minutes during the first 2 hours and each hour during the last 4 hours after the high-fat meal. Blood samples were taken for the assessment of glucose, insulin, free glycerol, plasma FFAs, circulating triglycerides and lactate. The samples were gently mixed and centrifuged at 3000 revolutions per minute (rpm) at 4° C for 10 min. Subsequently, plasma aliquots were collected in *Nunc cryotubes*, frozen in liquid nitrogen and stored at -80° C till further analysis. Measurements of glucose, free glycerol, plasma FFAs, circulating triglycerides and lactate

occurred with enzymatic assays automated on the *Cobas Fara/Pentra* (Roche Diagnostics, Basel, Switzerland). The concentration of insulin was determined by a radioimmunoassay (NucliLab, Ede, The Netherlands). In addition, blood samples were assembled to determine catecholamine levels at baseline and every 2 hours during the postprandial period. These blood samples were used to assess the effect of polyphenols on the sympathetic nervous system. Alterations in blood sample concentrations were calculated as *incremental area under the curve* (iAUC) using the trapezium method.

2.3.3 Adipose tissue biopsies

Finally, at the end of the clinical investigation day, an adipose tissue biopsy was taken. This biopsy was obtained from abdominal subcutaneous adipose tissue under local anaesthesia, using the needle biopsy technique, as performed previously by our group (109). The adipose tissue biopsy was frozen straight away in liquid nitrogen and stored at -80° C until further analysis. These specimens will be used to determine mRNA (RT-PCR) and protein levels (Western blot) of factors involved in lipolysis and fatty acid metabolism (hormone-sensitive lipase (HSL), adipose triglyceride lipase (ATGL), ATGL's co-activator comparative gene interaction-58 (CGI-58), its inhibitor G_0/G_1 *switch* gene 2 (GOS2) and lipid droplet covering protein perilipin).

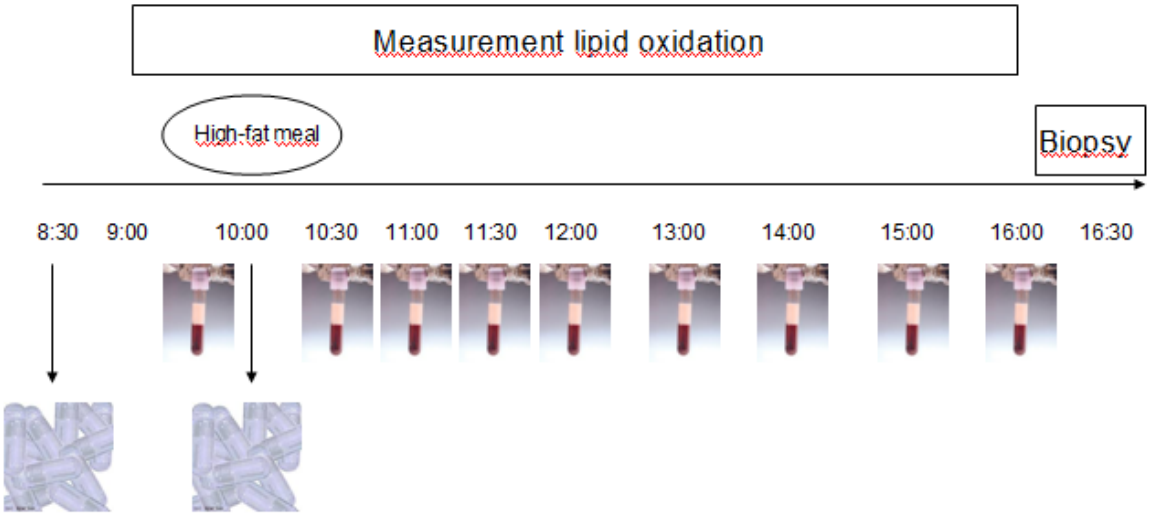

Figure 2. Clinical investigation day

2.4 Dietary intake and physical activity standardization

Subjects were encouraged to maintain their regular lifestyle, particularly dietary and physical activity patterns, throughout the entire study. Dietary intake was recorded with 2 (day 1 and 2 of the first treatment) food intake diaries to standardize food intake during each treatment

period. Furthermore, all participants were requested to consume a meal rich in carbohydrates (e.g pasta) the evening before each clinical investigation day. Subjects were instructed to come to the university by public transportation or car after an overnight fast on test days.

2.5 Supplements

The polyphenol supplements were commercially available and fabricated in capsular form by *Pure Encapsulations inc.* **Teavigo™** is a safe, highly purified extract from the leaves of green tea, including ≥ 94% EGCG on a dry weight basis. The use of 282 mg daily is based on pilot data (19). **Resveratrol EXTRA™** consists of 100 mg trans-resveratrol, grape seed extract and red wine polyphenols. Moreover, **Soy isoflavones 40™** contains 40 mg of total isoflavones with a high content of genistein (60-70%). These supplements were distributed by *Wellspring Clinical Services* (Europe, Doncaster, United Kingdom) and packaged into bottles. The placebo capsules were filled with microcrystalline cellulose (Avicel™) as inactive filler. All capsules were over-encapsulated to mask the content of the capsules (DBcaps® Capsules). At day 1 and 2 of each treatment, subjects were instructed to take one capsule of each bottle during breakfast and dinner to attain the appropriate dose (6 capsules daily, **Table 1**). At the 3rd day of each treatment period, the remaining supplements were taken at the university under supervision of the researcher. The investigator also tested the compliance of the subjects by counting the returned capsules. Compliance was obtained when 80% of the total amount of the provided supplements was taken by the subject. Subjects that were not compliant were withdrawn from the study.

Table 1. Polyphenol supplementation: Subjects ingest 6 capsules daily during each treatment period

Treatment	EGCG	RSV	GEN	Placebo	Total capsules
Daily dose	282 mg	200 mg	48 mg		
Dose/capsule	141 mg	100 mg	24 mg		
EGCG & RSV	2	2	-	2	**6**
EGCG, RSV & GEN	2	2	2	-	**6**
Placebo	-	-	-	6	**6**

2.6 Statistics

As the study is currently still running, statistical analyses were performed with preliminary data. Only the results of 6 subjects were accessible, because they had already undergone all treatments. The obtained *ventilated hood* data of these 6 subjects were completely examined. However, in blood plasma solely the concentrations of FFAs, free glycerol, triglycerides, glucose and lactate were studied. The plasma concentrations of insulin and catecholamines were not analyzed yet. Likewise, adipose tissue biopsies were not investigated so far. If data were normally distributed, differences between placebo treatment and treatment with a combination of 2 or 3 polyphenols were analyzed by using *repeated-measures analysis of variance* (ANOVA). The normal distribution of the used variables was assessed by means of the *Kolmogorov-Smirnov* test. If data were not normally distributed, they were log transformed. When statistically significant differences were found, post-hoc testing with *Bonferroni* was applied. Statistics were done with the use of SPSS version 17.0. Statistical significance was set at an α-level of 0.05.

3 Results

3.1 Subjects

The characteristics of the subjects are shown in **Table 2**. In total, 6 people (3 male and 3 female volunteers), completed all treatments. On average, the subjects were 38 ± 5 years old. The mean values of BMI (28,7 ± 0,8) and fat percentage (31,8 ± 3,6) were within the range of those of overweight people. Furthermore, as expected for healthy individuals, fasting glucose concentrations (4,8 ± 0,1) and HbA_{1c} values (5,3 ± 0,1) were within the normal range (3,9 - 5,5 mmol/L for fasting glucose; 4 – 6 % for HbA_{1c}). Moreover, mean blood pressure (122 – 82) was consistent with the blood pressure of healthy individuals. However, the *homeostatic model assessment of insulin resistance* (HOMA-IR) indicates a mean value of 3,2 ± 0,5. Given a cut-off point of 2,5, this finding implies that these subjects are slightly insulin-resistant (110).

Table 2. Characteristics of the subjects[1]

Characteristics	Mean ± SEM	Range
Sex (M/F)	3/3	
Age (years)	38 ± 5	22 - 48
BMI (kg/m^2)	28,7 ± 0,8	26,2 - 31,3
Fat (%)	31,8 ± 3,6	17,9 - 42,3
Waist-hip ratio	0,83 ± 0,03	0,73 - 0,90
Systolic blood pressure (mmHg)	122 ± 5	101 - 134
Diastolic blood pressure (mmHg)	82 ± 2	72 - 86
Fasting glucose (mmol/L)	4,8 ± 0,1	4,6 - 5,1
HOMA-IR	3,2 ± 0,5	1,8 - 5,0
HbA_{1c} (%)	5,3 ± 0,1	4,8 - 5,6

[1] Data are means ± SEM. The total range of the observed values is shown. Abbreviations: BMI: body mass index, HOMA-IR: homeostatic model assessment of insulin resistance, HbA_{1c}: glycated haemoglobin

3.2 Indirect calorimetry

3.2.1 Substrate oxidation

The time-course of RQ and substrate (carbohydrate/lipid) oxidation after treatment with placebo or a combination of polyphenols is shown in **Figure 3**, **4** and **5**, respectively. At baseline, no statistically significant differences in RQ, carbohydrate and lipid oxidation between test conditions were found. After ingestion of the high-fat meal, the RQ increased in all treatment groups (**Figure 3**), with an obvious peak one hour after the meal. This finding is

consistent with an increase in carbohydrate oxidation (**Figure 4**) and a decrease in lipid oxidation (**Figure 5**). Subsequently, RQ decreased in each test condition. This result is equivalent to a lower carbohydrate oxidation and a higher lipid oxidation.

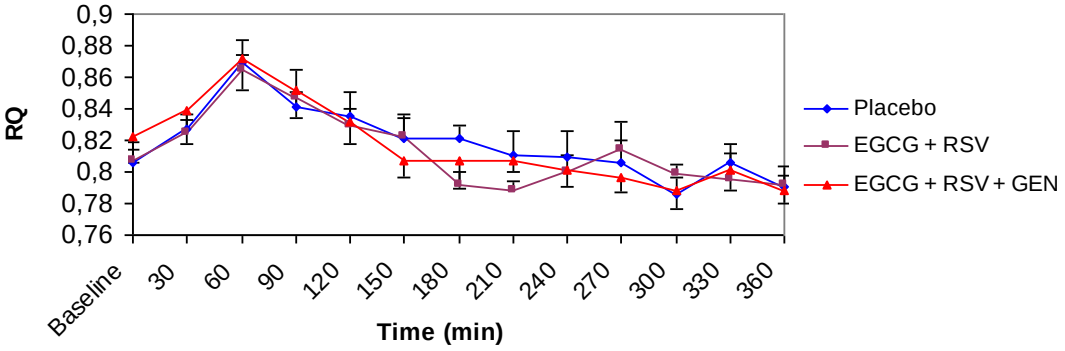

Figure 3. Mean (± SEM) RQ after treatment with placebo or a combination of polyphenols during baseline fasting conditions and for 6 hours after intake of a meal (n = 6).

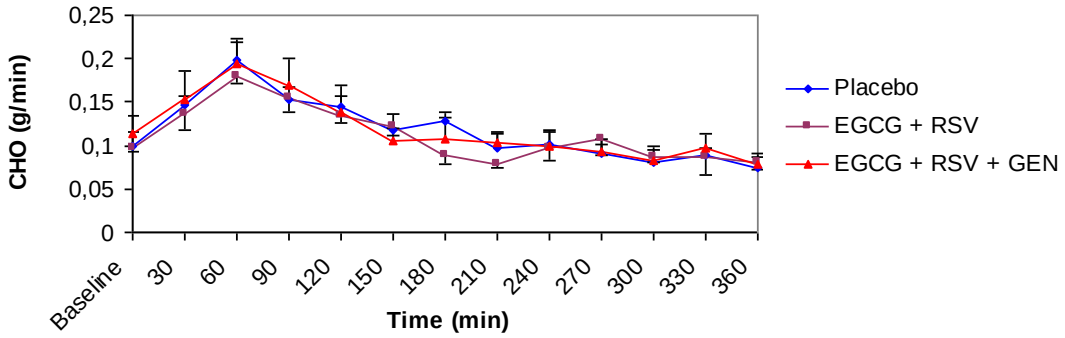

Figure 4. Mean (± SEM) carbohydrate oxidation after treatment with placebo or a combination of polyphenols during baseline fasting conditions and for 6 hours after intake of a meal (n = 6).

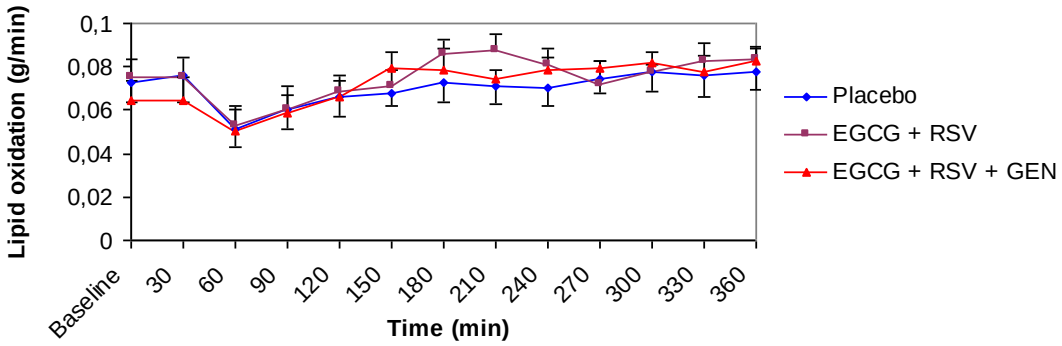

Figure 5. Mean (± SEM) lipid oxidation after treatment with placebo or a combination of polyphenols during baseline fasting conditions and for 6 hours after intake of a meal (n = 6).

After calculation of the total iAUC (**Table 3**), no statistically significant differences in RQ, carbohydrate and lipid oxidation between treatments were found.

Table 3. Total iAUC of RQ, carbohydrate and lipid oxidation after treatment with placebo or a combination of polyphenols[1]

	Total iAUC (6h)		
	PLA	**EGCG + RSV**	**EGCG + RSV + GEN**
RQ	5,0 ± 4,4	2,9 ± 1,4	-1,8 ± 3,3
CHO	7,5 ± 5,3	5,8 ± 1,7	2,1 ± 3,8
Lipid oxidation	-0,9 ± 2,5	-0,2 ± 1,1	2,6 ± 1,5

[1] All values are means ± SEM. Data were analyzed by means of repeated-measures ANOVA with treatment as within-subjects factor. No significant differences between treatments for any of these variables were noticed (RQ: **P = 0,40**; carbohydrate oxidation: **P = 0,64**; lipid oxidation: **P = 0,37**).

After dividing the postprandial period in an early (0 - 2h), a mid (2 - 4h) and a late (4 - 6h) postprandial phase, no significant differences in RQ, carbohydrate and lipid oxidation between placebo and polyphenols treatment were observed (**Table 4**).

Table 4. Early, mid and late iAUC of RQ, carbohydrate and lipid oxidation after treatment with placebo or a combination of polyphenols[1]

	Early iAUC (2h)		
	PLA	**EGCG + RSV**	**EGCG + RSV + GEN**
RQ	4,1 ± 2,0	3,8 ± 0,5	3,0 ± 1,4
CHO	6,7 ± 2,1	5,9 ± 0,6	5,6 ± 2,1
Lipid oxidation	-1,0 ± 1,2	-1,2 ± 0,4	-0,6 ± 0,6
	Mid iAUC (2h)		
RQ	1,6 ± 1,6	-0,3 ± 0,4	-1,6 ± 1,2
CHO	2,2 ± 2,2	0,5 ± 0,4	-0,6 ± 1,4
Lipid oxidation	-0,3 ± 0,8	0,6 ± 0,3	1,4 ± 0,5
	Late iAUC (2h)		
RQ	-0,7 ± 1,4	-0,7 ± 0,7	-3,3 ± 1,3
CHO	-1,4 ± 1,7	-0,6 ± 0,8	-2,9 ± 1,2
Lipid oxidation	0,4 ± 0,6	0,5 ± 0,5	1,8 ± 0,7

[1] All values are means ± SEM. Data were investigated by means of repeated-measures ANOVA with treatment as within-subjects factor. No statistically significant differences in RQ, carbohydrate and lipid oxidation between treatments were noticed.

3.2.2 Energy expenditure

The time-course of EE after treatment with placebo or a combination of polyphenols is illustrated in **Figure 6**. No differences in baseline EE between test conditions were detected. After intake of the high-fat meal, EE showed a similar increase in every test condition. A peak was detected 30 minutes after the meal, followed by a decrease in all test conditions.

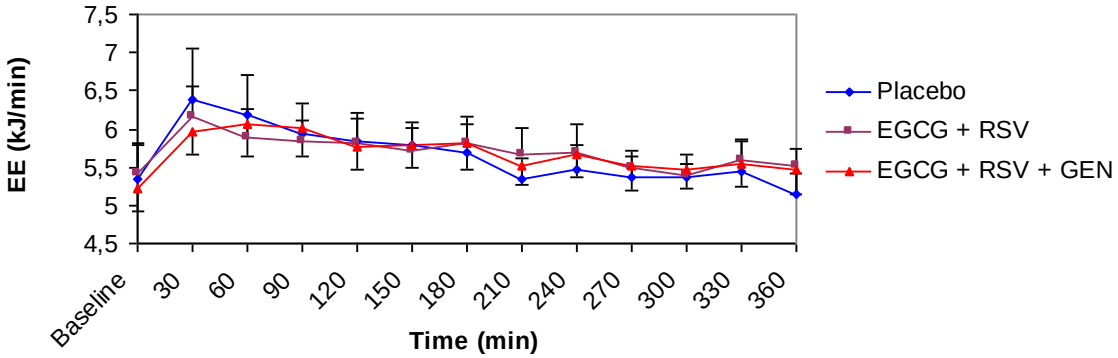

Figure 6. Mean (± SEM) EE after treatment with placebo or a combination of polyphenols during baseline fasting conditions and for 6 hours after intake of a meal (n = 6).

After calculating total iAUC (**Table 5**), no statistically significant differences in EE between test conditions were found.

Table 5. Total iAUC of EE after treatment with placebo or a combination of polyphenols[1]

	Total iAUC (6h)		
	PLA	**EGCG + RSV**	**EGCG + RSV + GEN**
EE	118 ± 71	102 ± 43	177 ± 45

[1] All values are means ± SEM. Data were analyzed by using repeated-measures ANOVA with treatment as within-subjects factor. No significant differences in EE between placebo and polyphenols treatment were detected (**P = 0,56**).

After dividing the postprandial period in stages of 2 hours, no statistically significant differences in EE between treatments were found (**Table 6**).

Table 6. Early, mid and late iAUC of EE after treatment with placebo or a combination of polyphenols[1]

	Early iAUC (2h)		
	PLA	**EGCG + RSV**	**EGCG + RSV + GEN**
EE	82 ± 38	54 ± 11	80 ± 18
	Mid iAUC/min (2h)		
EE	33 ± 22	38 ± 12	60 ± 17
	Late iAUC/min (2h)		
EE	3 ± 20	11 ± 24	37 ± 15

[1] All values are means ± SEM. Data were investigated by using repeated-measures ANOVA with treatment as within-subjects factor. No statistically significant differences in EE between treatments were detected in the early **(P = 0,74)**, mid **(P = 0,55)** and late **(P = 0,36)** postprandial phase.

3.3 Blood sampling

3.3.1 Lipolysis

The time-course of triglycerides, free glycerol and FFAs after treatment with placebo or a combination of polyphenols is depicted in **Figure 7**, **8** and **9**, respectively. At baseline, no statistically significant differences in triglycerides, free glycerol and FFAs concentrations between treatments were observed. After intake of the high-fat meal, the concentration of triglycerides increased in each condition (**Figure 7**). A peak of triglycerides was observed 2 hours after the meal. In the last 2 hours of the postprandial period, the concentration of triglycerides decreased in each test condition. Furthermore, the concentration of free glycerol and FFAs decreased after the meal (**Figure 8**, **Figure 9**). The lowest concentration of free glycerol and FFAs was seen after 90 minutes. After this, free glycerol and FFAs concentrations increased in every condition.

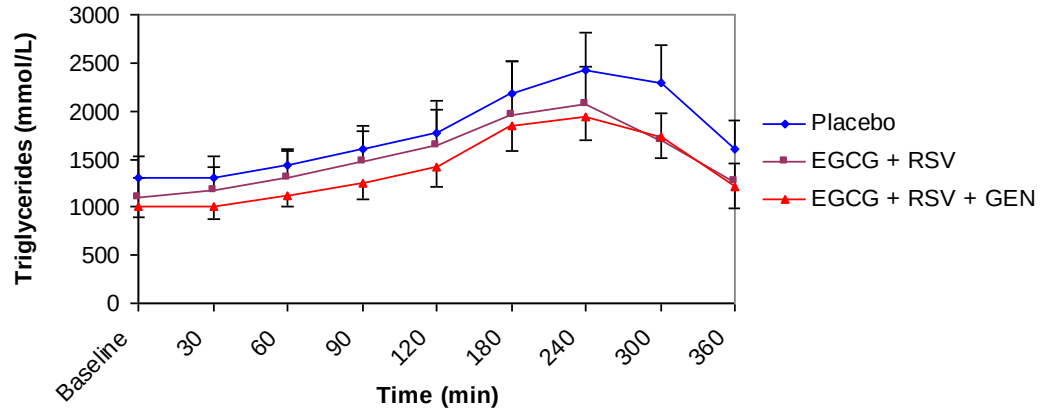

Figure 7. Mean (± SEM) concentration of triglycerides after treatment with placebo or a combination of polyphenols during baseline fasting conditions and for 6 hours after intake of a meal (n = 6).

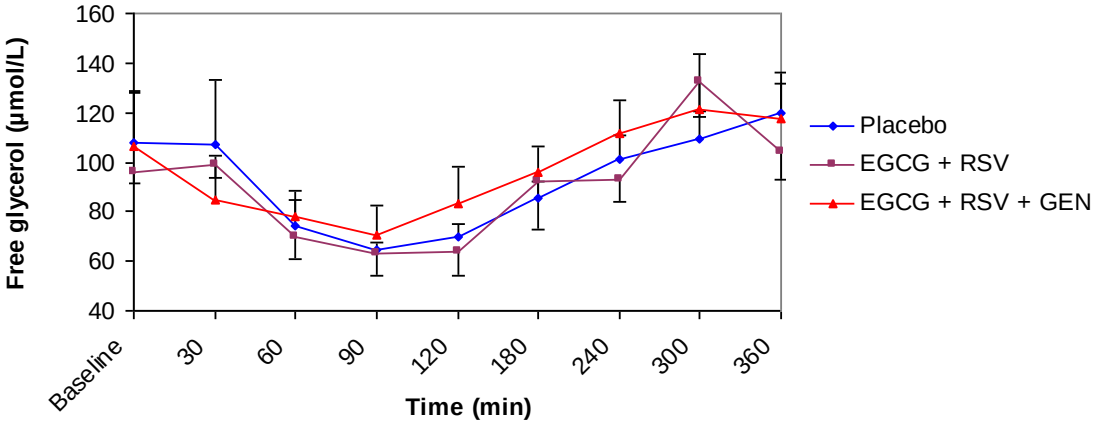

Figure 8. Mean (± SEM) concentration of free glycerol after treatment with placebo or a combination of polyphenols during baseline fasting conditions and for 6 hours after intake of a meal (n = 4).

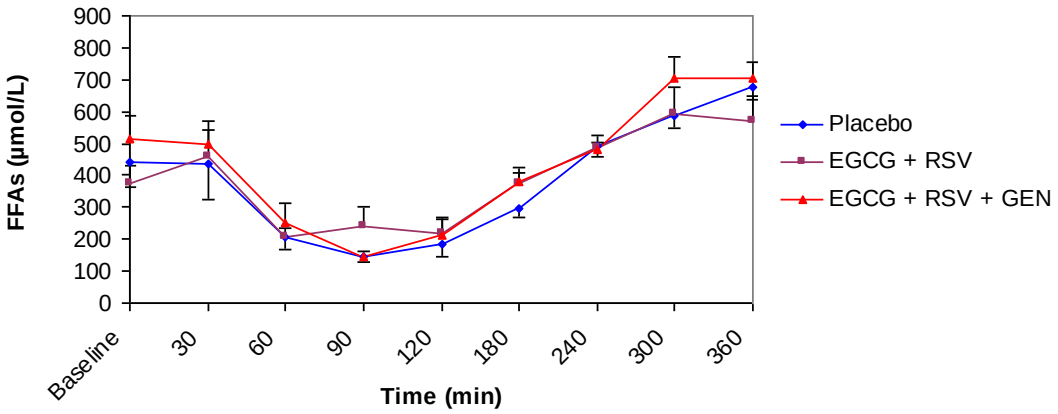

Figure 9. Mean (± SEM) concentration of FFAs after treatment with placebo or a combination of polyphenols during baseline fasting conditions and for 6 hours after intake of a meal (n = 6).

After calculating the total iAUC of triglycerides, free glycerol and FFAs (**Table 7**), no statistically significant differences between treatments were found.

Table 7. Total iAUC of triglycerides, free glycerol and FFAs after treatment with placebo or a combination of polyphenols[1]

	Total iAUC (6h)		
	PLA	**EGCG + RSV**	**EGCG + RSV + GEN**
Triglycerides	$2*10^5 \pm 6*10^4$	$2*10^5 \pm 6*10^4$	$2*10^5 \pm 4*10^4$
Free glycerol	$-5283 \pm 6*10^3$	$-1090 \pm 4*10^3$	$-2587 \pm 9*10^3$
FFAs	$-2*10^4 \pm 2*10^4$	$1*10^4 \pm 2*10^4$	$-3*10^4 \pm 3*10^4$

[1] All values are means ± SEM. Data were analyzed by means of repeated-measures ANOVA with treatment as within-subjects factor. No significant differences between treatments for any of these variables were noticed (triglycerides: **P = 0,89**; free glycerol: **P = 0,99**; FFAs: **P = 0,53**).

When the postprandial period was divided in stages of 2 hours, also no differences in triglycerides, free glycerol and FFAs concentrations between treatments were detected (**Table 8**).

Table 8. Early, mid and late iAUC of triglycerides, free glycerol and FFAs after treatment with placebo or a combination of polyphenols[1]

	Early iAUC (2h)		
	PLA	**EGCG + RSV**	**EGCG + RSV + GEN**
Triglycerides	18969 ± 14257	26773 ± 18144	16573 ± 8434
Free glycerol	-2885 ± 1006	-2173 ± 1003	-2873 ± 2338
FFAs	-20018 ±5738	-8924 ± 4655	-24243 ± 7053
	Mid iAUC (2h)		
Triglycerides	98891 ± 24745	95235 ± 38581	89851 ± 22296
Free glycerol	-2660 ± 2481	-1276 ± 1875	-1150 ± 3068
FFAs	-15029 ± 10633	-1515 ± 10671	-18408 ± 10404
	Late iAUC (2h)		
Triglycerides	100947 ± 28079	69688 ±16992	77390 ± 15303
Free glycerol	262 ± 2023	2360 ± 1622	1435 ± 3572
FFAs	17107 ± 7773	22303 ± 7404	16018 ± 11807

[1] All values are means ± SEM. Data were investigated by means of repeated-measures ANOVA with treatment as within-subjects factor. No statistically significant differences in triglycerides, free glycerol and FFAs between treatments were noticed.

3.3.2 Glucose

The time-course of the glycemic response after treatment with placebo or a combination of polyphenols is illustrated in **Figure 10**. At baseline, there was no significant difference in glucose concentrations between test conditions. After the meal, the concentration of glucose in the blood rose steeply. Subsequently, the plasma concentration of glucose decreased.

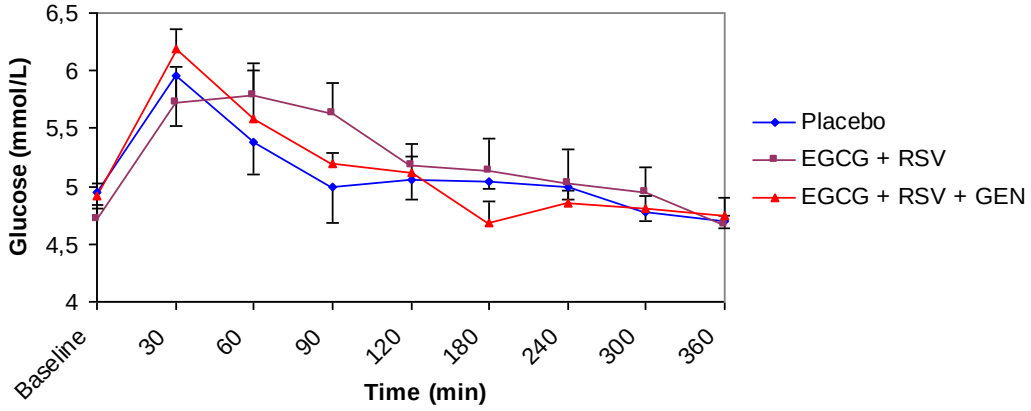

Figure 10. Mean (± SEM) concentration of glucose after treatment with placebo or a combination of polyphenols during baseline fasting conditions and for 6 hours after intake of a meal (n = 6).

After computing the total iAUC, no statistically significant differences in glucose concentrations between test conditions were found (**Table 9**).

Table 9. Total iAUC of glucose after treatment with placebo or a combination of polyphenols[1]

	Total iAUC (6h)		
	PLA	**EGCG + RSV**	**EGCG + RSV + GEN**
Glucose	40 ± 36	169 ± 53	48 ± 36

[1] All values are means ± SEM. Data were analyzed by using repeated-measures ANOVA with treatment as within-subjects factor. No significant differences in glucose concentrations between test conditions were detected (**P = 0,23**).

After dividing the postprandial period in stages of 2 hours, no statistically significant differences in glucose concentrations between treatments were found (**Table 10**).

Table 10. Early, mid and late iAUC of glucose after treatment with placebo or a combination of polyphenols[1]

	Early iAUC (2h)		
	PLA	**EGCG + RSV**	**EGCG + RSV + GEN**
Glucose	46 ± 11	98 ± 13	70 ± 18
	Mid iAUC (2h)		
Glucose	10 ± 17	49 ± 31	-9 ± 19
	Late iAUC (2h)		
Glucose	-16 ± 11	22 ± 29	-13 ± 8

[1] All values are means ± SEM. Data were investigated by means of repeated-measures ANOVA with treatment as within-subjects factor. No statistically significant differences in early, mid or late iAUC of glucose between treatments were noticed (early: **P = 0,11**; mid: **P = 0,33**; late: **P = 0,30**).

3.3.3 Lactate

The time-course of lactate concentrations after treatment with placebo or a combination of polyphenols is shown in **Figure 11**. At baseline, no significant differences between test conditions were observed. After the meal, plasma lactate concentrations increased in all test conditions, with a peak one hour after the meal. Subsequently, plasma lactate concentrations decreased in each test condition.

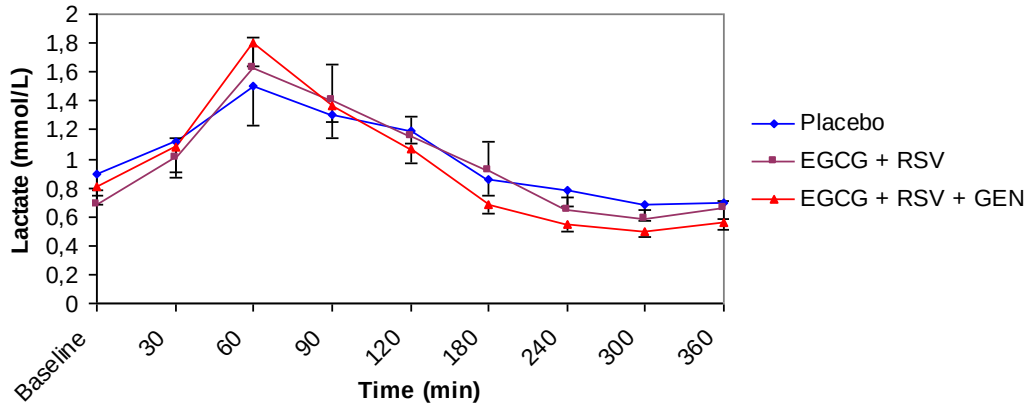

Figure 11. Mean (± SEM) concentration of lactate after treatment with placebo or a combination of polyphenols during baseline fasting conditions and for 6 hours after intake of a meal (n = 6).

After calculation of the total iAUC, lactate concentrations tended to be different between test conditions (**P= 0,06**; **Table 11**).

Table 11. Total iAUC of lactate after treatment with placebo or a combination of polyphenols[1]

	Total iAUC (6h)		
	PLA	**EGCG + RSV**	**EGCG, RSV + GEN**
Lactate	25 ± 37	85 ± 43	16 ± 38

[1] All values are means ± SEM. Data were analyzed by using repeated-measures ANOVA with treatment as within-subjects factor. Lactate concentrations tended to be different between test conditions (**P = 0,06**).

After dividing the postprandial period in stages of 2 hours, no statistically significant differences were found in early and mid iAUC between treatments (**Table 12**). However, late iAUC was significantly different between test conditions. In particular, lactate concentrations were significantly lower after additional supplementation with GEN compared to treatment with only EGCG and RSV in the late postprandial phase (**P = 0,02**). Nevertheless, no significant differences in lactate concentrations between polyphenols and placebo treatment were found.

Table 12. Early, mid and late iAUC of lactate after treatment with placebo or a combination of polyphenols[1]

	Early iAUC (2h)		
	PLA	**EGCG + RSV**	**EGCG + RSV + GEN**
Lactate	42 ± 23	66 ± 18	58 ± 15
	Mid iAUC (2h)		
Lactate	4 ± 9	27 ± 20	-8 ± 14
	Late iAUC (2h)		
Lactate	-21 ± 9	-8 ± 10	-34 ± 13

[1] All values are means ± SEM. Data were investigated by means of repeated-measures ANOVA with treatment as within-subjects factor. No statistically significant differences in early or mid iAUC of lactate between test conditions were noticed (early: **P = 0,54**; mid: **P = 0,12**). However, a significant difference in late iAUC of lactate between treatment with a combination of 2 polyphenols and treatment with a combination of 3 polyphenols was observed (**P = 0,005**). After post-hoc testing with *Bonferroni*, the P-value was 0,03.

4 Discussion

This study was designed to determine whether combining polyphenols, with distinct mechanisms of action, has short-term effects on adipose tissue lipolysis, lipid oxidation and mitochondrial function in overweight volunteers.

Preliminary data show that in a group of 6 subjects short-term supplementation with a combination of 2 (EGCG and RSV) or 3 (EGCG, RSV and GEN) polyphenols did not affect adipose tissue lipolysis, substrate oxidation and EE compared to placebo treatment. Furthermore, the combination of these polyphenols had no effect on glycemic control. However, lactate concentrations tended to be reduced after treatment with a combination of 3 polyphenols compared to treatment with a combination of 2 polyphenols. This effect on lactate concentrations was significantly different in the late postprandial period. These findings may suggest that supplementation with a combination of EGCG, RSV and GEN reduces the glycolytic pathways, shifting towards more lipid oxidation. Furthermore, glucose and free glycerol concentrations tended to be reduced after treatment with a combination of EGCG, RSV and GEN. Assuming that insulin concentrations are comparable, this may indicate a tendency towards an improved insulin sensitivity. These data need to be confirmed when analyzing the complete data set of 18 volunteers.

The main reason for our inconclusive results is the use of a very small sample size (n = 6). As the study is currently still running, statistical analyses were carried out with data of people, who had already undergone all treatments. Because of the very small sample size, statistical power was probably too low to observe differences between test conditions. Notably, the reduced concentrations of free glycerol, FFAs, glucose and lactate after treatment with a combination of EGCG, RSV and GEN suggest that metabolic effects did occur. These effects may be more pronounced when analyzing the total data set.

Another possible explanation for the lack of effects on lipolysis and substrate oxidation is that the chosen study population differs from that in other human studies. In particular, in this study overweight men and women were included, whereas in most studies only overweight men were allowed to participate (19, 21). Hence, gender differences in lipid metabolism might play a role in the inconsistent results. Moreover, it seems that most of the participating subjects are slightly insulin-resistant. A simple, reliable technique to assess *in vivo* insulin sensitivity in humans is *homeostatic model assessment of insulin resistance* (HOMA-IR) (111). The HOMA-IR is calculated as follows: (fasting plasma glucose concentration * fasting serum insulin concentration)/22,5. For this calculation, only one fasting blood sample is

required. In this study, the mean value of the HOMA scores was 3,2 ± 0,5. By using a cut-off point of 2.5, as previously shown (110), we can conclude that 4 out of 6 participating subjects are insulin-resistant. As mentioned before, the insulin-resistant condition is characterized by an impaired capability to increase lipid oxidation during fasting conditions and by a disturbed *switch* from lipid to carbohydrate oxidation after intake of a meal. This phenomenon is called *metabolic inflexibility*. The fact that the combination of the used polyphenols does not improve this phenomenon might explain why no differences in substrate oxidation were observed between test conditions (38, 112). However, a tendency towards an improved oxidative phenotype was observed after treatment with a combination of EGCG, RSV and GEN. *Metabolic flexibility* in response to meals is estimated by calculating the difference between baseline and stimulated RQ. Accordingly, this may originate from a lower stimulated RQ and/or an increased baseline RQ (112). A study of *Kelley* and colleagues suggests that baseline RQ is elevated in obese insulin-resistant subjects (35). Within few days prior to the test day, this baseline RQ is sensitive to differences in energy balance and diet composition (113). To minimize the variability in baseline RQ in this study, diet composition was standardized during each treatment period. In particular, subjects were instructed to fill in a food intake diary throughout the first test period. Also, they were asked to consume the same amounts of the same foods and drinks during the other test periods. Similarly, to exclude the effects of energy balance on baseline RQ, subjects were requested to refrain from heavy physical activity and exercise at least 3 days before testing. Therefore, if subjects followed our instructions, differences in baseline RQ could not be explained by inadequate control of these variables.

Besides the small sample size and differences in choice of the study population, the discrepancies between our results and findings of other studies that investigate polyphenols may be explained by many different factors. Possible explanations include treatment duration, the used dose of polyphenols and species-specific differences. In addition, testing the combination of polyphenols on lipid and energy metabolism in humans is a relatively new area of nutritional research. Consequently, it is possible that polyphenols do not only have synergistic effects but also antagonistic effects that are not known in humans at the present.

Recently, there has been considerable interest in the use of polyphenols to combat obesity and T2DM (25, 26). Current evidence has revealed that polyphenols have advantageous effects on both lipid and energy metabolism. However, the main findings concerning the action of polyphenols have been derived from *in vitro* experiments and studies in rodents, whereas limited data of human studies are available. For instance, *Skudelzka* et al. examined the effects

of varying concentrations of GEN (0,01; 0,1 and 1 mM) on lipolysis in isolated rat adipocytes and they found a significantly enhanced basal lipolysis (24). Furthermore, *Rayalam* and colleagues have shown that a 6 day-treatment with a combination of RSV (25µM) and GEN (25µM) increased lipolysis in isolated murine adipocytes. Recent data have revealed that both RSV and GEN regulate lipolysis through the activation of AMPK (20, 23). In particular, a recent study of *Lasa* et al. has provided novel evidence that RSV activates AMPK in human and murine adipocytes, as well as in white adipose tissue from mice. This leads to enhanced levels of ATGL, an enzyme contributing to lipolysis (23). In addition, *Cederroth* and colleagues have demonstrated that mice, fed with a soy-rich diet, manifest an improved lipid and glucose metabolism. It has been suggested that activation of the AMPK pathway by dietary soy is involved (20). In contrast with these affirmative results, we did not manage to find any effects of combining polyphenols on lipolysis. A remarkable fact is that the plasma free glycerol and FFAs concentrations are lower in the postprandial period after treatment with a combination of EGCG, RSV and GEN compared to treatment with solely a combination of EGCG and RSV. This finding might be explained by an increased insulin sensitivity of inhibition of lipolysis after treatment with the 3 polyphenols EGCG, RSV and GEN.

Another reason why our results do not seem to be entirely consistent with previous studies is that all aforementioned studies investigated the effects of polyphenols *in vitro* or in rodents, whereas there are limited human studies. Owing to ethical concerns, higher concentrations of polyphenols can be tested *in vitro* or in rodent studies when compared to human studies. Moreover, another explanation for our inconsistent results might be that genistein only affect lipid profiles of humans when consumed along with the protein part of soybeans (104).

In addition to the inconsistent results of lipolysis, we did not manage to find any effects of combining polyphenols on substrate oxidation and EE. Nevertheless, an interesting finding is a decrease in lactate concentrations in the late postprandial period after treatment with a combination of 3 polyphenols compared to treatment with a combination of 2 polyphenols. This result indicates a reduced glycolytic flux and a shift towards a more oxidative phenotype after treatment with a combination of 3 polyphenols. These data are consistent with findings of *Murase* et al. (90), who reported a decline in plasma lactate concentrations after long-term treatment with a green tea extract in mice that exert swimming exercises. Our obtained data are in contrast with a study of *Dulloo* et al. (19, 21). They investigated whether a green tea extract could augment 24-h EE and lipid oxidation in 10 healthy men. Subjects were randomly assigned to 3 treatments (green tea extract containing 150 mg caffeine and 270 mg

EGCG, 150 mg caffeine and placebo). A significant increase in 24h-EE has been reported after treatment with green tea extract compared to treatment with placebo or caffeine. Also, 24-h RQ was significantly lower after treatment with green tea extract compared to placebo or caffeine, indicative of an increased lipid oxidation. These results propose that EGCG and caffeine work synergistically (21). Albeit the mechanism of EGCG is not completely understood yet, it has been suggested that it affects lipid oxidation through inhibiting COMT, an enzyme responsible for the degradation of nodadrenalin. As a result, noradrenalin confers a prolonged interaction with adrenergic receptors, causing an increased lipid oxidation and EE (21, 114). Our findings are in contrast with a pilot study of *Boschmann* and colleagues. They examined the effect of a 3 day-treatment with 300 mg EGCG on fasting and postprandial EE and substrate oxidation in 6 overweight men. Postprandial RQ values were significantly lower after EGCG treatment when compared to placebo, representing an increased lipid oxidation. On the other hand, resting EE did not differ significantly between treatments (19). This potential of EGCG to augment lipid oxidation without influencing EE has already been reported in mice (115). This study suggests that changes in EE result from the caffeine present in green tea extracts. Although our study design is similar to the pilot study of *Boschmann* et al. (19) (the same dose of EGCG and the same treatment duration), we did not manage to find any effects on substrate oxidation. This discrepancy may be explained by the fact that the other polyphenols in our study (RSV and GEN) do not only have synergistic effects but also antagonistic effects. Although a total caffeine use of more than 300 mg/day was an exclusion criteria of this study, another possible explanation may be that our subjects did not restrict their habitual caffeine intake. Two studies, performed at our department, have revealed that supplementation with EGCG is only effective when regular caffeine consumption is low (17, 95). Thus, if our subjects consumed too much caffeine few days prior to the test day, it is possible that the effects of EGCG were masked. Finally, gender differences in lipid metabolism might play a role in the inconsistent results. The studies of *Boschmann* and *Dulloo* examined the effects of EGCG treatment in overweight men, whereas in our study both overweight men and women participated.

Besides reported beneficial effects on lipolysis and lipid oxidation, there is also evidence that long-term administration of polyphenols improves insulin resistance. A study of *Lagouge* and colleagues, in which mice received a daily dose of 400 mg RSV for a period of 15 weeks, has revealed an enhanced insulin sensitivity and mitochondrial function (22). It has been suggested that these outcomes are derived from an increased activity of both AMPK and SIRT1 (98). In particular, RSV phosphorylates AMPK and its downstream target ACC. As a

consequence, the activity of AMPK increases and insulin sensitivity improves (116). In addition to AMPK, RSV activates SIRT1 (98). Subsequently, SIRT1 deacetylates PGC-1α. This deacetylation causes an increased activity of PGC-1α and leads to induction of genes for oxidative phosphorylation and mitochondrial biogenesis. As RSV is a sirtuin activator, it mimics Sir2-dependent lifespan extension during caloric restriction. In addition, it has been shown that RSV increases cell survival by stimulating SIRT-1 dependent deacetylation of p53 (117). In contrast with these affirmative results, we did not manage to find any effects on insulin sensitivity in humans after a 3-day treatment with 200 mg RSV in combination with EGCG or with both EGCG and GEN. Particularly, no effect on glucose concentrations was seen after treatment with a combination of 2 or 3 polyphenols compared to placebo. Interestingly, plasma glucose concentrations seem lower after treatment with EGCG, RSV and GEN compared to treatment with only EGCG and RSV. This finding suggests an improved insulin sensitivity after treatment with a combination of EGCG, RSV and GEN. The differences in results between our human study and the animal studies might be explained by species-specific differences in lipid metabolism. Additional explanations might include duration of treatment and the used dose of polyphenols. It is possible that a treatment period of 3 days and the used dose of polyphenols are not sufficient to see beneficial effects on insulin sensitivity.

In summary, short-term supplementation with a combination of 2 (EGCG and RSV) or 3 (EGCG, RSV and GEN) polyphenols has no beneficial effects on postprandial adipose tissue lipolysis, substrate oxidation and EE in overweight subjects. In the late postprandial period, combining EGCG, RSV and GEN reduces lactate concentrations, implying a shift in substrate oxidation towards more lipid oxidation. In addition, the tendency to reduced glucose concentrations after treatment with a combination of EGCG, RSV and GEN indicates a trend towards an improved insulin sensitivity. These data need to be confirmed when analyzing the complete data set of 18 subjects.

5 Conclusion

This study showed a tendency to reduced lactate concentrations after treatment with a combination of EGCG, RSV and GEN compared to a treatment with only EGCG and RSV. This finding implies that supplementation with a combination of EGCG, RSV and GEN reduces the glycolytic pathways, shifting towards a more oxidative phenotype. Moreover, free glycerol and glucose concentrations tended to be decreased after treatment with a combination of EGCG, RSV and GEN. Assuming that insulin concentrations are comparable, this may suggest a tendency towards improved insulin sensitivity. However, because a very small sample size of 6 subjects was used for this interim analysis and therefore statistical power was probably too low to observe differences between test conditions, definite conclusions can only be drawn after analyzing the complete data set of 18 subjects.

Most studies investigated the effects of combining polyphenols *in vitro*, whereas there is very little evidence for synergy *in vivo*. Therefore, additional *in vivo* research on the appropriate combination of polyphenols to combat obesity and T2DM is needed in the near future. These upcoming studies also need to examine the suitable dose and duration of supplementation. In the future, a *follow-up* study will be performed. This double-blinded, randomized, placebo-controlled dietary intervention study will examine the long-term effects of the most promising combination of polyphenols on lipid oxidation, mitochondrial function and insulin sensitivity in 60 overweight subjects over a period of 12 weeks. If the effects of this combination on lipid and energy metabolism are convincing in the long-term, a functional food consisting of the examined polyphenols can be distributed on the market. This functional food would contribute to the prevention of obesity and T2DM. Many companies would be interested in manufacturing such functional foods, because the prevalence of obesity is reaching epidemic proportions in many Western countries. For that reason, there is an urgent need of a functional food to slow down the rate of progression of these diseases and to reduce dependence on costly medical treatment. In addition, this functional food would have far-reaching economic and medical implications with effects beyond obesity. However, the polyphenols must be safe for human use and the correct dose should be determined before distribution on the market.

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