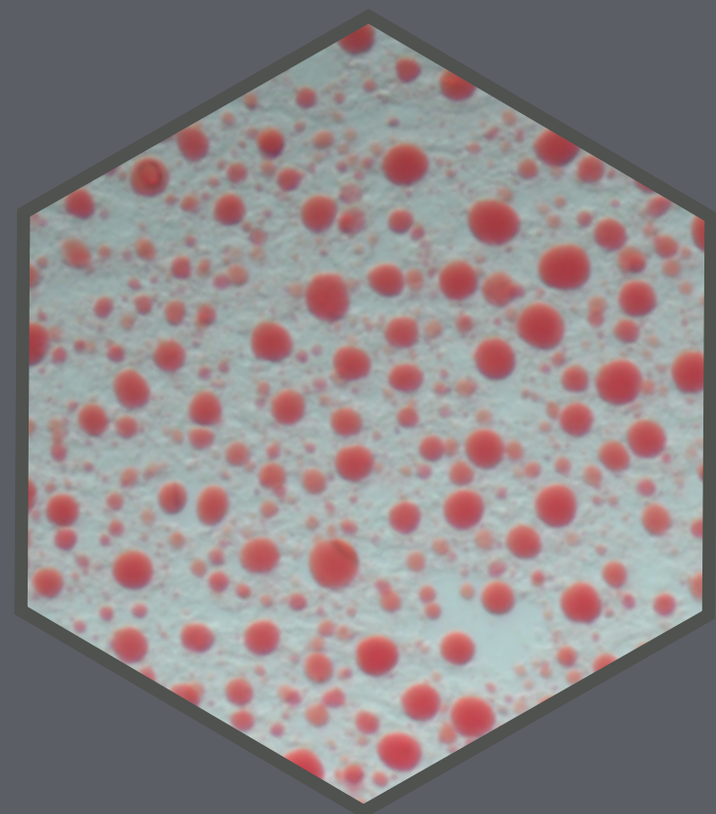

UNRAVELLING MECHANISMS OF DIETARY FLAVONOID-MEDIATED HEALTH EFFECTS

Elise Hoek – van den Hil

# UNRAVELLING MECHANISMS OF DIETARY FLAVONOID-MEDIATED HEALTH EFFECTS:

## effects on lipid metabolism and genotoxicity

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Elise F. Hoek – van den Hil

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# UNRAVELLING MECHANISMS OF DIETARY FLAVONOID-MEDIATED HEALTH EFFECTS: EFFECTS ON LIPID METABOLISM AND GENOTOXICITY

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# CHAPTER 1

## GENERAL INTRODUCTION

# 1.1 FLAVONOIDS

## Vitamin P

In the 1930s, Szent-Györgyi identified a substance in lemon peel that reduced capillary permeability, and called it 'vitamin P' [1]. This vitamin status was never confirmed. Later, he reported that the substance contained, among others, the flavonoid hesperidin [2]. Flavonoids belong to the large group of polyphenols which are abundantly present in all plant foods. Polyphenols may contribute to the beneficial health effects of fruits and vegetables, and therefore there is substantial interest in these dietary compounds. Flavonoids have been mostly studied for their preventive role against several diseases, like cancer and cardiovascular diseases (CVD) [3]. However, still no conclusive underlying mechanism has been found which is targeted by these dietary compounds.

## Flavonoid structure

The basic flavonoid structure is an aglycone that is characterized by two aromatic rings, called A and B that are linked by a heterocyclic pyrane ring (C). Flavonoids can be divided into six subclasses: anthocyanins, flavan-3-ols, flavanones, flavones, flavonols, and isoflavones, which are all present in our diet (Figure 1.1). The differences between the subclasses are determined by differences in oxidation state and pattern of substitution of the C ring. The individual flavonoids within a subclass differ in the level of substitution on the A and B rings [3,4].

## Presence in food and intake of flavonoids

Anthocyanins, flavanones, flavones, flavonols, and isoflavones are present as glycosides in our food. In these glycosides sugars are covalently bound to the aglycone, in general at the hydroxyl moiety at position 3 or 7. The attached compound can be a rhamnose, glucose, glucorhamnose, galactose, arabinose or another sugar. Some flavonoids are found in all plant food products, such as fruits, vegetables, cereals, tea, and wine, whereas other flavonoids are mainly found in specific types of foods. For

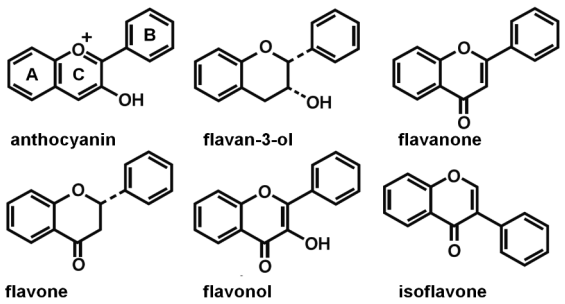


**Figure 1.1 Basic structures of the six flavonoid subclasses.**

example, isoflavones are mainly present in soy and flavanones in citrus fruits. However, most foods contain a mixture of various flavonoids [4,5].

It is difficult to estimate flavonoid intake from food intake. This is because the amount of flavonoids may vary widely within a product type. For example, within different varieties of apples and between yellow and red onions a 2-3 fold difference in the amount of flavonoids was found [6]. Furthermore, in most foods flavonoids are not evenly distributed, with the peel or surface commonly containing most of the compounds [6,7]. Furthermore, processing and storage will influence the amount of flavonoids in food [3]. Table 1.1 shows the different subclasses of flavonoids and their main flavonoids, examples of foods in which they are present, and an estimation of the flavonoid concentrations in these foods. The estimated total dietary intake of flavonoids is between 100 and 1000 mg/day [4]. However, when supplements are used the intake will be much higher. For example, for some quercetin containing supplements the intake can be about 1500 mg/day, which is far beyond the estimated dietary intake of quercetin of 16 mg/day [8].

## 1.2 ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION OF FLAVONOIDS

For systemic effects of flavonoids it is essential that the flavonoids will enter the bloodstream, therefore the absorption, distribution, metabolism and excretion of flavonoids will be described in this paragraph.

**Table 1.1** Presence of flavonoids in foods[a].

| Flavonoid Subclasses | Flavonoids | Dietary Sources | Concentrations (mg/kg) or (mg/L) |
|---|---|---|---|
| Anthocyanins | Cyanidin<br>Delphinidin<br>Malvidin<br>Pelargonidin | Aubergine, Blackberry, Blueberry, Cranberry, Elderberry, Plum, Strawberry | 20-10,000 |
| Flavan-3-ols | Catechin<br>Epicatechin<br>Epigallocatechin | Chocolate, Cocoa,<br>Tea (black/white), Wine (red/white) | 60-800 |
| Flavanones | Hesperetin<br>Naringenin | Grapefruit, Orange | 100-650 |
| Flavones | Apigenin<br>Luteolin | Celery, Olive, Parsley | 20-1850 |
| Flavonols | Myricetin<br>Kaempferol<br>Quercetin | Apple, Beans, Blueberry, Broccoli, Elderberry, Onion, Spinach, Tea | 30-1200 |
| Isoflavones | Daidzein<br>Genistein | Soya Beans | 200-900 |

[a]Based on [4,9].

## Absorption

When foods are ingested flavonoids are mostly present in the form of glycosides. There are some indications that already in the saliva of the mouth hydrolysis of the glycosides can take place [10]. It was shown in rats that some flavonoids in their aglycone form can be absorbed in the stomach [11,12]. Nevertheless, the intestines are the most important organs in absorption of flavonoids. In the small intestine, flavonoid glycosides can mostly not be absorbed, while aglycones and glucosides can be absorbed. Flavonoid glycosides are hydrophilic, which prevents passive diffusion across the intestinal wall. The $Na^+$ dependent glucose co-transporter SGLT1 was shown to be able to transport quercetin glucoside across the small intestine [13]. Furthermore, lactose phloridzin hydrolase (LPH) attached to the outside of the small intestinal cells was shown to work as a glucosidase, facilitating subsequent absorption of the aglycone [14]. The flavonoid glycosides which cannot be transported via these mechanisms in the small intestine arrive in the colon. Here, glycosides should be hydrolysed by enzymes of the colonic microflora before they can be absorbed [15]. However, it was also shown that aglycones can be (partly) degraded by the colonic microflora [16].

## Tissue distribution and plasma concentrations

After consumption of flavonoid rich-foods, flavonoids first enter the gastro-intestinal tract, where the highest concentrations of flavonoids are expected. This was shown in studies with radiolabeled flavonoids which were found at the highest levels in stomach, intestine and liver [17-19]. Studies on the tissue distribution of quercetin in pig and chicken found also the highest levels in the small intestine, followed by significant levels in liver and kidneys [20,21]. Long-term exposure of rats to quercetin revealed appreciable amounts in lung and testis [22]. Overall, flavonoids have been detected in very different tissues like brain, heart, kidney, lung, spleen, pancreas, prostate, uterus, ovary, mammary gland, testes, bladder, muscle, bone and skin [4]. After uptake in the gastro intestinal tract, flavonoids enter the circulation via the portal vein and the liver. Alternatively, it was shown that some flavonoids can be absorbed into the circulation via the lymphatic system [23]. The amount of flavonoids found in plasma after consumption of foods varies between nM and, rarely, low µM concentrations, consisting mainly of flavonoid conjugates, since flavonoids are intensively metabolised in the human body. For anthocyanins the plasma levels are even much lower, they can only be found in the low nM range in plasma [4,24,25].

## Metabolism

Metabolism including especially conjugation of flavonoids takes place in the intestinal enterocytes and thereafter also in other organs. There are three types of conjugation that are relevant for flavonoids: glucuronidation by UDP-glucuronyltransferases (UGTs), sulfation by sulfotransferases (SULTs), and methylation by catechol-O-methyltransferases (COMTs) [26]. Glucuronidation of flavonoids by UGTs occurs at the

hydroxyl moieties at the 7, 3, 3' and/or 4' positions of the aglycone, and UGT activity for flavonoid conjugation was found in intestines, liver and kidney. During sulfation of flavonoids in liver and intestines a sulfate moiety is added likely to the hydroxyl moieties at position 3' or 7 of the aglycone [27]. Both SULTs and UGTs consist of a family of different isoforms of which some are predominately responsible for conjugation at a specific site or present in a specific tissue [3]. Methylation will result in the addition of a methyl group mainly at the hydroxyl moieties at the 3' and 4' position of flavonoids. Methylation of flavonoids by COMTs was found in liver, kidney and small intestine. Conjugation can also occur at more than one position which normally leads to formation of di- or tri-conjugates containing different types of conjugating groups attached to the aglycone [3]. The metabolism of flavonoids is dependent on the flavonoid, the dose, species, and sex. In general, the main conjugated forms found in human plasma are flavonoid glucuronides, however, numerous different glucuronidated, sulphated and methylated flavonoids are identified in plasma of rodents as well as in humans [4].

### Excretion

Flavonoids present in the circulation can be excreted via the urine and via bile into the faeces. The main route can be different for different flavonoids. Urinary excretion has been shown in human studies for several flavonoids, excretion via urine are regularly the small conjugates for example the mono-sulfates. Furthermore, unabsorbed flavonoids are excreted via the faeces, these are mostly the large conjugated metabolites [4,28].

## 1.3 BENEFICIAL HEALTH EFFECTS OF FLAVONOIDS

### Flavonoid-mediated health effects in human studies

Consumption of foods containing flavonoids has been associated with several beneficial health effect [29]. Flavonoids are suggested to reduce the risk of CVD, cancer, diabetes, inflammatory and neuro generative disorders [3,30-32]. Epidemiological studies have shown indications for the protective effects of flavonoids against cancer and CVD [30,33]. Altogether available epidemiological data showed that most evidence was found for beneficial health effects of flavonoids on CVD [30]. Epidemiological studies with foods rich in flavonoids suggested that daily consumption of three or more cups of tea per day decreased the risk of coronary heart disease by 11% or the risk of stroke by 21% [34,35]. Similarly, high cocoa intake was associated with lowered mortality due to cardiovascular events by 40-50% [36,37]. However, another study did not found such an effect [38]. Most observational studies that calculated the intake of individual flavonoids focused on flavonols. A meta-analysis of these flavonol studies concluded that high flavonol intakes could reduce the incidence of stroke by 20% [39]. Observational studies with other subclasses of flavonoids showed no clear indication of reduced risk on CVD [38,40-42]. However, the number of studies is limited, which makes it difficult to draw

conclusions about the effects on CVD of these flavonoid subclasses [9]. Randomized placebo controlled intervention studies were in general performed with flavonoid-rich foods or extracts and not with individual flavonoids. The studied risk factors related to CVD were blood pressure, endothelial dysfunction, and serum lipid profile. A meta-analysis of 130 trials found that cocoa reduced blood pressure and that soy protein rich in isoflavones reduced LDL cholesterol [43]. Overall, these human studies suggested effects by flavonoids on CVD risk factors, however the effects and possible underlying mechanisms are far from being completely known.

## Mechanisms underlying flavonoid-mediated health effects

Several mechanisms are proposed to explain the health effects of flavonoids. Effects like scavenging free radicals, regulation of nitric oxide, decreasing leukocyte immobilization, induction of apoptosis, inhibition of cell proliferation and angiogenesis, and estrogenic activity were shown *in vitro* and *in vivo* [44]. Mostly, the antioxidant and free radical scavenging activity of flavonoids were brought up as explanation for the health effects. Indeed, flavonoids showed strong antioxidant activities *in vitro*. However, it was demonstrated that these effects could not explain disease prevention *in vivo*, because absorption of flavonoids is low and they are extensively metabolised, which further reduces their antioxidant impact in the body [9]. Therefore, other properties of flavonoids should underlie the beneficial health effects especially on CVD. Considering the low absorption and extensive metabolism of polyphenols, only biological effects that can be caused by low concentrations should be considered as relevant. Such effects may include effects on regulatory pathways, ultimately resulting in altered serum lipids or gene expression levels.

## Flavonoids and serum lipids in rodents

Some observations in humans suggested an effect of flavonoids on serum lipid profiles. Lipid lowering effects of flavonoids are more established in rodents. Table 1.2 shows an overview of effects of individual pure flavonoids on serum and hepatic lipids found in rodents. Two studies showed that exposure of rats for several weeks to hesperetin or its glycoside hesperidin lowered serum triglycerides and cholesterol as well as hepatic lipids [45,46], while other hesperetin studies did not show such results in mice and rats [47,48]. Naringenin and its glycoside naringin, also showed serum triglycerides and total cholesterol lowering effects in rodents [48-51]. In some of these rodent naringenin studies hepatic triglycerides were not lowered, while hepatic cholesterol levels were lowered in some cases [49-51]. Quercetin showed some lowering effects on serum and hepatic triglycerides in mice [52,53]. Some other quercetin studies in mice did find lowering effects on other serum lipid fractions [53,54]. For all of these compounds, these effects were not consistently found [37,38,43]. The effects on serum and hepatic lipids could be (partly) responsible for the CVD preventive effects of flavonoids, because high lipid levels in serum and liver are suggested risk factors for CVD [55-57]. However,

**Table 1.2** Flavonoid-mediated effects on serum and hepatic lipids in rodents.

| Flavonoids | Flavonoid in diet (w/w) | Weeks | Diet | Animal | Serum lipids[a] | Hepatic lipids[a] | Ref |
|---|---|---|---|---|---|---|---|
| Hesperidin | 1% | 4 | Normal | Streptozotocin-induced marginal type 1 diabetic rats | ↓TG<br>↓TC<br>↑HDL<br>↓ LDL | ↓Hepatic lipids | 45 |
| Hesperetin | 0.02 % | 6 | High cholesterol | Sprague-Dawley rat | ↓TG<br>↓TC | ↔Hepatic lipids<br>↓Hepatic TC | 46 |
| Hesperetin | 1% | 3 | Normal | ICR mice | ↔TG<br>↔ TC<br>↔PL | ↔Hepatic lipids | 48 |
| Hesperetin | 1% | 1.5 | Orotic acid (1%) | Sprague-Dawley rat | ↔TG<br>↔TC<br>↓PL | ↓Hepatic lipids | 47 |
| Hesperetin | 1% | 1.5 | Normal | Sprague-Dawley rat | ↔TG<br>↔ TC<br>↔PL | ↔Hepatic lipids | 47 |
| Naringin | 0.02% | 3,6 | High-fat high-cholesterol | Sprague-Dawley rat | ↓TG<br>↓TC (week 6) | ↔Hepatic lipids<br>↓Hepatic TC | 51 |
| Naringenin | 0.003,0.006, 0.012% | 6 | Normal | Male Long-Evans hooded rats | ↓TG<br>↓TC (0.006%)<br>↔FFA | ↔Hepatic TG<br>↓Hepatic TC (0.006%) | 49 |
| Naringenin | 0.02% | 5 | High-cholesterol | Sprague-Dawley rat | ↓TG<br>↓TC<br>↑HDL | ↔Hepatic TG<br>↓Hepatic TC | 50 |
| Naringenin | 1% | 3 | Normal | ICR mice | ↓TG<br>↓TC<br>↓FFA<br>↓PC | ↔Hepatic lipids<br>BW ↔ | 48 |
| Quercetin | 0.025% | 9 | High-fat | C57BL/6J mice | ↓TG | ↓Hepatic TG<br>↓Total hepatic lipids | 52 |
| Quercetin | 0.05% | 4,8,20 | High-fat | C57BL/6J mice | ↓TG<br>↓FFA (wk 8,20) | ↓Hepatic TG (wk 20)<br>↓Hepatic TC (wk 20) | 53 |
| Quercetin | 0.03% | 4 | High-fat | Wistar rats | ↓TG<br>↑FFA | ND | 61 |
| Quercetin | 1% | 2 | Normal | ICR mice | ↔TG<br>↓TC<br>↓PL | ND | 54 |
| Quercetin | 0.05% | 4,8,20 | Normal | C57BL/6J mice | ↔TG<br>↔TC<br>↔FFA | ND | 53 |

[a]Effects could be overestimated, due to interference of flavonoids in the used enzymatic assays, as will be described in chapter 2. FFA free fatty acids, TG triglycerides, TC total cholesterol, PL phospholipids.

the results are possibly compromised by interference of flavonoids with the enzymatic assays used, as described in chapter 2. For most flavonoids the lipid lowering effects were not conclusive and the mechanisms behind these effects are hardly understood.

## 1.4 SAFETY OF FLAVONOIDS

In line with the observed beneficial health effects of flavonoids, flavonoid intake is generally regarded as safe because these compounds are naturally present in food. However, since supplements of flavonoids are freely available in Western Europe and the United States, ingested doses of flavonoids may increase substantially. A human intervention trial with antioxidant vitamin supplements showed an increased mortality risk in supplement users [58]. Therefore, the safety of flavonoids, especially at high dose levels, is an item of concern and should also be taken in consideration.

### Adverse effects of flavonoids

Different types of potential adverse effects have been described for flavonoids. One potential adverse effect is based on the interaction of various flavonoids with trace elements. Interaction with iron, for example resulted in decreased iron absorption [59]. This may be especially relevant in relation to flavonoid supplement use in people susceptible for deficiency in iron or other divalent metals.

Another potential adverse effect concerns especially isoflavones. Isoflavonoids can exert estrogenic and goitrogenic activities [60]. The estrogenic properties were shown to lead to cancer in rodents [60]. Effects on thyroid hormone biosynthesis and goitric effects are seen in humans [59]. However, some human interventions showed no increased risk for hypothyroidism [59,60]. Possible adverse effects of isoflavones and inconsistencies in the health benefits lead still to some concern for the use of isoflavone supplements.

Finally, effects of flavonoids on phase 1 metabolism, such as cytochrome P450s and phase 2 metabolism enzymes, have been observed. This can cause complications due to interactions with drugs and may affect the detoxification capacity of the body [59].

### Genotoxic properties of flavonoids

Overall, genotoxic properties of flavonoids are of highest concern with regard to safety of flavonoids. Some flavonoids tested positive in various *in vitro* genotoxicity tests. For example genotoxic properties were shown in the Ames test, a bacterial reverse mutation test, also after metabolic activation of flavonoids [62-66]. Furthermore, in several cultured human and rodent cells, formation of micronuclei, DNA single strand breaks, and chromosomal aberrations were observed after flavonoid exposure [65,67-70]. It was also shown that quercetin can generate DNA adducts in different cell types *in vitro* [71]. This genotoxicity of flavonoids has been related to the quinone-quinone methide chemistry, implicitly connected to the structure of some flavonoids. Structural

requirements were shown to contribute to this effect; a free hydroxyl group at the 3-position, a double bound at the 2,3 position, and a keto-group at the 4-position [62]. The flavonoid quercetin meets all these requirements and has been shown to form quinone methide intermediates, which can bind to DNA. This is likely the mechanism of the observed genotoxicity of quercetin *in vitro* [72].

*In vivo*, genotoxicity of flavonoids was not shown in rodents after oral quercetin administration measured by, among others, micronuclei, chromosomal aberrations, and unscheduled DNA synthesis in bone marrow, liver, and gastric mucosa cells [73-77]. However, quercetin was shown to be carcinogenic in two studies in rats fed a quercetin diet of 0.1%-4%, where tumours in intestine, bladder and/or kidney were found [78,79]. The increased tumour incidence after flavonoid exposure observed in these studies have been criticized. Re-evaluations of the study of the National Toxicology Program which showed an increase of kidney tumours after quercetin exposure only in male F344/N rats [78] led to the conclusions that the observed tumours were specific for the rat strain used and probably related to α2u-globulin nephropathy, which occurs only in male rats [80,81]. The other *in vivo* quercetin study [79] was also criticized [82] because the observed carcinogenicity could possibly be ascribed to the use of a specific rat strain and a whole grain diet in the study, which is however not fully understood. Moreover, several other long-term studies with even higher doses of quercetin (0.2%-10%), did not show carcinogenicity in rodents [83-86]. An acute toxicity study in rodents with a proanthocyanidin extract also showed no toxicity [87]. Finally, for green tea catechins no genotoxic or carcinogenic indications were found in rodents [88].

However, taking the high intake of supplementary flavonoids into account, the potential genotoxic activity of flavonoids, which is shown *in vitro*, still leads to concern for a possible genotoxic effect of flavonoids *in vivo* especially for tissues of first contact exposed to relatively high levels upon oral intake such as intestine and liver.

## 1.5 MECHANISTIC APPROACH

Several indications for health effect of flavonoids are found, however the mechanisms behind these effects are not fully elucidated. In studying the possible health effects of flavonoids, several important issues have to be considered. Human studies showing indications of flavonoid-mediated health effects are mostly based on flavonoid-rich food products, which could also contain other substances influencing the effects, like theobromine, and caffeine [89]. Therefore, it is important that the pure individual compounds are taken along, since there is now only limited information on effects of the individual flavonoids. Furthermore, the absorption and metabolism of flavonoids should be taken into account when studying possible health effects of flavonoid in humans or animal models.

Several health effect of flavonoids are associated with effects related to lipid metabolism. These include reducing effects of flavonoids on circulating lipids, lipid

accumulation in the liver and obesity, which are known to be associated with a lower risk for CVD [55-57]. Liver is a key effector organ of lipid metabolism. Furthermore, reasonable concentrations of flavonoids are found in liver, and next to this the liver is involved in flavonoid metabolism. Therefore, studying the gene expression (transcriptomic) effects of flavonoids in liver will offer a good approach towards identification of mechanisms underlying health effects of flavonoids. Transcriptomic tools, which are nowadays widely used, allow for an unbiased genome-wide approach to identify effects in specific organs.

Since analysis of liver tissue is not easily arranged in human subjects, the mouse is a widely used alternative model. The C57BL/6J mouse strain is a respected model for diet-induced obesity, insulin resistance, cardiovascular diseases, and hepatic lipid accumulation [90-92], in which effects on lipid metabolism can be adequately studied [93-95]. When using such an animal model it is important to consider that kinetics of flavonoids can be different between species. Various di- and tri-conjugates are found for quercetin in the circulation of rodents as well as in humans [96], suggesting a comparable pattern of conjugates formed after flavonoid metabolism in mice and humans. Mice are small and relatively cheap, there are many research tools available and suitable for use in mouse models (like gene expression microarrays and antibodies), highly controlled studies are possible, and the mouse genome has an overlap of over 90% with the human genome [97], making it a widely accepted and human relevant model for this type of research.

A combination of transcriptomics and physiological data obtained in mice exposed to pure flavonoids will substantially contribute to the unravelling of the possible underlying mechanisms of the metabolic health effects of flavonoids. Furthermore, the identified targets could be used for translation into biomarkers for effects of flavonoids in animals and in humans.

## 1.6 AIMS AND OUTLINE OF THIS THESIS

### Aims

The first aim of this thesis was to identify mechanisms underlying potential beneficial health effects by flavonoids in mice, with a specific focus on hepatic lipid metabolism and circulating lipids. These lipid metabolism related markers are associated with a reduction of CVD risk by flavonoids. Secondly, we aimed to study the potential *in vivo* genotoxic effects of quercetin in liver and small intestine, since these represent the tissues of first contact exposed to relatively high levels upon oral intake of flavonoids.

### Outline of this thesis

Serum lipid parameters are important CVD related risk markers that will be assessed in this thesis. Circulating lipids are in general determined with fast and simple enzyme based assays. We have first studied whether these assays can also be used in flavonoid research in a sensitive and reliable manner. This is described in **chapter 2**.

To unravel the underlying mechanisms of potential beneficial effects of flavonoids to CVD related risk factors, mechanistic and physiological effects on hepatic lipid metabolism and circulating lipids in mice were first studied for the flavonoid quercetin, and results obtained are described in **chapter 3**.

Subsequently, the effects of quercetin on hepatic lipid metabolism were studied in mice by using a high-fat background diet. This diet was used, because it induces body weight gain and hepatic lipid accumulation, enabling potentially more sensitive detection of effects of quercetin on hepatic lipid metabolism. In **chapter 4** the physiological and mechanistic effects of quercetin on hepatic lipid metabolism are described, with a focus on the influence of the background diet on the observed effects.

In **chapter 5** we investigated if flavonoids from other subclasses could exert the same effects on hepatic lipid metabolism as quercetin showed. Furthermore, we have extended the investigations by studying whole body energy balance to obtain a complete overview of the metabolic effects of the different flavonoids. The effects of flavonoids representing five flavonoid subclasses were compared in a fully controlled intervention study in mice.

Apart from beneficial health effects of flavonoids, safety aspects have to be considered, because of potential genotoxic effects found for quercetin. In **chapter 6** possible genotoxic effects of quercetin were studied by transcriptome analyses in liver and small intestine of quercetin exposed mice.

Finally, in **chapter 7**, the general discussion, the outcomes of this thesis are discussed and recommendations for future research are given.

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# CHAPTER 2

## INTERFERENCE OF FLAVONOIDS WITH ENZYMATIC ASSAYS FOR THE DETERMINATION OF FREE FATTY ACIDS AND TRIGLYCERIDES LEVELS

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## ABSTRACT

Flavonoids are bioactive food compounds with potential lipid lowering effects. Commercially available enzymatic assays are widely used to determine free fatty acids (FFA) and triglycerides (TG) levels, both *in vivo* in plasma or serum and *in vitro* in cell culture medium or cell lysate. However, we have observed that various flavonoids interfere with peroxidases used in these enzymatic assays, resulting in incorrect lower FFA and TG levels than actually present. Furthermore, addition of isorhamnetin or the major metabolite of the flavonoid quercetin in human and rat plasma, quercetin-3-O-glucuronide, to murine serum, also resulted in a significant reduction of the detected TG levels, while a trend was seen for FFA levels. It is concluded that when applying these assays vigilance is needed and alternative analytical methods, directly assessing FFA or TG levels, should be used for studying the biological effects of flavonoids on FFA and TG levels.

# 2.1 INTRODUCTION

Free fatty acids (FFA) and triglycerides (TG) plasma levels are important factors which can give an indication of the metabolic health status and are therefore generally used as markers of lipid metabolism. Commercially available enzymatic kits are widely used to determine FFA and TG levels. There are many advantages for the use of these assays, including small sample volume, no need for extraction, and high sensitivity [1]. Flavonoids are natural food compounds which are suggested to have beneficial lowering effects on lipid status [2]. Several studies investigated the effects of flavonoids on FFA and TG levels after dietary intervention or cell culture exposure and used these enzymatic kits to determine the lipid levels in serum cq. cell culture medium [3-9]. FFA and TG assays are based on enzymatic reactions. Independent of the supplier of the kits, peroxidase catalyzes the final quantitative conversion to the colored compound, which is subsequently measured by absorbance in both of these assays. Furthermore, also other frequently used enzymatic assays for the determination of for example cholesterol and glucose are based on peroxidase reactions.

In the FFA assay, FFA are transformed into Acyl-coA, which is oxidized and generates hydrogen peroxide. In a quantitative oxidation reaction, hydrogen peroxide together with 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline and 4-aminoantipyrine (4-AA) are converted into a blue purple product by peroxidase activity. Absorbance is used to quantify the FFA levels. In the TG assay, TG are hydrolyzed with lipases generating glycerol, which is converted into glycerol-3-phosphate and oxidized generating hydrogen peroxide. Hydrogen peroxide and 4-AA are converted into quinoneimine catalyzed by peroxidase. Absorbance of quinoneimine is used to quantify the TG levels. It is clear that peroxidase has an important role in both assays, because it catalyzes the final quantitative reaction. Importantly, flavonoids are known to be inhibitors of peroxidase activity [10-12]. Nevertheless, these assays are widely used in (nutritional) studies focusing on the effects of flavonoids. The inhibition of peroxidase by flavonoids may result in reduced peroxidase mediated color formation, which will result in apparently lower FFA and TG levels than actually present. Therefore, the aim of the present study was to investigate whether at physiologically relevant concentrations flavonoids do indeed interfere with these widely applied peroxidase-based assays for the quantification of FFA and TG levels.

# 2.2 MATERIALS AND METHODS

## Sample preparation

Cell culture medium samples were prepared in 1x DMEM high glucose without phenol red (Invitrogen, Breda, The Netherlands), with 10% fetal bovine serum charcoal/dextran treated (Thermo Scientific, Logan, Utah, USA), 2% penicillin-streptomycin (Invitrogen), and 1% non-essential amino acids (Invitrogen). Medium was supplemented with 80

μM linoleic acid-oleic acid-albumin (Sigma, Zwijndrecht, The Netherlands) and 20 mg/dL triglycerides standard. Quercetin, (+)-catechin (Sigma), naringenin, luteolin, kaempferol, and genistein (Extrasynthese, Lyon, France) were individually spiked to cell culture medium samples in different concentrations prior to measurements.

Serum samples obtained from Harlan Laboratories (Horst, The Netherlands) were pooled serum of C57BL/6JOlaHsd male mice. Quercetin, quercetin-3-O-glucuronide (Phytolab, Vestenbergsgreuth, Germany) and isorhamnetin (Extrasynthese) were spiked to the murine serum.

All flavonoids were dissolved in DMSO, except quercetin-3-O-glucuronide which was dissolved in ethanol, for controls only the solvent was added at equal volumes.

### Free fatty acids assay

FFA kit reagents (Wako NEFA-HR(2) kit, Sopachem BV, Ochten, The Netherlands) were used according to the manufacturer's protocol with some small adaptations. The protocol mentions several usually present compounds in serum or plasma that may interfere with the assay; however, information on flavonoids or other bioactive food ingredients is not provided. Briefly, 5 or 15 μl of sample plus 100 μl of the first reagent were incubated for 10 minutes at 37 °C. Then 50 μl of the second reagent was added and incubated for another 10 minutes at 37 °C. Oleic acid (Wako NEFA calibrator solution) was used for calibration curves. The absorbance was read at 550 nm using a Synergy HT plate reader (Biotek Instruments, Winooski VT, USA).

### Triglycerides assay

TG kit reagent (Triglycerides liquicolor kit, Human, Wiesbaden, Germany) was used according to the manufacturers protocol. The protocol mentions several usually present compounds in serum or plasma that may interfere with the assay; however, information on flavonoids or other bioactive food ingredients is not provided. Briefly, 5 μl of sample plus 100 μl reagent were incubated for 10 minutes at room temperature. Triglycerides standard from the kit was used for calibration curves. The absorbance was read at 500 nm.

### Statistical analysis

Statistical analysis was performed by One-Way ANOVA followed by Dunnett's post-hoc test, $p\text{-value}<0.05$ was considered significant.

## 2.3 RESULTS AND DISCUSSION

Individual spiking of quercetin, kaempferol and (+)-catechin to the cell culture medium prior to measurements resulted in a significant reduction in the FFA levels detected (Figure 2.1). The same flavonoids as well as luteolin caused also significant reductions in the TG levels detected. The apparent reduction in the levels of FFA and TG in these

assays was already present at concentrations (10-100 μM) which are generally used in *in vitro* studies, indicating that these peroxidase-based assays should be used with extreme caution under these circumstances. Different flavonoids are known to be able to inhibit peroxidase activity [10-12], and thus cause this unintentional reduction in the levels of FFA and TG detected by these assays.

Inhibition of myeloperoxidase activity is dependent on the presence of hydroxyl group at the 3, 5 and 4′- positions in the structure of flavonoids and the $C_2$-$C_3$ double bond [12]. As a consequence, quercetin, kaempferol and luteolin showed inhibition of myeloperoxidase in the following order: quercetin > kaempferol > luteolin [12]. This corresponds with our results, which also showed an identical interference capacity in the FFA and TG assays. However, genistein can also inhibit the activity of peroxidase [13], while we found no interference of genistein in both assays (Figure 2.1).

*In vivo*, mainly metabolites of flavonoids are present in the circulation due to extensive intestinal and hepatic metabolism. Therefore, we analyzed the effect of individual spiking of quercetin, isorhamnetin and the metabolite quercetin-3-O-glucuronide in murine serum. This also resulted in a significant reduction of the FFA and TG levels detected (Figure 2.2). Quercetin-3-O-glucuronide is the major metabolite found in human and rat plasma [12,14,15]. It was previously shown that plasma concentrations of quercetin metabolites could inhibit myeloperoxidase [12]. This result corresponds with our finding that besides quercetin also its metabolite quercetin-3-O-glucuronide interferes with the TG and FFA assays. Addition of quercetin-3-

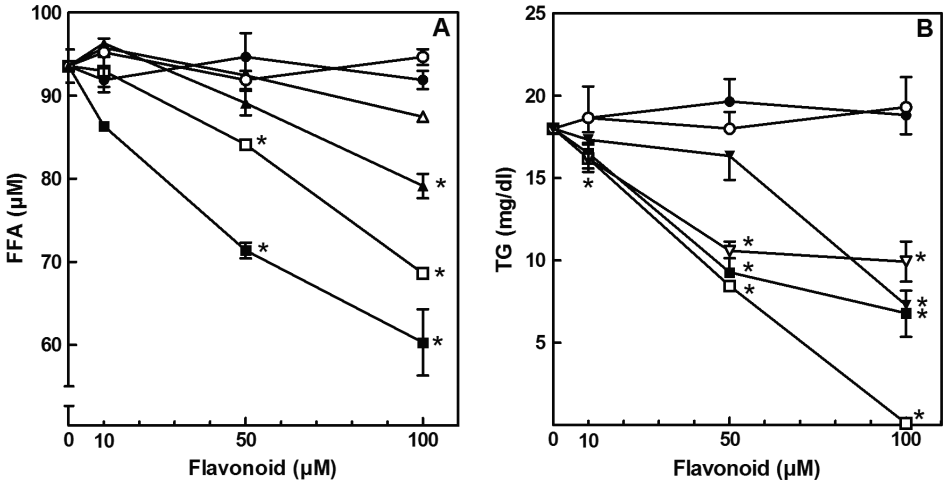


**Figure 2.1 Interference of different flavonoids on the FFA (A) and TG (B) levels in cell culture medium measured by enzymatic assays.** (+)-Catechin (■), quercetin (□), kaempferol (▲), luteolin (Δ), genistein (●), and naringenin (○). Results are mean ± SEM, n = 3 for FFA and n = 4 for TG. * indicates a significant difference ($p<0.05$) from the control (0 μM); B: quercetin has already a significant effect at 10 μM.

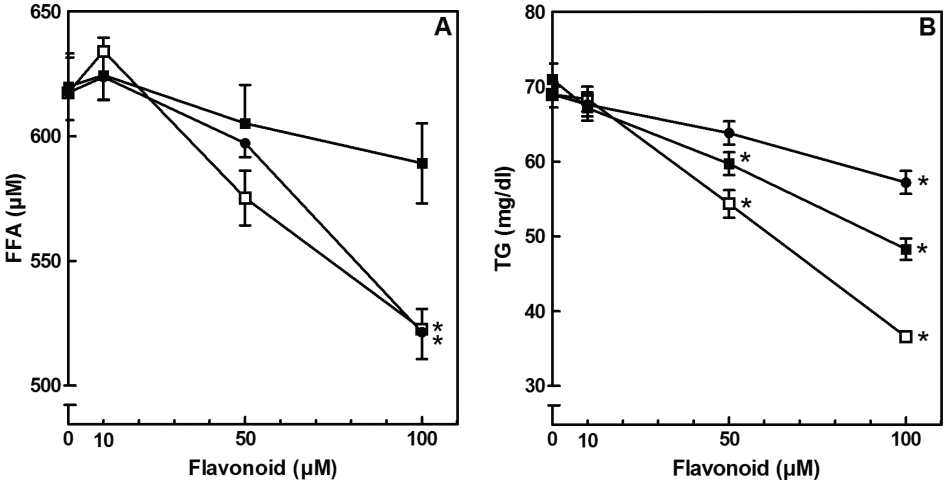


**Figure 2.2 Interference of quercetin (□) and its major metabolite quercetin-3-O-glucuronide (■), and isorhamnetin (●) in murine serum on FFA (A) and TG (B) levels measured by enzymatic assays.** Results are mean ± SEM, n = 8. * indicates a significant difference ($p<0.05$) from the control (0 µM).

O-glucuronide resulted in a significant reduction in the detected TG levels, while at similar concentrations it did not have such a strong effect on the FFA assay. Furthermore, isorhamnetin seems to have a stronger effect on the FFA assay than on the TG assay at similar concentrations. Since the concentrations of quercetin-3-O-glucuronide resulting in these reductions of FFA and TG levels detected are in the range that can be observed in *in vivo* serum samples upon oral intake of quercetin [16], these observations show that when these enzymatic assays are used for *in vivo* studies the results can also be unintentionally influenced.

There are several recent rodent nutritional studies with quercetin supplementation using these enzymatic assays to measure FFA and/or TG levels in serum or plasma [3-9]. The reported serum quercetin levels in these studies were between 10 µM and 45 µM. All studies reported decreased serum levels of FFA and/or TG after dietary quercetin supplementation, which could at least in part be due to the interference of quercetin with the FFA and TG assays as shown in the present study. The peroxidase-based assays for FFA and TG have also been used in human flavonoid intervention studies, although most of them found no lipid lowering effects, which could be partly due to lower plasma flavonoid levels [2,17]. The present study showed a dose-dependent interference of various flavonoids and of an important *in vivo* quercetin metabolite with peroxidase-based assays for the detection of FFA and TG levels. Therefore, results of studies reporting a reduction in FFA and TG levels upon *in vivo* flavonoid-supplementation should be interpreted with caution when these assays are used. Nevertheless, this does not exclude the biological effects on FFA and TG levels by flavonoids, as effects are also perceived by the use of other analytical techniques [18,19].

In conclusion, peroxidase-based enzymatic FFA and TG assays should be applied with caution in studies focusing on the effects of flavonoids. Interference of flavonoids and their metabolites with peroxidases used in these assays results in apparently lower levels of FFA and TG detected than actually present. Most likely this will also hold for other enzymatic kits using peroxidase reactions, such as total cholesterol and glucose assays. Vigilance is needed and alternative methods including LC-MS, gas chromatography, or $^{1}$H-NMR should be used in studies focusing on the effects of flavonoids on lipid status.

## 2.4 REFERENCES


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# CHAPTER 3

## QUERCETIN INDUCES HEPATIC LIPID OMEGA-OXIDATION AND LOWERS SERUM LIPID LEVELS IN MICE

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## ABSTRACT

Elevated circulating lipid levels are known risk factors for cardiovascular diseases (CVD). In order to examine the effects of quercetin on lipid metabolism, mice received a mild-high-fat diet without (control) or with supplementation of 0.33% (*w/w)* quercetin for 12 weeks. Gas chromatography and $^{1}$H nuclear magnetic resonance were used to quantitatively measure serum lipid profiles. Whole genome microarray analysis of liver tissue was used to identify possible mechanisms underlying altered circulating lipid levels.

Body weight, energy intake and hepatic lipid accumulation did not differ significantly between the quercetin and the control group. In serum of quercetin-fed mice, triglycerides (TG) were decreased with 14% ($p<0.001$) and total poly unsaturated fatty acids (PUFA) were increased with 13% ($p<0.01$). Palmitic acid, oleic acid, and linoleic acid were all decreased by 9-15% ($p<0.05$) in quercetin-fed mice. Both palmitic acid and oleic acid can be oxidized by omega (ω)-oxidation. Gene expression profiling showed that quercetin increased hepatic lipid metabolism, especially ω-oxidation. At the gene level, this was reflected by the up-regulation of cytochrome P450 (Cyp) 4a10, Cyp4a14, Cyp4a31 and Acyl-CoA thioesterase 3 (Acot3). Two relevant regulators, cytochrome P450 oxidoreductase (Por, rate limiting for cytochrome P450s) and the transcription factor constitutive androstane receptor (Car; official symbol Nr1i3) were also up-regulated in the quercetin-fed mice.

We conclude that quercetin intake increased hepatic lipid ω-oxidation and lowered corresponding circulating lipid levels, which may contribute to potential beneficial effects on CVD.

## 3.1 INTRODUCTION

Cardiovascular diseases (CVD) are globally the most important cause of mortality. High consumption of fruits and vegetables are thought to be protective against CVD [1]. These protective effects have been suggested to be mediated by the flavonoid content of fruits and vegetables [2]. Various classes of flavonoids are common in plant foods, one being the flavonols. Quercetin is the major dietary flavonol in the Western diet, which is present in, for example, apples, tea, red wine and onions. Epidemiological studies have shown that the intake of this dietary flavonoid is associated with a reduction of CVD risk [3-5].

Elevated circulating levels of free fatty acids (FFA) and triglycerides (TG) are known risk factors for CVD [6-9]. In particular, increased levels of FFA and TG are associated with atherosclerosis, ischemic damage, pro arrhythmia, myocardial infarction, and heart failure. Accumulation of toxic lipid intermediates, suppression of glucose usage, or mitochondrial dysfunction potentially play a role in these effects [9]. Several studies showed that supplementation of quercetin to the diet decreased serum FFA and/or TG levels in rodents [10-12]. The cardio protective properties of quercetin may therefore be explained by the lipid lowering effect of quercetin. However, in these studies the FFA and TG levels were measured with enzyme-based assays. Recently, we have shown that flavonoids interfere with these enzymatic FFA and TG assays, which will lead to incorrect, apparently lower FFA and TG levels [13]. Therefore, it can be questioned whether quercetin has a true biological effect on lipid metabolism. Since various fatty acids are differently associated with CVD risk, it is also important to examine whether quercetin changes specific lipids [14]. Furthermore, based on gene expression analysis some genes have been put forward to explain the effects of quercetin on lipid metabolism [11,12], but these results are not conclusive. The aim of the present study was to re-examine the effects of quercetin on lipid metabolism, with state-of-the-art analytical techniques, to exclude any interference of quercetin in the measeruments. Gas chromatography (GC) and a novel technique, $^{1}$H-nuclear magnetic resonance ($^{1}$H-NMR) lipid profiling of mouse serum (based on [15,16]), were used to profile and quantify different serum lipids. In addition, whole genome microarray gene expression analysis of liver tissues was applied to unravel the possible underlying mechanisms. For this gene expression analysis the liver was chosen as target organ, since it is one of the major effector organs of lipid metabolism. This principal combination of profiling of serum lipids and gene expression were used to investigate the mechanisms of action of quercetin on lipid metabolism.

The results show that a quercetin supplemented mild-high-fat diet in mice increased hepatic lipid metabolism, especially omega (ω)-oxidation and reduced corresponding circulating lipid levels. These results contribute to the understanding of the protective properties of quercetin on CVD.

## 3.2 MATERIALS AND METHODS

### Animals and treatments

Twenty-four male C57BL/6JOlaHsd mice (Harlan Laboratories, Horst, The Netherlands) were individually housed and maintained under environmentally controlled conditions (temperature 21°C, 12 h/12 h light-dark cycle, 45% humidity). The mice had *ad-libitum* access to food and water. The food was a pelletized diet (Research Diets Services B.V., Wijk bij Duurstede, the Netherlands) with a mild-high-fat content of 30 energy % (en%). The fat content (en% and fat composition) corresponds to the average human intake in the Netherlands (Dutch Food Consumption Survey, 1998). The mice entered the experiment at 10 weeks of age. After two weeks of adaptation, the quercetin group (n=12) received the mild-high-fat diet supplemented with 0.33% (w/w) quercetin (Sigma, Zwijndrecht, the Netherlands) for twelve weeks. The control group (n=12) was given the mild-high-fat diet without quercetin. The body weight and food intake of individual mice were monitored on a weekly basis. After 12 weeks of intervention all mice were fasted for two hours before anesthetisation by inhalation of 5% isoflurane. Blood was sampled via orbital extraction in collect serum tubes (Greiner Bio-one, Longwood, USA), which were centrifuged for 10 min at 3000 g 4 °C to obtain serum, which was stored at -80°C. After blood collection, the mice were killed using cervical dislocation and the liver was dissected, weighted and snap frozen in liquid nitrogen and stored at -80°C. The experiment was performed according to the Dutch Animal Experimentation Act (1996) and the experimental protocol was approved by the Animal Welfare Committee of Wageningen University, Wageningen, The Netherlands (DEC 2007080).

### HPLC analysis of quercetin serum levels

HPLC with a coulometric array detector was used to measure the amount of quercetin in serum. For this 35 µl of serum was hydrolyzed by incubation with 15 µl of 12.5 mg/ml β-glucuronidase/sulfatase in 0.5 M sodium acetate (pH =5) with 5g/l ascorbic acid for two hours at 37°C to obtain deconjugated quercetin, isorhamnetin and tamarixetin. Subsequently, all samples were deproteinized by mixing with 100 µL acetonitrile and 50 µL 20% $H_3PO_4$, with 3g/L ascorbic acid and centrifugation for 10 min at 13500 rpm at 5°C. Twenty µl of the supernatant was analyzed on a HPLC system consisting of two pumps (model L-2100; Hitachi, Tokyo, Japan), an autosampler (Model L-2200, Merck Hitachi), a CoulArray Module (Model 5600, ESA, Chelmsford, MA, USA) with electrochemical channels using carbon electrodes arranged in line and set to increasing specified potentials (1=20 mV; 2=100 mV; 3=250 mV; 4=500 mV) and a thermostatic column/cell chamber set at 30°C. The chromatography was performed on a Platinum C18 column (EPS; 150x4.6mm, 3µ, Grace Davison Sciences, Deerfield, IL, USA) equipped with a MPLC Newguard precolumn (Brownlee RP18 7µm 15x3.2mm, Perkin Elmer, Shelton, CT, USA), using a gradient elution with two mobile phases. Mobile phase A consisted of 15% acetonitrile in 25 mM $H_3PO_4$ buffer (pH 2.4). Mobile

phase B consisted of 50% acetonitrile in 25 mM $H_3PO_4$ buffer (pH 2.4). The gradient, at a flow rate of 1.0 ml/min, was as follows: 0–20 min, linear gradient from 0% to 100% mobile phase B; 20–22 min, isocratic at 100% B; 22– 30 min, linear return from 100 to 0% B; the total runtime was 30.0 min. Quercetin, isorhamnetin and tamarixetin were quantified using calibration curves made with commercially available standards.



## Hepatic lipid staining with Oil Red O

Frozen liver sections (7 µm) were fixed with 3.7% buffered formalin. Neutral lipids were stained with Oil red O (Sigma). The stained areas were quantified based on a described method [17] using Photoshop software (version 12.0.4, Adobe). Briefly, contrast was enhanced with automatic contrast tool, red pixels were selected with the colour range selecting tool, and total selected area was measured in $\mu m^2$. Ten to 16 pictures per animal were quantified (n=6-8). Controls of hepatic lipid accumulation were liver of C57BL/6JOlaHsd mice fed a normal-fat diet (10 en% of fat) or a high-fat diet (40 en% of fat) for twelve weeks.

## Serum lipid analysis with enzymatic assays

FFA assay (Wako NEFA-HR(2) kit, Sopachem BV, Ochten, The Netherlands) and TG assay (TG liquicolor kit, Human, Wiesbaden, Germany) were performed as described previously [13].

## Serum fatty acid analysis with GC

Total serum fatty acids were extracted from 50 µl serum as described [18], using dichloromethane instead of chloroform [19]. Ten µg of nonadecanoic acid methyl ester (NuCheck Prep, USA) was added to each sample before extraction, as an internal standard. Samples were transmethylated to fatty-esther methyl esthers (FAME) by incubation in 1M sodium methoxide in dry methanol for 20 min at 80°C. The reaction mixture was then cooled, acidified with 98% sulphuric acid and incubated for 1 hour at room temperature to methylate free acids. Lipid methyl esters were extracted with hexane, and the hexane extracts were subsequently dried under a nitrogen flow. Next, the residue was dissolved in 100 µl of n-heptane and stored at -20°C under nitrogen until analysed. All reactions were performed under nitrogen atmosphere. GC was performed with a Trace-GC gas chromatograph combined with AS 2000 autosampler (ThermoFinnigan, USA), equipped with a capillary split/splitless injector and a flame ionization detector. Analyses of FAME were performed on a fused-silica capillary column coated with chemically bond stationary phase CP-Sil 88 CB (100 m, 0.32 mm I.D.). The oven temperature was programmed as follows: from 80°C to 260°C at 2°C/min, then to 280°C at 10°C/min, where it was maintained for 45 min. The injector and detector temperatures were set at 250°C and 270°C, respectively. Hydrogen carrier gas was maintained at a head pressure of 70 kPa and total flow of 44 ml/min,

with a split ratio of 1:35. Integration software Clarity version 2.4.1.57 (Data Apex Ltd. Prague, Czech Rep.) was used for data acquisition and handling.

## Serum lipid analysis with $^{1}$H-NMR



Total serum lipids were extracted from 20 µl of blood serum as described [18] based on [15,16] with some adaptations to optimise the protocol. Briefly, 20 µl of 150 mM sodium phosphate buffer with 0.04% azide (pH =3) was added per serum sample. Then 900 µl chloroform:methanol (2:1) v/v and 900 µl 0.15 M NaCl (pH =3) were added to the sample. Samples were shaken for 5 minutes on a horizontal shaker and centrifuged for 10 minutes at 4500 g to separate the organic and water phase. The lower organic phase was recovered and the aqueous layer was extracted again with 500 µl chloroform. The collected organic layers were combined and evaporated to dryness using argon. The samples were vacuum and freeze dried and dissolved in chloroform containing 0.03% tetramethylsilane. $^{1}$H NMR was measured on a Bruker AVANCE spectrometer operating at 600 MHz. For each spectrum 256 (Free induction decays (FID)) transients were collected with a flip angle of 90°, with an acquisition time of 1.82 s, a relaxation delay of 4 s, a spectral width of 30 ppm and a standard noesy 1D pulse sequence, at 25°C, and four dummy scans were used. The FID with 64K data points were once zero-filled and multiplied by an exponential window function with a 0.2 Hz Line-broadening before a subsequent Fourier transformation. To all spectra a baseline correction was applied and the spectra were aligned on the chloroform peak at 7.24 ppm.

The nature of the various resonances was based on the $^{1}$H NMR spectra as reported [15, 16]. The regions selected to quantify the different lipid fractions were as follows; TG (4.300-4.250 ppm), total FFA (1.380-1.198 ppm), mono unsaturated fatty acids (MUFA) and PUFA (-CH=CH-)(5.41 to 5.27 ppm), other PUFA than 18:2 FA (2.862-2.768 ppm), 18:2 FA including linoleic acid (18:2(n-6)) (2.767-2.721 ppm), 18:1 and 16:1 FA including oleic acid (18:1(n-9)) (2.050-2.011 ppm), 22:6 FA including docosahexaenoic acid (22:6(n-3)) (2.379-2.342 ppm), ω-3 FA (0.957-0.947 ppm), phophoglycerides (PGLY) (5.258-5.190 ppm), phosphatidylcholine (PC) (3.787-3.738 ppm), esters of cholesterol (EC) (4.651-4.539 ppm) and total cholesterol (TC) (0.902-0.895 ppm).

## Fatty acid composition of diets

Fatty acids from the diets were extracted with accelerated solvent extraction according to the manufacturer's protocol (Thermo Scientific) and dissolved in toluene. Subsequently, toluene was evaporated under nitrogen at 40°C and fatty acids were dissolved in iso-octane (5 ml) and 200 µl 2 M KOH in methanol was added and the mixture was shaken for one minute. For neutralization, $NaHSO_4$ was added and samples were shaken for 1 minute. The iso-octane fraction (1 µl) was injected in the gas chromatograph equipped with a capillary split injector (split ratio 1:40) and flame ionization detector. Analyses were performed on a CP select column for FAME (50 m x 0.25 mm ID). The oven temperature was programmed from 100°C to 230°C at 6°C/min.

## RNA isolation

For RNA isolation, liver was homogenized in liquid nitrogen, total RNA was isolated using TRIzol reagent (Invitrogen, Breda, The Netherlands) followed by purification with RNeasy columns (Qiagen, Venlo, The Netherlands). RNA concentration and purity were measured using a Nanodrop spectrophotometer (IsoGen Life Science, Maarsen, The Netherlands); all RNA samples were of high purity. RNA quality was additionally checked on the Experion automated electrophoresis system (Bio-Rad, Veenendaal, The Netherlands).

## Microarray analysis

For global transcriptome analysis of liver samples, 4 x 44K Agilent whole-mouse genome microarrays (G4122F, Agilent Technologies Inc., Santa Clara, CA) were used. Preparation of the samples and the microarray hybridizations were carried out according to the manufacturer's protocol with a few modifications as described previously [20]. All materials and reagents were from Agilent Technologies, Palo Alto, USA unless stated otherwise. Briefly, cDNA was synthesized for each animal from 1 µg RNA using the Agilent Low-RNA Input Fluorescent Linear Amplification Kit without addition of spikes. Thereafter, samples were split into two equal amounts, to synthesize Cyanine 3-CTP (Cy3) and Cyanine 5-CTP (Cy5) labelled cRNA, using half the amounts per dye as indicated by the manufacturer. Labelled cRNA was purified using RNeasy columns (Qiagen). All samples had a cRNA yield higher than 825 ng and a specific activity of at least 8.0 pmol Cy-dye per µg cRNA. Cy3-labeled cRNA samples were pooled on an equimolar basis and used as a common reference pool. Individual 825-ng Cy5-labeled cRNA and 825-ng pooled Cy3-labeled cRNA were fragmented in 1x fragmentation and 1x blocking agent at 60°C for 30 min and thereafter mixed with GEx hybridization buffer (HI-RPM) and hybridized in a 1:1 ratio at 65°C for 17h in the Agilent Microarray Hybridization Chamber rotating at 10 rpm. After hybridization, slides were washed according to the manufacturers' wash protocol. Arrays were scanned with an Agilent scanner with 10 and 100% laser-power intensities.

## Normalisation and microarray data analysis

Signal intensities for each spot were quantified using Feature Extraction version 9.1 (Agilent Technologies). Median density values and background values of each spot were extracted for both the experimental samples (Cy5) and the reference samples (Cy3). Quality control for every microarray was performed visually by using 'Quality control graphs' from Feature extraction and M-A plots and box plots, which were made using limmaGUI in R (Bioconductor, Wettenhal, 2004). Data were imported into GeneMaths XT 2.0 (Applied Maths, Sint-Martens-Latem, Belgium). Spots with an average Cy5 and Cy3 signal twice above background were considered expressed and log transformed. The Cy5 signal was normalized against the Cy3 intensity as described before [21]. Pathway analysis was performed using MetaCore (GeneGo, St. Joseph, Michigan, USA) and Ingenuity Systems (Ingenuity, Redwood City, California,

USA). Fold change was expressed as the ratio of the quercetin group versus the control group. Microarray data has been deposited in NCBI Gene Expression Omnibus (GEO) under accession number GSE39140.

### Real time quantitative polymerase chain reaction (RT-qPCR)



RT-qPCR was performed using the RNA of the liver samples to validate the microarray data. One microgram of RNA of all individual samples was used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad). RT-qPCR reactions were performed with iQ SYBR Green Supermix (Bio-Rad) using the MyIQ single-colour real-time PCR detection system (Bio-Rad). Individual samples were measured in duplicate. Data were normalized against reference genes beta-2 microglobulin (B2m) and hypoxanthine phophoribosyltransferase 1 (Hprt1) which were chosen based on stable gene expression levels (geNorm, Ghent University Hospital, Ghent, Belgium) and the microarray data. Primers were designed using the NCBI Primer-Blast (NCBI Web site). Sequences of the used primes were as follows: cytochrome P450 4a14 (Cyp4a14); 5′-TTCTTTCGCCTGCGGAATGC-3′ and 5′-CACTCCATCTGTGTGCTC GTGA-3′, cytochrome P450 4a10 (Cyp4a10); 5′-TCTACCCACCTGTCCCAGGC-3′ and 5′-ACACCTCTGGATTTGGCCACA-3′, acyl-CoA thioesterase 3 (Acot3); 5′-GC TGTGACCTACCTGCTCAGTCA-3′and5′-ATATAGAGCCATTGATGATGACAGCGG-3′, cytochrome P450 oxidoreductase (Por); 5′-CGAGGGCAAGGAGCTGTACC-3′ and 5′-CACAGGTGGTCGATGGGTGG-3′, constitutive androstane receptor (Car; official gene symbol Nr1i3); 5′-CCGTGTTGCCTCTGCTCACA-3′ and 5′-GGTTAGGGACC GGAAGAGCG -3′, beta-2-microglobulin (B2m); 5′-CCCCACTGAGACTGATACATAC GC-3′ and 5′-AGAAACTGGATTTGTAATTAAGCAGGTTC-3′, hypoxanthine-guanine phosphoribosyltransferase (Hprt1); 5′-TGACACTGGTAAAACAATGCAAACTTTG-3′ and 5′-GAGGTCCTTTTCACCAGCAAGCT -3′.

### Statistical analysis

For microarray analysis, Student's *t* tests were used with false discovery rate (FDR) adjustment for multiple testing correction according to Benjamini-Hochberg [22]. GraphPad Prism version 5.03 (Graphpad Software, San Diego, USA) was used for other statistical analysis, with Student's *t* test being used to compare the two groups. Two-way ANOVA was used for analysis of the lipid profiles in serum and diets, followed by a Bonferroni post hoc test. P-values smaller than 0.05 were considered statistically significant.

## 3.3 RESULTS

### Body weight, energy intake and quercetin uptake

Body weight (BW) and energy intake of the adult male mice, which were fed a mild-high-fat diet with or without quercetin supplementation, were not significantly different

between the quercetin and the control group during all 12 weeks. Final body weight was 27.9 ± 1.9 and 28.5 ± 1.6 (mean ± SD) gram, and cumulative total energy intake was 4580 ± 172 and 4636 ± 207 kJ for the quercetin and control group, respectively.

The calculated quercetin intake for the quercetin-fed mice was ~ 400 mg/kg BW/day. The sum of quercetin and isorhamnetin after deconjugation in serum was 13.5 ± 3.1 µM expressed as aglycone (quercetin was 6.7 ± 0.9 µM, isorhamnetin was 6.8 ± 2.6 µM, and no tamarixetin was found). No quercetin was found in serum of the control animals.

Relative liver weight was significantly lower in the quercetin-fed mice (3.80% ± 0.20; p=0.007) compared to the control mice (4.08%± 0.26), while no significant differences were found for other organs, including white adipose tissue, brown adipose tissue, lung, heart, muscles (data not shown). Hepatic lipid staining showed no significant differences between the quercetin and control group (Figure 3.1a and b); the Oil red O recorded areas were 480 ± 493 µm$^2$ and 321 ± 440 µm$^2$, respectively. The hepatic lipid levels were much lower than a positive control of hepatic lipid accumulation (13,151 ± 4,410 µm$^2$) and in the same range of hepatic lipid levels found in liver of mice fed a normal-fat diet (516 ± 271 µm$^2$) (Figure 3.1c and d).

## Serum lipids as determined by enzymatic assays

Quantification of serum FFA and TG levels was performed using the enzymatic FFA and TG assays, which showed a significant decrease of 13% FFA ($p<0.05$) and 27% TG ($p<0.05$) due to the quercetin diet. However, since quercetin has been shown to interfere with these enzymatic assays resulting in incorrect, apparently lower FFA and TG levels [13], two additional analytical techniques were applied to assess serum lipid

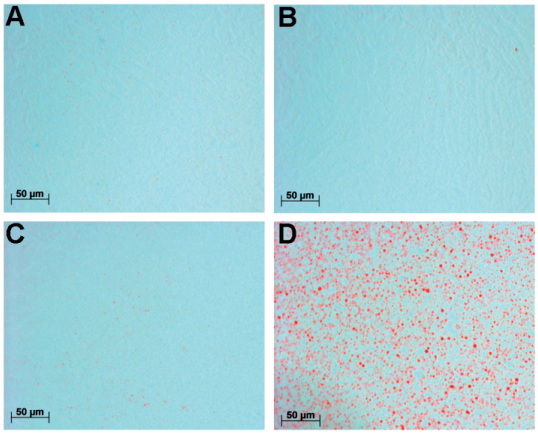


**Figure 3.1 Representative pictures of hepatic lipid staining with Oil red O.** There were no significant differences in lipid accumulation between the control (A) and the quercetin (B) group. The lipid levels were comparable to mice fed a normal-fat diet (C) and much lower than the positive control of hepatic lipid accumulation from mice fed a high-fat diet (D).

profiles, and to check if the decreased FFA and TG levels detected by the enzymatic assays represent real biological effects.

## Serum fatty acid profile as determined by GC

GC fatty acid profiles reveales fatty acids originating from TG, FFA, cholesteryl esters and phospholipids. The serum fatty acid profile showed a total decrease of 7% ($p<0.001$) in the quercetin-fed mice. The levels of palmitic acid (16:0), oleic acid (18:1(n-9)) and linoleic acid (18:2(n-6)) were 9-15% lower ($p<0.001$) in the quercetin group (Figure 3.2). These are the main fatty acids in the quercetin diet and the control diet, which were similar in terms of fatty acid composition (Table 3.1). All other fatty acids that were present in the serum showed a tendency of decreased levels due to the quercetin treatment, except for some poly unsaturated fatty acids (PUFA), such as arachidonic acid (20:4(n-6)) and docosahexaenoic acid (22:6(n-3)) which were slightly, but non significantly, increased in the serum of quercetin-supplemented mice.

## Serum lipid profile as determined by $^1$H NMR

$^1$H NMR measurement reveals total TG, FFA, cholesterol and phospholipids that are present in serum, separately. Figure 3.3 presents the $^1$H NMR difference spectrum, that is composed of the $^1$H NMR spectra of serum samples from mice exposed to quercetin (n=12) minus the $^1$H NMR spectra of the sera from control mice (n=12). The different

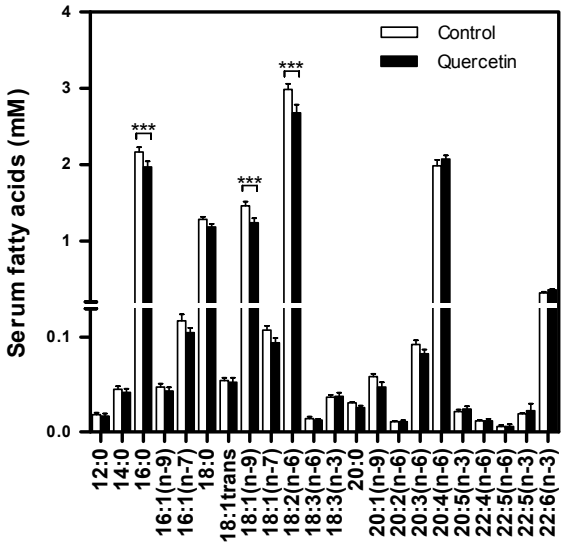


**Figure 3.2 Cumulative serum profile of fatty acids originating from total lipids.** Fatty acids were measured with GC. The levels of palmitic acid (16:0), oleic acid (18:1(n-9)), and linoleic acid (18:2(n-6)) were significant lower in the quercetin group. Data is presented as mean ± SEM. Asterisks indicates a significant difference between the control and the quercetin group; *** $p < 0.001$.

**Table 3.1** Fatty acid composition of the control and quercetin diet in percentages.

| Lipids | Control diet (%) | Quercetin diet (%) |
|---|---|---|
| C12:0 | 0.09 | 0.08 |
| C14:0 | 1.11 | 1.09 |
| C14:1 | 0.06 | 0.06 |
| C15:0 | 0.08 | 0.08 |
| C16:0 | 22.40 | 22.26 |
| C16:1 | 1.25 | 1.25 |
| C17:0 | 0.30 | 0.30 |
| C17:1 | 0.19 | 0.15 |
| C18:0 | 10.79 | 10.87 |
| C18:1 trans | 0.46 | 0.47 |
| C18:1 | 33.87 | 33.94 |
| C18:1 | 1.60 | 1.61 |
| C18:2 | 24.23 | 24.21 |
| C18:3(n-6) | 0.01 | 0.01 |
| C18:3 | 0.71 | 0.72 |
| C20:0 | 0.23 | 0.22 |
| C20:1 | 0.55 | 0.56 |
| C20:2 | 0.29 | 0.30 |
| C20:3(n-6) | 0.05 | 0.05 |
| C22:0 | 0.06 | 0.06 |
| C20:3(n-3) | 0.20 | 0.20 |
| C24:0 | 0.06 | 0.06 |
| C22:5(n-3) | 0.00 | 0.07 |
| C22:6(n-3) | 0.02 | 0.03 |
| Saturated FA | 35.12 | 35.02 |
| MUFA | 37.98 | 38.04 |
| PUFA | 25.51 | 25.59 |

regions (based on [15,16]) were selected to obtain information on several subsets of FFA and/or TG, as shown in Figure 3.3. Integration of the respective peak areas in the $^{1}H$ NMR spectra of the individual serum samples resulted in the amounts of the various lipids. The data are presented in Figure 3.4 as the mean ratio of percentages of lipids present in serum of quercetin-fed mice as compared to control mice. From these data it follows that upon quercetin exposure the levels of TG are significantly decreased with 14% ($p<0.001$), while some specific poly unsaturated FFA levels were increased

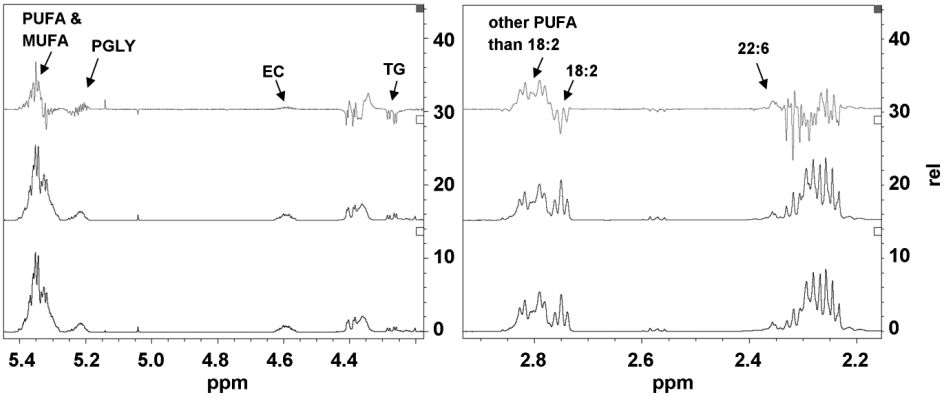


**Figure 3.3 $^{1}$H NMR difference spectrum of the quercetin-fed mice minus the control mice.** Serum samples from mice exposed to quercetin minus the $^{1}$H NMR spectra of the sera from control mice is represented by the top line. The control group is represented by the middle line and the quercetin group is represented by the lowest line. Two representative parts of the spectrum are presented in the figure. PUFA, poly unsaturated fatty acids; MUFA, mono unsaturated fatty acids; FA, fatty acids; TG, triglycerides; PGLY, phosphoglycerides; PC phosphatidylcholine; EC, esterified cholesterol; TC total cholesterol.

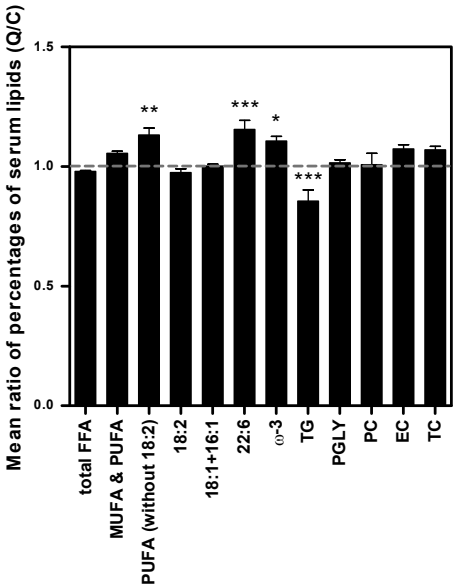


**Figure 3.4 Percentages of lipids present in serum per mouse plotted for quercetin mice to control mice.** Lipids were measured with $^{1}$H NMR. Data is presented as the mean ratio of percentages of lipids present in serum per mouse plotted for quercetin-fed (Q) mice over control (C) mice. Total FFA were not changed, while other PUFA than 18:2 FA, 22:6 FA, and, w-3 FA were significantly increased. TG were significantly decreased by the quercetin diet. Data is presented as mean ± SEM. Asterisks indicates a significant difference between the control and the quercetin group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. PUFA, poly unsaturated fatty acids; MUFA, mono unsaturated fatty acids; FA, fatty acids; TG, triglycerides; PGLY, phosphoglycerides; PC phosphatidylcholine; EC, esterified cholesterol; TC total cholesterol.

with 11-16%; these were PUFA other than 18:2 FA ($p<0.01$), 22:6 FA ($p<0.001$), and ω-3 FA ($p<0.05$). The total amount of FFA was the same in both groups, the levels of PGLY and PC showed no change and the EC and TC showed a slight increase, although not significant. This implies that the overall decrease in lipid levels that are observed in the GC analysis are due to a decrease in TG.

### Quercetin altered the expression of genes involved in lipid metabolism

Gene expression was analysed using whole genome gene expression microarrays. Of the 23,256 probes being expressed, 415 probes were significantly differently expressed by quercetin treatment ($p<0.05$, FDR-adjusted). Regulation of lipid metabolism by quercetin was found by pathway analysis of the differently expressed genes using the two major analysis programs, Metacore and Ingenuity. 'Phospholipid metabolism' was the most significantly regulated pathway in Metacore with a p-value of 4.8E-05 (with 3 down-regulated and 1 up-regulated gene out of 33 genes). Ingenuity pathway analyses identified: 'LPS/IL-1 Mediated Inhibition of RXR Function' as the most regulated pathway (p-value of 5.46 E-05, with 13 up-regulated genes out of 187).

In this pathway Car is the central transcription factor and the genes, in particular cytochromes P450, overlap partly with the 'linoleic acid pathway', which is the number 3 pathway (p-value of 1.7E-03 with 4 up-regulated genes out of 83 genes) in Metacore. Although, in each of these pathways a relative small number of genes were regulated out of the total number of genes present, it was clear that these regulated genes corresponded with the top significantly regulated genes. The ten most regulated genes (absolute fold change > 1.75 and a FDR adjusted p-value < 0.01), where almost all involved in lipid metabolism, particularly in ω-oxidation of fatty acids (Figure 3.5, Table 3.2). These genes involved in ω-oxidation included Cyp4a14, Cyp4a10, Cyp4a31, Acot3 and Por. Altogether, lipid metabolism, and in particular ω-oxidation, were identified as being regulated by quercetin in the liver.

### Confirmation with RT-qPCR

The quercetin induced changes in expression of Cyp4a14, Cyp4a10, Acot3, Car, and Por that were identified by microarray analysis, were confirmed with RT-qPCR (Figure 3.6). Cyp4a14, Cyp4a10, Acot3 and Por were significantly up-regulated in the quercetin group, while the up regulation of Car followed the same trend, but did not reach significance.

## 3.4 DISCUSSION

This study showed that chronic intake of quercetin in mice lowered serum lipid levels which are risk factors for CVD. Microarray analysis indicated that hepatic genes involved in lipid metabolism, in particular in ω-oxidation of fatty acids, could be responsible for these quercetin-induced effects.

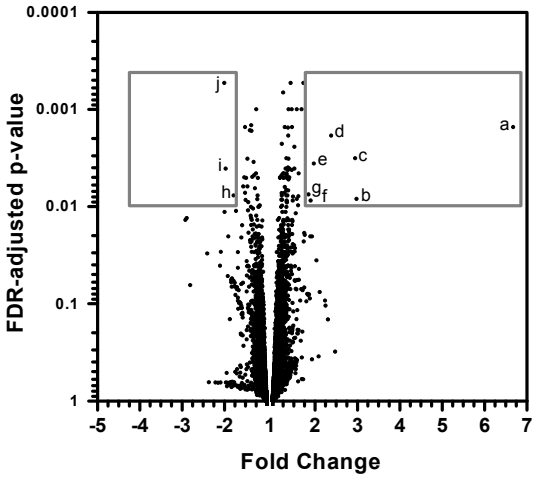


**Figure 3.5 Volcano plot of all expressed probes by global hepatic gene expression analysis.** Volcano plot of all probes showing statistics FDR-adjusted p-values plotted against the fold change of each probe (quercetin vs. control). Frames outline genes that are regulated with absolute fold change >1.75 and a FDR-adjusted p-value <0.01; these gene symbols, names and functions are also represented in table 3.1.

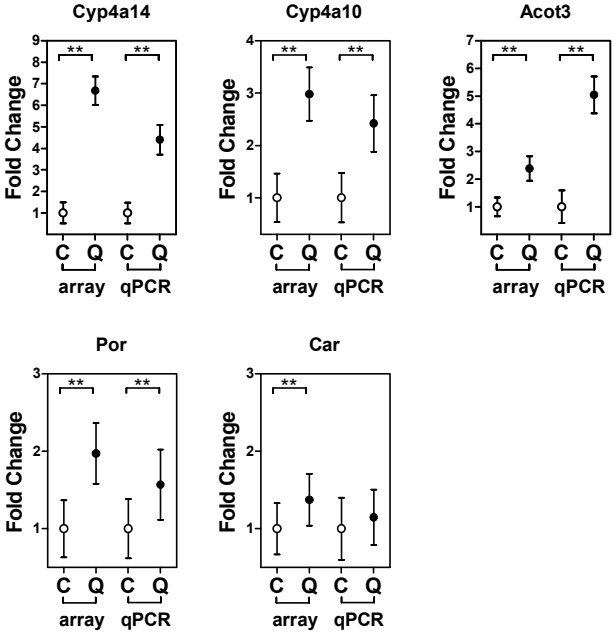


**Figure 3.6 Microarray confirmation by RT-qPCR.** The quercetin (Q) regulated genes Cyp4a14, Cyp4a10, Acot3, Car (Nr1i3) and Por, compared to the control (C) found by microarray analysis were confirmed with RT-qPCR. Data is presented as mean ± SEM (n=12). Asterisks indicates a significant difference between the control and quercetin group; ** p<0.01.

**Table 3.2** Regulated hepatic genes with an absolute fold change >1.75 and FDR-adjusted p-value <0.01.

| Gene symbol | Gene name | Fold change | FDR adjusted p-value | Function |
|---|---|---|---|---|
| I. Cyp4a14 | Cytochrome P450 4a14 | 6.68 | 0.0015 | ω-oxidation of medium-chain fatty acids |
| II. Cyp4a10 | Cytochrome P450 4a10 | 2.98 | 0.0083 | ω-oxidation of medium-chain fatty acids |
| III. Usp2 | Ubiquitin specific peptidase 2 | 2.95 | 0.0032 | regulation of intracellular protein breakdown, cell cycle regulation and stress response |
| IV. Acot3 | Acyl-CoA thioesterase 3 | 2.38 | 0.0018 | catalysator of hydrolysis of acyl-CoAs (C12-C16) after ω-oxidation to FFA and coenzyme A |
| V. Por | P450 (cytochrome) oxidoreductase | 1.97 | 0.0036 | electron donor for the microsomal cytochrome P450 mixed-function oxidase system |
| VI. Cyp4a31 | Cytochrome P450 4a31 | 1.90 | 0.0086 | ω-oxidation of medium-chain fatty acids |
| VII. Coq10b | Coenzyme Q10 homolog B | 1.85 | 0.0074 | an essential electron carrier and proton translocator in the mitochondrial respiratory chain |
| VIII. Insig2 | Insulin-induced gene 2 | -1.78 | 0.0076 | lipid and cholesterol metabolic process |
| IX. Spon2 | Spondin 2, extracellular matrix protein | -1.98 | 0.0040 | essential in the initiation of the innate immune response |
| X. Chka | Choline kinase alpha | -2.00 | 0.0005 | phosphatidylcholine biosynthesis |

Other studies have also observed that supplementation of quercetin to a high-fat diet decreases serum FFA and/or TG levels in mice [10-12]. However, these circulating FFA and TG levels were measured with commercial enzymatic assays, which have recently been found to be sensitive to interference of quercetin and its major metabolite quercetin-3-O-glucuronide, resulting in apparently incorrect lower detected levels [13]. Here, besides these enzymatic assays, we also used two independent analytical methods for quantification of serum lipid profiles; GC and $^{1}$H NMR techniques. The observed effect of quercetin on lipid levels measured with the enzymatic FFA and TG assays (FFA -13% and TG -27%) was higher than measured with the two analytical techniques (GC: total fatty acids -7% and $^{1}$H NMR: FFA -2%, TG -14%).

This confirms interference of quercetin in the enzyme based assays [13] in the physiological range of quercetin exposure and as a consequence overestimate the lipid lowering effect of quercetin. Nevertheless, with GC and $^{1}$H NMR a significant reduction in serum lipid levels was found, proving that lipid lowering is a real biological effect of quercetin. The GC data revealed that the specific serum fatty acids palmitic acid (16:0), oleic acid (18:1(n-9)) and linoleic acid (18:2(n-6)), originating from total lipids, were all significantly decreased in the quercetin-fed mice. Moreover, with $^{1}$H NMR, serum lipids were measured separately, which revealed that serum TG levels of the quercetin group were significantly decreased, while total FFA, cholesterol and phospholipid levels remained unchanged. This indicates that the decreased levels of palmitic acid (16:0), oleic acid (18:1(n-9)) and linoleic acid (18:2(n-6)) found by GC originated from TG. Moreover, the $^{1}$H NMR data showed unchanged levels of total FFA and increased levels of PUFA in the serum of the mice on the quercetin diet, which indicate a shift from saturated fatty acids to PUFA, which are known as the more healthy fatty acids. Together, these data proved that quercetin significantly reduced serum lipid levels and resulted in a more beneficial lipid profile.

The increased levels of PUFA and the decreased levels of saturated fatty acids cannot be fully explained by the microarray data. Genes involved in beta oxidation or specific desaturases were not differentially regulated by the quercetin diet.

There were no significant differences found in the serum phospholipid levels, while pathway analysis revealed phospholipid metabolism as a regulated pathway. However, based on gene expression it was not clear how phospholipid metabolism would be affected, since up as well down regulated genes were observed in different parts of this pathway, and a relative small number of genes of the total pathway was regulated. Therefore, it was concluded that this was not a crucial pathway in this study. Quercetin induced a decrease in relative liver weight in our study. This decrease cannot be explained by a decrease in hepatic lipid accumulation, because hepatic lipid levels were not affected by quercetin. Other studies [11,12] have shown a decrease in lipid accumulation in liver upon dietary administration of quercetin and thus seem to be in contrast with our study. While a study with mulberry leaves, high in quercetin, did report unmodified lipid accumulation in the liver [23] and is thus in line with our data. The differences may be explained by the diets used in the different studies. We have used

a mild-high-fat diet rich in unsaturated fatty acids, which did not result in extensive lipid accumulation in the liver, since the found hepatic lipid levels were in the same range as found for mice fed a normal-fat diet. The other studies that show a quercetin induced decrease in lipid accumulation used a high saturated fatty acid rich diet which induced lipid accumulation in the liver [11,12]. This suggests that quercetin may prevent lipid accumulation in the liver under adverse dietary conditions, but not with relatively healthy diets. In general, quercetin induced altered lipid metabolism on a mild-high-fat diet (our study), a normal-fat diet [10], and different high-fat diets [11,12]. Suggesting, that quercetin can affect lipid metabolism independent of the diet, although the impact of this effect can be different.

Using whole genome microarrays and confirmation by RT-qPCR, we showed that quercetin up-regulates Cyp4a10, Cyp4a14, Cyp4a31, Acot3, Por, and, possibly Car. An integration of these genes into a single 'hepatic pathway' differentially expressed by quercetin treatment is proposed in Figure 3.7.

Normally, fatty acids are mainly metabolized by β-oxidation first in peroxisomes (very long chain FFA) and subsequently in mitochondria (long, medium, and short chain FFA). Another type of fatty acid oxidation is ω-oxidation, which occurs in the endoplasmatic reticulum by members of the cytochrome P450 4A family [24]. Omega-oxidation becomes more important during periods of increased influx of fatty acids

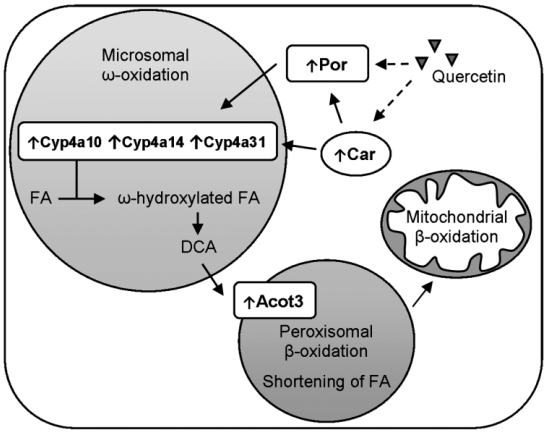


**Figure 3.7 Schematic representation of the quercetin-regulated genes involved in ω-oxidation.** Microarray and RT-qPCR analysis showed an up regulation of Cyp4a14, Cyp4a10, Acot3, Por and Car. Quercetin is suggested to activate Car and/or Por (dashed arrow). Activation of the transcription factor CAR can induce the microsomal cytochrome P450 enzymes, CYP4a14, CYP4a10 and CYP4a31, which are important enzymes involved in ω-oxidation. POR is the electron donor for the microsomal cytochrome P450 mixed-function oxidase system. Formed DCA by ω-oxidation are further degraded by peroxisomal β-oxidation to shorter chain fatty acids. ACOT3 is involved in the transport of DCA into the peroxisomes by hydrolysis of long-medium chain fatty acyl-CoA esters to FFA, which can be further transported out of peroxisomes to mitochondria for β-oxidation or excreted in the urine. FA, fatty acids; DCA, dicarboxylic acids.

into the liver, for example in our high-fat diet mice study, in obesity, and when the mitochondrial oxidation system is insufficient to metabolize fatty acids [25,26]. In these situations ω-oxidation can prevent lipid toxicity [27]. Fatty acids oxidized by ω-oxidation result in ω-hydroxy fatty acids which are then dehydrogenated to a dicarboxylic acid in the cytosol. These dicarboxylic acids are further degraded by peroxisomal β-oxidation to shorter chain dicarboxylic fatty acids, which can be excreted in the urine, metabolized by the peroxisomal oxidation system to succinate and acetyl CoA, or completely oxidized after transport into the mitochondrial β-oxidation system [28]. A small increase of ketone bodies was found in the quercetin-fed mice suggesting an increase of β-oxidation (292.5 ± 199.2 versus 185.6 ± 118.1 μM, p=0.12).

Acot3 was also up-regulated in our study, and the enzyme ACOT3 hydrolyses long-medium chain fatty acyl-CoA esters to FFA, and thus facilitate transport into peroxisomes. The FFA can subsequently be transported out of peroxisomes to mitochondria for further β-oxidation [29,30].

It has been described that, among others, palmitic acid (16:0) and oleic acid (18:1(n-9)) can be hydroxylated by CYP4A11, the human variant of murine Cyp4a10 [31]. This is especially consistent with the serum fatty acid profile obtained in the present study (Figure 3.2), where levels of palmitic acid (16:0) and oleic acid (18:1(n-9)) were significantly lower in the quercetin-fed mice. The significant up regulation of Cyp4a10, Cyp4a14, Cyp4a31 and Acot3 therefore explains the observed reduced serum levels for these specific fatty acids.

In humans, various polymorphisms are described in the genes of cytochromes P450s and they can be considered as one of the major determinants of individual susceptibility to CVDs [32]. Allelic variations in CYP4A11 are suggested to result in an increased risk for hypertension [25,32]. Hypertension can be caused by increased serum lipid levels[6], which were decreased by quercetin in our study with concomitant up regulation of Cyp4a genes.

The up regulation of the Cyp4a genes is consistent with the significant, 1.97 fold up regulation of Por by quercetin. POR is an enzyme that is required for electron transfer to cytochrome P450 enzymes and is therefore rate limiting for P450 enzymes. Deletion of the Por gene in a mouse model reduced hepatic P450 activity by more than 95%. Moreover, hepatic Por knockout (Por-KO) mice showed decreased CYP4A protein levels, and an enlarged and fatty liver. Based on these observations, it was concluded that the P450 system plays a major role in regulating lipid homeostasis and hepatic lipid levels [33,34]. Two to three-fold more genes were significantly regulated when WT mice were exposed to quercetin compared to Por-KO mice. These genes were, among others, involved in fatty acid metabolism pathways. This suggests that hepatic POR mediates many of the biological effects of quercetin, including fatty acid metabolism [35]. These results underscores our data, which showed an up regulation of Por.

It is also suggested that P450 expression can be mediated via a CAR-dependent signaling pathway [36]. CAR is a transcription factor that is highly expressed in the liver. It is

shown that ligand dependent activation of CAR increased lipid metabolism in rodents [37,38] and it is also shown that this can lead to specifically increased expression of genes involved in ω-oxidation [39]. Furthermore, exposure of quercetin to HepG2 cells transfected with CAR showed that CAR can be activated by quercetin [40,41]. Our data showed significant up regulation of Car (FC = 1.37, FDR adjusted p-value = 0.005), which suggests that Car has an important role in quercetin mediated regulation of lipid metabolism.



This study used male mice, therefore caution is needed in translating these data to female mice. It is known that there are sex differences in the sensitivity to CAR activators and also Cyp4a genes can be under sex-dependent control [42,43].

In conclusion, quercetin can affect hepatic lipid metabolism, especially ω-oxidation. This is shown by the up regulation of Cyp4a10, Cyp4a14, Cyp4a31, Acot3, Por and the transcription factor Car. These effects are associated with decreased corresponding circulating lipid levels, which may contribute to potential beneficial effects on CVD.

### Acknowledgments

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# CHAPTER 4

## QUERCETIN DECREASES HIGH-FAT DIET-INDUCED BODY WEIGHT GAIN AND ACCUMULATION OF HEPATIC AND CIRCULATING LIPIDS IN MICE

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## ABSTRACT

Dietary flavonoids may protect against cardiovascular diseases (CVD). Elevated circulating lipid levels and hepatic lipid accumulation are known risk factors for CVD. The aim was to investigate the effects and underlying molecular mechanisms of the flavonoid quercetin on hepatic lipid metabolism in mice with high-fat diet-induced body weight gain and hepatic lipid accumulation.

Adult male mice received a 40 energy% high-fat diet without or with supplementation of 0.33% *(w/w)* quercetin for 12 weeks. Body weight gain was 29% lower in quercetin fed mice ($p<0.01$), while the energy intake was not significantly different. Quercetin supplementation lowered hepatic lipid accumulation to 29% of the amount present in the control mice ($p<0.01$). $^{1}$H nuclear magnetic resonance serum lipid profiling revealed that the supplementation significantly lowered serum lipid levels. Global gene expression profiling of liver showed that cytochrome P450 2b (*Cyp2b*) genes, key target genes of the transcription factor constitutive androstane receptor (*Car*; official symbol *Nr1i3*), were down-regulated.

Quercetin decreased high-fat diet-induced body weight gain, hepatic lipid accumulation and serum lipid levels. This was accompanied by regulation of cytochrome P450 2b genes in liver, which are possibly under transcriptional control of CAR. The quercetin effects are likely dependent on the fat content of the diet.

# 4.1 INTRODUCTION

High consumption of fruits and vegetables is protective against cardiovascular diseases (CVD) [1]. Flavonoids in fruits and vegetables are suggested to contribute to these protective effects [2]. Moreover, epidemiological studies have shown that the intake of the flavonoid quercetin is associated with a reduction of CVD risk [3-5]. In the Western diet, quercetin is the major dietary flavonol, a subclass of the flavonoids, and is present in, for example, apples, tea, red wine, and onions. Our previous results in mice showed that a quercetin supplemented mild-high-fat diet, increased hepatic lipid metabolism, especially omega (ω)-oxidation, and reduced corresponding circulating lipid levels [6]. The cardiovascular protective properties of quercetin might therefore be explained by the lipid lowering effect of quercetin, since elevated circulating levels of free fatty acids and triglycerides are known risk factors for CVD [7-9]. Furthermore, growing evidence shows that increased lipid accumulation in the liver also increased the risk of CVD [10,11].



The mechanisms behind these effects of quercetin are neither fully understood nor conclusive. Other rodent studies with quercetin supplementation that investigated lipid lowering effects showed reduction in body weight, serum lipid levels, hepatic lipid accumulation, and/or white adipose tissue mass. However, these effects were not seen in all studies and were sometimes conflicting [6,12-17]. It is not clear which combination of factors, like dose of quercetin, dietary en% of fat or duration causes these effects on lipids. Previously, we have observed an effect of quercetin on serum lipid lowering and hepatic lipid ω-oxidation using a mild-high-fat diet (30 en%) for 12 weeks [6]. Here, we examined whether the same effects would be observed using the same amount of quercetin and the same duration, but using a high-fat diet (40 en%), which is expected to induce body weight gain as well as hepatic lipid accumulation. This high-fat diet is a fully standardised diet with a fatty acid composition that provides a balance of essential fatty acids and a healthy polyunsaturated to saturated ratio [18]. The main focus was on the effects of quercetin on hepatic lipid metabolism, since the liver is the major effector organ of lipid metabolism. We assessed hepatic lipid accumulation and profiled serum lipids. Based on previous results, expression of genes involved in ω-oxidation was studied. In addition, we performed whole genome gene expression analysis to obtain an overview of the molecular changes induced by quercetin on this dietary background.

# 4.2 MATERIALS AND METHODS

## Animals and treatments

Twenty-four male C57BL/6JOlaHsd mice (Harlan Laboratories, Horst, The Netherlands) were individually housed and maintained under environmentally controlled conditions (temperature 21°C, 12 h/12 h light-dark cycle, 55 ± 15 % humidity), with ad libitum access to food and water. At arrival, the mice were 9 weeks old and were adapted for 3 weeks. The first five days of adaptation were on standard chow diet, which was

followed by a standardised semi-synthetic normal fat diet (10 en% fat) with the same dietary constituents as the control high-fat diet [18,19] (Research Diets Services B.V., Wijk bij Duurstede, the Netherlands; Supplementary Table S4.1). During the intervention, the mice (n=12) received high-fat diet (40en% fat) without or with supplementation of 0.33% *(w/w)* quercetin (Sigma, Zwijndrecht, the Netherlands). The percentage of quercetin in the diet was identical to that used in our previous study [6]. Body weight and food intake of individual mice were weekly monitored. Faeces was collected in weeks 11 and 12 (n=4). One quercetin fed mouse was excluded from all analyses, because a nasal abscess developed in week 6. After 12 weeks of intervention, all mice were fasted for 2-4 hours during the light phase and anesthetized by inhalation of 5% isoflurane using $O_2$ as a carrier. Blood was sampled via orbital extraction in collect serum tubes (Greiner Bio-one, Longwood, USA), kept on ice for max 2 hours, and centrifuged for 10 min at 3000g at 4 °C to obtain serum, aliquoted, and stored at -80°C. After blood collection, mice were killed by cervical dislocation, and liver was dissected, weighted and snap frozen in liquid nitrogen and stored at -80°C. The experiment was performed according to the Dutch Animal Experimentation Act (1996) and the experimental protocol was approved by the Animal Welfare Committee of Wageningen University, Wageningen, The Netherlands (DEC 2011079).

## Energy content of faeces and diet

Bomb calorimetry was used to determine energy content of diet and faeces (n=4) (Calorimeter C7000, IKA, Staufen, Germany). Measured faecal energy content was extrapolated to calculate with the measured dietary energy content and the weekly measured food intake the total digestible energy intake over 12 weeks. Digestible energy intake is assumed to be comparable with metabolisable energy intake, as dietary protein content is equal for both diets and no differences in urinary energy losses were expected.

## HPLC analysis of quercetin levels in serum

Quercetin levels in serum (n=6) were measured using HPLC with coulometric array detection as described [6]. Before analysis, samples were hydrolysed by β-glucuronidase/sulfatase to obtain deconjugated quercetin, isorhamnetin and tamarixetin.

## Serum and tissue lipid measurements

Because quercetin was previously shown to interfere with commonly used commercially available enzymatic lipid assays [20], alternative methods were used. Neutral lipids were stained in frozen liver sections (n=6) with Oil red O (Sigma) and quantified as described [6]. Ten to 16 pictures per animal were quantified (n=6). Serum lipids were extracted and analysed with $^1$H nuclear magnetic resonance ($^1$H-NMR) as described [6].

Serum insulin levels were quantified by sandwich-type ELISA (Shibayagi Co., Ltd., Gunma, Japan) and blood glucose levels using ADC Freestyle blood glucose system

(Abbott Diabetes Care, Hoofddorp, the Netherlands). The homoeostasis model assessment–insulin resistance (HOMA-IR) was calculated using the following formula: fasting plasma insulin (mIU/l) × fasting glucose (mmol/l) / 22.5. Taurine levels in serum and hepatic tissues were quantified as described [21] using the aTRAQ® reagent (aTRAQ™ Reagent Kit, ABSciex, Foster City, USA).

## Real time quantitative polymerase chain reaction (RT-qPCR)

RNA from liver was isolated using RNeasy columns (Qiagen, Venlo, The Netherlands) and used for RT-qPCR and microarray analysis. RNA purity and quality was verified using a Nanodrop spectrophotometer (IsoGen Life Science, Maarsen, The Netherlands) and on Experion automated electrophoresis system (Bio-Rad, Veenendaal, The Netherlands). RT-qPCR was performed as previously described [6] according to the MIQE guidelines [22]. Data were normalised against reference genes beta-2 microglobulin *(B2m)* and hypoxanthine phophoribosyltransferase 1 *(Hprt1)* which were chosen based on stable gene expression levels (geNorm, Ghent University Hospital, Ghent, Belgium). Sequences of the used primers can be found in Supplementary Table S4.2.

## Microarray analysis

For global transcriptome analysis liver samples of individual mice and 8x60K Agilent whole-mouse genome microarrays (G4852A, Agilent Technologies Inc., Santa Clara, CA) were used according to the manufacturer's protocol with a few modifications as described previously [23]. cDNA was synthesised for each animal from 200 ng RNA. Normalisation and data analysis were performed as published [24] using Feature Extraction version 10.7.3.1 (Agilent Technologies). Based on visual inspection three arrays were excluded in which hybridisation was not homogenous. Fold change was expressed as ratio of quercetin group (n=10) versus control group (n=11). Pathway analysis was performed using MetaCore (GeneGo, St. Joseph, Michigan, USA). Microarray data have been deposited in NCBI Gene Expression Omnibus under accession number GSE51343.

## Statistical analysis

GraphPad Prism version 5.03 (Graphpad Software, San Diego, USA) was used for statistical analysis, with Student's *t* test being used to compare the two groups if normally distributed. Two-way ANOVA (repeated measures, matched values) followed by a Bonferroni post hoc test was used for body weight in time analysis. Two way ANOVA (no repeated measures) was used for analysis of the lipid profiles in serum. *P*-values smaller than 0.05 were considered significantly different.

## 4.3 RESULTS

### Quercetin lowered high-fat diet-induced body weight gain and food efficiency

Mice received a 40 en% high-fat diet without (control) or with supplementation of quercetin for 12 weeks. Body weight of the mice was significantly lower upon quercetin supplementation compared to the high-fat diet from week 7 onwards (Figure 4.1a). Total body weight gain after 12 weeks was 29% lower in the quercetin fed mice compared to the control mice ($p$<0.01; Figure 4.1b), while digestible (equals metabolisable) energy intake over 12 weeks was not significantly different (Figure 4.1c). Consequently, the calculated food efficiency was 26% lower for the quercetin fed mice ($p$<0.001) (Figure 4.1d). The weekly food intake and energy content of faeces used for the calculation is provided in Supplementary Figure S4.1.

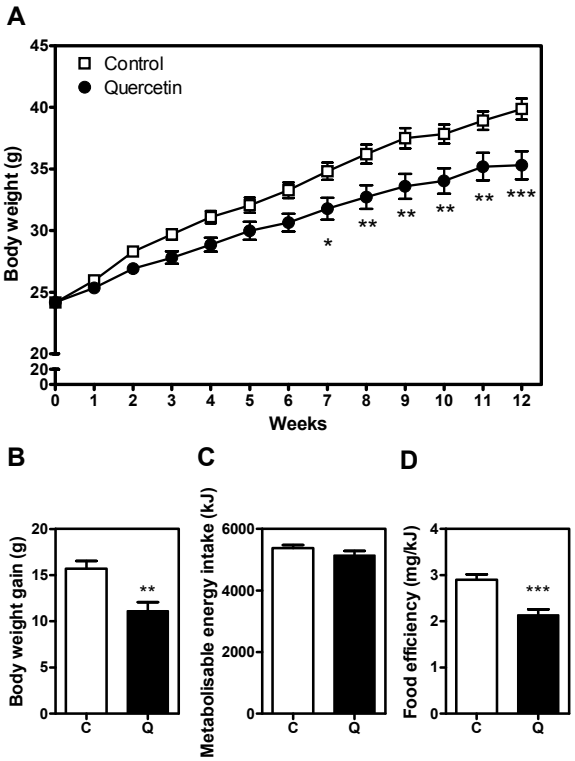


**Figure 4.1 Quercetin reduces body weight gain and food efficiency.** Body weight of control (n=12) and quercetin (n=11) fed mice during 12 weeks on high-fat diet (A). Cumulative body weight gain over 12 weeks (B). Cumulative metabolisable energy intake over 12 weeks (C). Calculated total food efficiency in body weight gain per kJ consumed (D). Data are presented as mean ± SEM. White bars indicate the control group (C) and black bars indicate the quercetin group (Q). Asterisks indicates a significant difference between both groups, *$p$<0.05, **$p$<0.01, ***$p$<0.001.

## Concentration of quercetin in serum

The sum of quercetin and isorhamnetin after deconjugation in serum was 6.5 ± 1.4 μM (quercetin 2.8 ± 1.4 μM, isorhamnetin 3.7 ± 0.8 μM, and no tamarixetin was found). No quercetin was found in serum of control animals. The calculated quercetin intake based on the food intake of the quercetin fed mice was ~325 mg $kg^{-1}$ body weight $day^{-1}$ .

## Quercetin decreased high-fat diet-induced serum and hepatic lipid levels



Because quercetin was previously shown to interfere with many commercially available enzymatic assays including the ones commonly used for measurement of serum and hepatic lipids [20], we quantified various serum lipid fractions with $^{1}$H NMR and hepatic lipid accumulation with histological Oil red O staining. Two-way ANOVA analysis revealed that quercetin supplementation has a significant lowering effect on the high-fat diet-induced serum lipid levels (Figure 4.2a).

Relative liver weight was not significantly different between both groups. Oil red O stained liver sections showed that hepatic lipid accumulation in quercetin fed mice was significantly lower amounting to 29% (measured as area) of the value observed for control mice fed the high-fat diet, and lipid droplet number in the quercetin fed mice was 69% ($p$<0.05) of the value observed in control mice (Figure 4.2).

Serum insulin (0.60 ± 0.46 and 0.98 ± 0.58 ng/ml, resp.), blood glucose (8.4 ± 1.2 and 9.1± 0.7 mM, resp.) and calculated HOMA-IR (5.9 ± 4.8 and 10.5 ± 6.8, resp) were not significantly different between the quercetin and the control group.

## Quercetin regulated hepatic gene expression

First, hepatic expression levels of genes involved in ω-oxidation, identified in our previous study where quercetin was supplemented to a mild high-fat diet [6], were studied. However, RT-qPCR analysis indicated no significant regulation of *Cyp4a14*, *Cyp4a10*, *Acot3*, nor *Por* (Table 4.1).

Subsequently, we profiled gene expression in the liver using whole genome microarrays, since we observed large differences in hepatic lipid accumulation between

**Table 4.1** Gene expression of genes related with lipid ω-oxidation measured with RT-qPCR in liver.

| Gene symbol | Gene name | Accession number | RT-qPCR | |
|---|---|---|---|---|
| | | | FC | p |
| Acot3 | acyl-CoA thioesterase 3 | NM_134246 | 1.07 | 0.68 |
| Cyp4a10 | cytochrome P450, family 4a10 | NM_010011 | -1.09 | 0.82 |
| Cyp4a14 | cytochrome P450, family 4a14 | NM_007822 | 1.11 | 0.47 |
| Por | P450 (cytochrome) oxidoreductase | NM_008898 | 1.08 | 0.57 |

Fold Changes are depicted as the expression values of quercetin over control animals. FC, Fold Change; p, p-value.

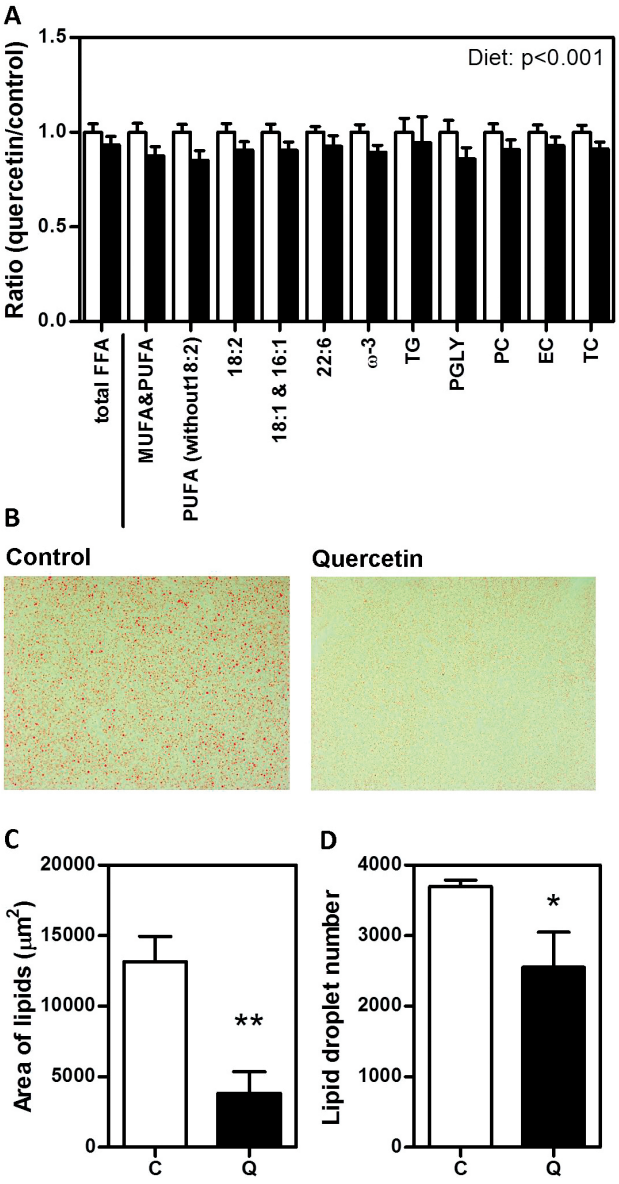


**Figure 4.2 Quercetin decreased high-fat diet-induced serum lipid levels and hepatic lipid accumulation.** A. Serum lipid fractions are shown as ratio of quercetin fed mice (n=11) over the average value of the control mice (n=12). Two-way ANOVA; diet significant ($p<0.01$). B. Representative pictures of hepatic lipid staining with Oil red O for control and quercetin fed mice on a high-fat diet (n=6). Quantification of mean total area of lipids per picture (C) and mean lipid droplet number per picture (D). Data are presented as mean ± SEM. White bars indicate the control group (C) and black bars indicate the quercetin group (Q). Asterisks indicate a significant difference between both groups, *$p<0.05$, **$p<0.01$. PUFA, poly unsaturated fatty acids; MUFA, mono unsaturated fatty acids; FA, fatty acids; TG, triglycerides; PGLY, phosphoglycerides; PC phosphatidylcholine; EC, esterified cholesterol; TC total cholesterol.

the two groups. Of the 34,373 probes showing expression, 462 probes showed differential expression upon quercetin supplementation to a high-fat diet as compared to the high-fat diet alone ($p$<0.01). Similar to RT-qPCR, microarrays did not show regulation of the ω-oxidation related genes. Pathway analysis of the differentially expressed genes revealed no reliable regulated pathways. In the volcano plot, four major regulated genes (absolute fold change >2.0 with $p$<0.01), were observed (Figure 4.3). Cysteine sulfinic acid decarboxylase (*Csad)*, encoding the rate limiting enzyme in taurine biosynthesis, was up-regulated with a fold change of 2.3. However, taurine levels in serum and in hepatic tissue were not significantly affected by the quercetin diet (Supplementary Table S4.3).



Three cytochrome P450 enzyme encoding genes, *Cyp2b9*, *Cyp2b10*, and *Cyp2b13*, were all down regulated with fold changes between -2.3 and -2.6. These genes are known to be transcriptionally regulated by *Car* (official symbol *Nr1i3)*, which we previously postulated to be an important transcription factor affected by quercetin supplementation [6]. The top regulated genes ($p$<0.01 and absolute fold change >1.5) and confirmation of these genes by RT-qPCR are shown in 4.2. This list includes another cytochrome P450 and two genes involved in lipid metabolism: fatty acid binding protein 5 (*Fabp5*) and hydroxyacid oxidase 2 (*Hao2*).

## 4.4 DISCUSSION

This study shows that quercetin attenuated the increase of circulating lipids, hepatic lipid accumulation, and body weight gain, all dietary risk factors for CVD, caused by

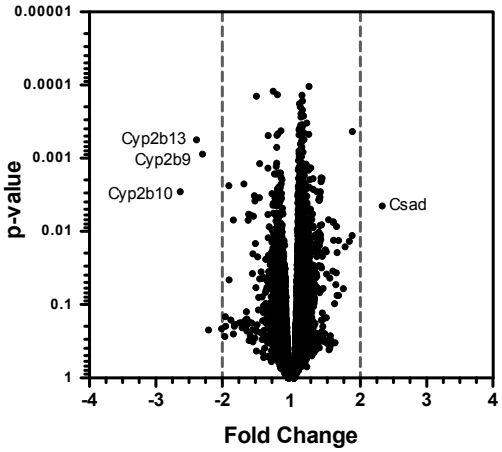


**Figure 4.3 Volcano plot of all expressed probes by global hepatic gene expression analysis.** P-values of all probes are plotted against the fold change of each probe, quercetin (n=10) vs. control (n=11). Dotted lines indicate used cut-off value of an absolute fold change >2.0. Selected gene symbols are depicted in the figure; cytochrome P450 (Cyp) enzymes *Cyp2c9*, *Cyp2c10*, and *Cyp2c13*, and cysteine sulfinic acid decarboxylase (*Csad*).

**Table 4.2** Top regulated genes from microarray analysis in liver with an absolute fold change >1.5 and $p<0.01$.

| Gene symbol | Gene name | Accession number | Microarray | | RT-qPCR | |
|---|---|---|---|---|---|---|
| | | | FC | p | FC | p |
| Csad | Cysteine sulfinic acid decarboxylase | NM_144942 | 2.33 | 0.0045 | 2.73 | 0.001 |
| Cib3 | Calcium and integrin binding family member 3 | NM_001080812 | 1.88 | 0.0004 | n.a. | |
| Fabp5 | Fatty acid binding protein 5, epidermal | NM_010634 | 1.59 | 0.0075 | 1.37 | 0.173 |
| Tfrc | Transferrin receptor | NM_011638 | 1.52 | 0.0069 | 1.45 | 0.016 |
| Cyp3a59 | Cytochrome P450, subfamily 3a59 | NM_001105160 | -1.51 | 0.0038 | n.a. | |
| Hao2 | Hydroxyacid oxidase 2 | NM_019545 | -1.67 | 0.0022 | -2.81 | 0.006 |
| Cyp2b9 | Cytochrome P450, family 2b9 | NM_010000 | -2.30 | 0.0009 | -8.2 | 0.0003 |
| Cyp2b13 | Cytochrome P450, family 2b13 | NM_007813 | -2.39 | 0.0006 | n.a. | |
| Cyp2b10 | Cytochrome P450, family 2b10 | NM_009999 | -2.63 | 0.0028 | n.a. | |

Not assigned probes were not mentioned in the table; NAP111439-1 (FC=1.64, p=0.009), NAP114472-1 (FC=1.59, p=0.007), Gm10804 (FC=-1.83, p=0.0069), A_55_P2071906 (FC=-1.9, p=0.002). Fold Changes are depicted as the expression values of quercetin over control animals. FC, Fold Change; p, p-value; n.a., not assessed.

a chronic high-fat diet. These effects of quercetin were accompanied by regulation of cytochrome P450s.

The significant lower body weight gain of mice induced by high-fat diet upon 0.33% quercetin supplementation cannot be explained by a lower food intake or higher faecal energy losses. The quercetin fed mice gained less weight with a similar digestible energy intake resulting in a significantly lower calculated food efficiency. The high-fat (40 en%) diet-induced body weight gain and hepatic lipid accumulation. In case of a normal fat (10 en%) diet, body weight gain over 12 weeks was 40-50% of the body weight gain by high-fat diet feeding [18,25]. The observed body weight gain in mice fed the high-fat diet supplemented with quercetin was lowered with 29% compared to high-fat diet feeding, thus decreasing it towards a more normal level. Hepatic lipids level after normal fat feeding for 12 weeks was only around 4% of the value after high-fat feeding and the high-fat supplemented with quercetin decreased the hepatic lipid level to 27% of the high-fat values. Both parameters show that quercetin supplementation reduced the effects of the high-fat diet substantially, changing these parameters into the direction of values that are observed upon a more normal healthy (10 en%) fat diet.

Our results were in line with other studies performed with supplementation of 0.025% and 0.05% quercetin to 40 en% high-fat diets [13,17], which also resulted in lowering of body weight gain and hepatic lipid accumulation. Other studies with doses of quercetin ranging between 0.05% and 1% were shorter or used a diet with a lower energy% of fat. Consequently, body weight gain was lower in these studies and as a result,

quercetin did not show a decrease in bodyweight gain [6,12-16]. Our previous data (30en% fat diet) [6], showed also no effects on body weight gain and hepatic lipid accumulation, which study was identical to the present study except for the used background diet. Taking this together, we conclude that the observed effects of quercetin on body weight gain and hepatic lipid accumulation are dependent on the en% fat in the diet. The magnitude of body weight gain and hepatic lipid accumulation caused by the diet, which is dependent on the dietary fat content, seems to be important for the outcomes obtained in these quercetin supplementation studies.



In our previous study we have shown that quercetin induced hepatic lipid ω-oxidation and lowered corresponding serum triglyceride levels, possibly under transcriptional control of CAR [6]. These specific changes in serum triglyceride le vels and genes involved in lipid ω-oxidation were not seen in the present study, which also suggests an influence of the used diet on these effects. Omega-oxidation is known to be induced by a high-fat diet and in case of induced nonalcoholic fatty liver disease [26]. Here, the high-fat diet-induced a fatty liver, thus ω-oxidation and especially *Cyp4a* transcripts are expected to be up regulated, already in the high-fat control group, possibly diminishing further stimulation by quercetin.

Whole genome microarray analysis of liver revealed that *Cyp2b9*, *Cyp2b10*, and *Cyp2b13* were the most evident genes regulated by quercetin. These three genes comprise the hepatic *cyp2b* genes of mice. Besides metabolism of endogenous and xenobiotic compounds, cytochrome P450s are also important in hepatic lipid homeostasis [27]. Importantly, these *Cyp2b* genes are known to be under control of CAR [27,28]. Thus, although differences in liver gene expression are seen in this study compared to our previous study [6], in both cases lipid metabolizing genes that are under control of CAR were modulated, and the changes were accompanied by changes in serum lipids.

CAR is involved in the regulation of genes which are involved in lipid homoeostasis. CAR mediates the induction of cytochrome P450 enzymes, including *Cyp2b10*, dietary linoleic acid is shown to be a regulator of P450 expression via CAR [29]. Linoleic acid is also abundantly present in the high-fat diet used in the present study, at double the amount compared to our previous study (Supplementary Table S4.1). *Fabp5*, the fourth gene among the top up regulated genes ($p<0.01$) with a fold change of 1.59, can bind and transport long-chain fatty acids and it has a high affinity for linoleic acid [30]. This may suggest that linoleic acid transport into the liver of quercetin fed animals is increased. Furthermore, activation of CAR as well as induction of Cyp2b mRNA by the known inducer phenobarbital can be attenuated by PUFAs [31,32]. Altogether, this information suggests that possible activation of CAR by quercetin can be possibly attenuated by dietary linoleic acid. Although, this has to be further investigated in detail, our results indicate that it is important to take the composition of the dietary background into account in evaluation effects of quercetin and, most likely, of other polyphenols. Indeed, also for the polyphenol resveratrol, marked differences in functional effects were observed dependent on standard or high-fat dietary backgrounds [33].

In conclusion, high-fat diet-induced hepatic lipid accumulation, circulating lipids and weight gain were reduced by chronic intake of quercetin in mice. Also cytochrome P450s were regulated in liver, which are under transcriptional control of the nuclear receptor CAR. Our data newly suggest that these effects may depend on dietary fat content and composition. This novel notion may provide an explanation for the apparent contradictions in the outcomes of studies with quercetin and potentially has important implications for the analysis and interpretation of human studies.



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**Table S4.1** Dietary composition of the diets used in previous and present study.

| Components | Previous study[a] | | | | Present study[b] | | | |
|---|---|---|---|---|---|---|---|---|
| | % of total amount of lipids, proteins or carbohydrates | g/kg diet | en% | kcal/ kg diet | % of total amount of lipids, proteins or carbohydrates | g/kg diet | en% | kcal/ kg diet |
| Lipids | | | | | | | | |
| C8:0 | - | - | | | 1.3 | 2.73 | | |
| C10:0 | - | - | | | 1.1 | 2.31 | | |
| C12:0 | 0.09 | 0.13 | | | 8.5 | 17.85 | | |
| C14:0 | 1.11 | 1.61 | | | 3.4 | 7.14 | | |
| C14:1 | 0.06 | 0.09 | | | - | - | | |
| C15:0 | 0.08 | 0.12 | | | - | -- | | |
| C16:0 | 22.40 | 32.48 | | | 6.7 | 14.07 | | |
| C16:1 | 1.25 | 1.81 | | | - | - | | |
| C17:0 | 0.30 | 0.44 | | | - | - | | |
| C17:1 | 0.19 | 0.28 | | | - | - | | |
| C18:0 | 10.79 | 15.65 | | | 3.9 | 8.19 | | |
| C18:1 trans | 0.46 | 0.67 | | | - | - | | |
| C18:1 (n-9) | 33.87 | 49.11 | | | 21.8 | 45.78 | | |
| C18:1 | 1.60 | 2.32 | | | - | - | | |
| C18:2 (n-6) | 24.23 | 35.13 | | | 47.0 | 98.70 | | |
| C18:3(n-6) | 0.01 | 0.01 | | | - | - | | |
| C18:3 (n-3) | 0.71 | 1.03 | | | 6.0 | 12.60 | | |
| C20:0 | 0.23 | 0.33 | | | - | - | | |
| C20:1 | 0.55 | 0.80 | | | - | - | | |
| C20:2 | 0.29 | 0.42 | | | - | - | | |
| C20:3(n-6) | 0.05 | 0.07 | | | - | - | | |
| C22:0 | 0.06 | 0.09 | | | - | - | | |
| C20:3(n-3) | 0.20 | 0.29 | | | - | - | | |
| C24:0 | 0.06 | 0.09 | | | - | - | | |
| Not identified | 1.41 | 2.04 | | | - | - | | |
| Total saturated FA | 35.1 | 50.9 | | | 24.9 | 52.3 | | |
| Total MUFA | 38.0 | 55.1 | | | 21.8 | 45.8 | | |
| Total PUFA | 25.5 | 37.0 | | | 53.0 | 111.3 | | |
| Total lipids | 100 | 145 | 29.8 | 1305 | 100 | 210 | 40.2 | 1890 |

**Table S4.1** Continued

| Components | Previous study[a] | | | | Present study[b] | | | |
|---|---|---|---|---|---|---|---|---|
| | % of total amount of lipids, proteins or carbohydrates | g/kg diet | en% | kcal/ kg diet | % of total amount of lipids, proteins or carbohydrates | g/kg diet | en% | kcal/ kg diet |
| Proteins | | | | | | | | |
| Casein | 98.7 | 220 | | | 98.9 | 267 | | |
| L-Cystein | 1.4 | 3 | | | 1.1 | 3 | | |
| Total protein | 100 | 223 | 20.4 | 892 | 100 | 270 | 23.0 | 1080 |
| Carbohydrates | | | | | | | | |
| Maltodextrin | 20.6 | 112.2 | | | 23.1 | 100 | | |
| Dextrose | - | - | | | 11.6 | 50 | | |
| Sucrose | 15.7 | 85.5 | | | 23.1 | 100 | | |
| Vit mix | 1.8 | 10 | | | 2.3 | 10 | | |
| Starch (70-30) | 61.9 | 336.7 | | | 40.0 | 172.5 | | |
| Total carbohydrates | 100 | 544.4 | 49.8 | 2178 | 100 | 432.5 | 36.8 | 1730 |
| Others | | | | | | | | |
| Mineral mix | | 35 | | | | 35 | | |
| Cellulose | | 50 | | | | 50 | | |
| Choline bitartrate | | 2.5 | | | | 2.5 | | |
| Cholesterol | | 97 mg[c] | | | | 97 mg | | |
| total | | | 4375 | | | | 4700 | |

[a]Lipids from previous study originated from 70% lard and 30% corn oil, presented lipid composition is analysed with GC [6]. [b]Lipids from present study are calculated amount of fatty acids originating from 70% sunflower, 18% coconut oil, and 12% flaxseed oil [19]. [c]Based on theoretically present amount of cholesterol in lard.

**Table S4.2** Sequences of the primers used for RT-qPCR.

| Gene symbol | Forward primer (5′-->3′) | Reverse primer (5′-->3′) | Anealing temp (°C) |
|---|---|---|---|
| Acot3 | GCTGTGACCTACCTGCTCAGTCA | ATATAGAGCCATTGATGATGACAGCGG | 60 |
| B2m | CCCCACTGAGACTGATACATACGC | AGAAACTGGATTTGTAATTAAGCAGGTTC | 62 |
| Csad | CCATGTGGATGCTGCCTGGGG | AAGAGCGGAGCACTGCAGCC | 56.7 |
| Cyp2b9 | ACCACAAGCGCCACCCTCCA | TGGTAGCCGGTGTGAGCCGAT | 55.7 |
| Cyp4a10 | TCTACCCACCTGTCCCAGGC | ACACCTCTGGATTTGGCCACA | 58 |
| Cyp4a14 | TTCTTTCGCCTGCGGAATGC | CACTCCATCTGTGTGCTCGTGA | 62 |
| Fabp5 | GTGGCGCCTGATGGAAAGCC | TCCACGATCATCTTCCCATCCTTCA | 58 |
| Hao2 | TGTTGAAGGCACTAGCCCTTGGAG | AGGTCTGGACTGATCTCAGCAACTG | 58 |
| Hprt1 | TGACACTGGTAAAACAATGCAAACTTTG | GAGGTCCTTTTCACCAGCAAGCT | 62 |
| Por | CGAGGGCAAGGAGCTGTACC | CACAGGTGGTCGATGGGTGG | 62 |
| Tfrc | CCTTGCACTCTTTGGACATGCTCATC | AACCCTGATGACTGAGATGGCGG | 60 |

Acot3, acyl-CoA thioesterase 3; B2m, Beta-2 microglobulin; Csad, Cysteine sulfinic acid decarboxylase; Cyp2b9, Cytochrome P450 family 2b9; Cyp4a10, Cytochrome P450 family 4a10; Cyp4a14, cytochrome P450 family 4a14; Fabp5, Fatty acid binding protein 5 epidermal; Hao2, Hydroxyacid oxidase 2; Hprt1, hypoxanthine phophoribosyltransferase 1; Por, P450 (cytochrome) oxidoreductase; Tfrc, transferrin receptor.

**Table S4.3** Taurine measurements in serum and in liver.

| | Control | Quercetin |
|---|---|---|
| Serum (μM) | 564.9 ± 114.08 | 509.4 ± 62.93 |
| Liver (μmol/g protein). | 376.4 ± 96.6 | 332.8 ± 127.1 |

Data are presented as mean ± SD.



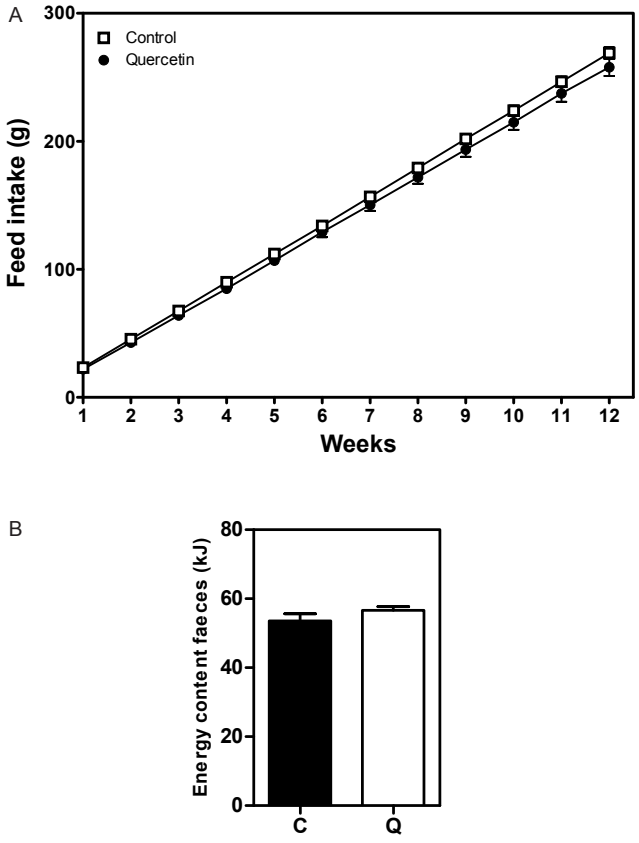


**Figure S4.1 Cumulative feed intake during the 12 weeks intervention period (A) and total energy content of faeces in two weeks (B).** Faeces was collected in weeks 10 and 11. Data are presented as mean ± SEM. White bars indicate the control group (C) and black bars indicate the quercetin group (Q).

# CHAPTER 5

## DIRECT COMPARISON OF METABOLIC HEALTH EFFECTS OF THE FLAVONOIDS QUERCETIN, HESPERETIN, EPICATECHIN, APIGENIN AND ANTHOCYANINS IN HIGH-FAT DIET FED MICE

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## ABSTRACT

Dietary flavonoid intake is associated with reduced risk of cardiovascular diseases, possibly by affecting metabolic health. The relative potency of different flavonoids in causing beneficial effects on energy and lipid metabolism has not been investigated.

Effects of quercetin, hesperetin, epicatechin, apigenin and anthocyanins, in mice fed a high-fat diet (HF) for 12 weeks were compared, relative to normal-fat diet. HF-induced body weight gain was significantly lowered by all flavonoids (17-29%), but most by quercetin. Quercetin significantly lowered HF-induced hepatic lipid accumulation (71%). Mesenteric adipose tissue weight and serum leptin levels were significantly lowered by quercetin, hesperetin, and anthocyanins. Adipocyte cell size and adipose tissue inflammation were not affected.

The effect on body weight and composition could not be explained by individual significant effects on energy intake, energy expenditure or activity. Lipid metabolism was not changed as measured by indirect calorimetry or expression of known lipid metabolic genes in liver and white adipose tissue. Hepatic expression of *Cyp2b9* was strongly down regulated by all flavonoids.

In conclusion, all flavonoids lowered parameters of HF-induced adiposity, with quercetin being most effective.

# 5.1 INTRODUCTION

Fruit and vegetable consumption is regarded as protective against cardiovascular diseases (CVD) [1]. Flavonoids in fruits and vegetables are suggested to be responsible, at least partly, for these protective effects. This is supported by epidemiological studies that have shown that an increased intake of flavonoids is associated with a reduction of CVD risk [2]. Nowadays flavonoid supplements are widely available resulting in even increasing intakes of flavonoids. Elevated circulating lipid levels, a fatty liver and obesity are associated with a higher risk of CVD [3-5]. Previously, we have shown that the flavonoid quercetin can lower serum lipid levels by affecting hepatic lipid metabolism [6], and that quercetin reduced high-fat diet-induced body weight gain and hepatic lipid accumulation [7].



Quercetin is one of the many flavonoids in our diet and is the major representative of the flavonoid subclass of flavonols. Other flavonoid subclasses are flavanones, flavan-3-ols, flavones, isoflavones, and anthocyanins, which all are present in our diet. However, it is not known whether these flavonoids exert similar effects as quercetin on body weight homeostasis and lipid metabolism, and if so, whether they affect the same metabolic pathways. Human intervention studies with fruits or vegetables or extracts rich in flavonols, flavanones, flavan-3-ols, or anthocyanins showed beneficial effects on CVD related parameters such as blood pressure, vascular function or blood lipid profiles [8-10]. Also, flavones, mostly present in herbs and cereals, are suggested to have beneficial effects on CVD risk factors [11]. Therefore, it is interesting to compare the effects of flavonoids from different subclasses on CVD risk factors.

The observed potential beneficial effects of flavonoids in humans are mostly based on studies using flavonoid-rich foods or extracts [12,13]. Therefore, also other components can be responsible for the observed effects. For instance, caffeine was presumably present in studies with green tea catechin extracts, which could be underlying the reduction in body weight gain [14]. Functional effects of flavonoids should therefore be investigated with fully characterized pure compounds. Furthermore, we have shown that effects of quercetin on body weight depended on the percentage and/or composition of fat in the background diet [7]. It is therefore important to directly compare effects using the same diet. A high-fat diet that induces body weight gain and hepatic lipid accumulation seems best suited to reveal the lipid lowering properties of flavonoids.

Flavonoids were selected from five subclasses, based on potential CVD protective effects, and presence in food [8-11,15]. In addition to quercetin (flavonol), hesperetin (flavanone), epicatechin (flavan-3-ol), apigenin (flavone), and a purified extract of mainly cyanindin-3-O-β-glucoside and delphinidin-3-O- β-glucoside (anthocyanins) were selected. The aim of this study was to investigate whether other flavonoids exert similar effects as quercetin, which reduced high-fat induced body weight gain and hepatic lipid accumulation [7]. To better understand the effects, we extend the assessment with analysis of whole body energy balance and other metabolic health related analyses. A comprehensive analysis of whole body energy balance was

performed, including quantification of parameters such as body weight gain, faecal energy loss, energy intake, and activity. Indirect calorimetry was used to evaluate the type and rate of substrate utilization and energy expenditure (EE). Furthermore, circulating lipids, hepatic lipid accumulation and hepatic gene expression patterns were studied. Additionally, the effects of the flavonoids on gene expression in white adipose tissue (WAT) were assessed, together with WAT-secreted serum leptin levels. Leptin is a peptide hormone which regulates body weight gain.

## 5.2 MATERIALS AND METHODS



### Animals and treatments

Eighty-four male C57BL/6JOlaHsd mice (Harlan Laboratories, Horst, The Netherlands) were individually housed and maintained under environmentally controlled conditions (temperature 21°C, 12h/12h light-dark cycle, 55 ± 15 % humidity), with *ad-libitum* access to food and water. At arrival, the mice were 9 weeks of age. During the first five days of a three weeks adaptation period, mice were fed a standard Harlan chow diet, followed by a standardized semi-synthetic normal-fat diet (NF, 10 energy % (en%) fat) with the same dietary constituents as the intervention high-fat diet (HF, 40en%) in which carbohydrates were substituted with fats [16] (Research Diets Services B.V., Wijk bij Duurstede, The Netherlands). At the start of the 12-week intervention period, mice were stratified based on body weight over 7 groups (n=12). One group of mice continued on NF, while the other six groups of mice received HF with or without supplementation of different flavonoids (HF+flavonoids). A subset of data of the control HF and HF+quercetin group were published before (body weight, energy intake, serum quercetin levels, and serum and hepatic lipid levels) [7]. Flavonoids were added in equimolar amounts to HF (0.01 mol/kg diet), amounts were based on our previous results, which showed effectiveness of quercetin at this concentration [6,17]; 0.33% (w/w) quercetin (Sigma, Zwijndrecht, The Netherlands), 0.33% hesperetin (Bioconnect, Huissen, The Netherlands), 0.32% epicatechin (Sigma), 0.29% apigenin (Fuzhou Corona Science & Technology Development Co., Ltd., Fuzhou Fujian, China), and 0.5% anthocyanins, a purified anthocyanin extract from bilberry and black currant consisting of mainly cyanindin-3-O-β-glucoside and delphinidin-3-O- β-glucoside ([18], Medox, Polyphenols Laboratories, Sandnes, Norway). Sufficient amounts of individual anthocyanins were not available. Body weight and food intake were monitored weekly. Faeces were collected in weeks 11 and 12. One HF+quercetin (HF+Q) fed mouse was excluded from all analyses, because of a nasal abscess. At the end of the intervention, all mice were fasted for 2-4 h during the light phase and anesthetized by inhalation of 5% isoflurane using $O_2$ as a carrier. Blood was sampled via orbital extraction in collect serum tubes (Greiner Bio-one, Longwood, USA) and stored at -80°C after obtaining serum. After blood collection, mice were killed by cervical dislocation, and liver, epididymal and mesenteric white adipose tissues (epiWAT and mesWAT, resp.) were dissected, weighted and snap frozen in liquid nitrogen and stored at -80°C. The experiment was performed

according to the Dutch Animal Experimentation Act (1996) and the experimental protocol was approved by the Animal Welfare Committee of Wageningen University, Wageningen, The Netherlands (DEC 2011079).

## HPLC analysis of flavonoid levels in serum and diet

Flavonoid levels in serum were measured using HPLC with coulometric array detection as described [6]. Anthocyanins could not be detected by our method. Before analysis, samples were hydrolyzed by β-glucuronidase/sulfatase to obtain deconjugated flavonoids. Flavonoid levels in the diets were also measured with HPLC to confirm presence and stability.



## Energy content of faeces and diet

Bomb calorimetry was used to determine energy content of diet and faeces (Calorimeter C7000, IKA, Staufen, Germany) as described [7]. Total digestible energy intake over 12 weeks was calculated based on weekly energy intake and faecal energy loss, by multiplying weekly food intake by the measured dietary gross energy content minus the extrapolated faecal energy loss. Digestible energy intake was assumed to be comparable with metabolisable energy intake, as dietary protein content was equal for all diets and no differences in urinary energy losses were expected.

## Indirect calorimetric and activity measurements

Indirect calorimetry and activity were measured in weeks 1, 5 and 11. Indirect calorimetry was performed by an open circuit LabMaster Metabolism Research Platform (TSE systems GmbH, Bad Homburg, Germany) and analysed as described previously [19] with minor adaptations. A reference cage was measured and then rates of oxygen consumption (VO2) and carbon dioxide production (VCO2) were measured during 1 minute every 12 minutes for 48h, of which the last 24h were used. To avoid any influence of initial stress and adaptation, only the data of the last 24h of a 48h measurement were used for analysis. Respiratory exchange ratio (RER) is defined as $VCO_2$ divided by $VO_2$, and EE was calculated using the equation [3.815 + (1.232 x RER)] x $VO_2$ [20]. Carbohydrate and lipid oxidation rates were calculated using [21]. During indirect calorimetry measurements, activity was continuously measured with the ActiMot system (TSE systems GmbH) in eight cages. Infrared beam breaks in horizontal plane (x and y direction) over the last 24hof a 48h period were used.

## Motor performance

Balance and motor coordination was assessed by rotarod (IITC life science, Woodland Hills, USA) in week 9. Latency to fall was recorded on an accelerating rod (3-38 rpm in 300s); mice were placed on the rod four times with an inter trial rest period of 30 minutes; the average of two longest runs per animal were used for analysis.

Several parameters of gait were assessed in week 10 by CatWalk analysis (Noldus Information Technology, Wageningen, The Netherlands) using the reflection of light projected on a glass walking area. Each mouse made at least six compliant runs, being defined as a maximum speed variation of 40%, a minimum run duration of 0.5 seconds and a maximum run duration of 10 seconds. Quantitative gait parameters were analysed using the CatWalk XT 10.0 software (Noldus Information Technology).

## Lipid determination in serum and liver

Because flavonoids were previously shown to interfere with commonly used commercially available enzymatic lipid assays [22], alternative methods were used to measure the amount of lipids in serum and liver, as described [6]. Serum lipids were extracted and analysed with $^{1}$H nuclear magnetic resonance ($^{1}$H-NMR), and neutral lipids were stained in frozen liver sections with Oil red O (Sigma) and quantified.

## RT-qPCR

RNA from liver was isolated using RNeasy columns (Qiagen, Venlo, The Netherlands),RNA from epiWAT was extracted with Trizol (Invitrogen, Breda, The Netherlands) and quality was verified (as published [6,7]). RT-qPCR was performed and analysed as described [6]. Data were normalized using reference genes beta-2 microglobulin (*B2m*) and hypoxanthine phophoribosyltransferase 1 (*Hprt1*) for liver and *Hprt1* and Ribosomal protein S15 (*Rps15*) for epiWAT, chosen based on stable gene expression levels as determined with GeNorm (GeNorm, Ghent University Hospital, Ghent, Belgium). Primer sequences can be found in Supplementary Table S5.1.

## Serum leptin levels

Serum leptin levels were determined using a leptin ELISA kit (Crystal Chem Inc., Chicago, IL, USA) according to the manufacturer's instructions.

## Histology of epididymal white adipose tissue

Paraffin embedded epididymal white adipose tissue (epiWAT) was cut into 5 µm sections and stained using Periodic Acid Schiff Haematoxylin (PASH). Per animal, circumference of at least 400 adipocytes was measured using AxioVision software v4.8 (Carl Zeiss Microscopy GmbH, Jena, Germany). To estimate macrophage infiltration as a marker for tissue inflammation, a MAC-2 staining was performed and analysed as published [23]. Macrophage infiltration is expressed as total number of crown likes structures (CLS) per 100 adipocytes; CLS are formed by macrophages around dying or dead adipocytes.

## Statistical analysis

All statistical analyses were in principle done based on all 12 mice per group with the following exceptions: HF+Q only for 11 mice; for indirect calorimetry measurements (n=9),

histological stainings (n=6), faeces collection (n=4), and serum flavonoid measurements (n=6), subsets of mice as indicated between the brackets were randomly selected, because of limited equipment or practical reasons. GraphPad Prism version 5.03 (Graphpad Software, San Diego, CA, USA) was used for statistical analysis. Data were checked for normality and if needed log transformed (*Acot3*, *Cyp4a14*, *Por*, *Fasn* and *Cyp2b9* in liver and *Cpt1a* in EpiWAT). One-Way ANOVA was used to compare the groups, followed by Dunnett's post hoc test to compare the different HF+flavonoid groups to HF, and HF to NF. For the analysis of data of liver weight, hepatic gene expression of *Acacb* and *Cpt1a*, and WAT gene expression of *Pparg*, a Kruskal-Wallis test was used, because also after log transformation these data were not normally distributed. Curve fitting was used to analyse body weight gain during the 12 week intervention period using Proast software [24]. Two-way ANOVA (no repeated measures) was used for analysis of the lipid profiles in serum and for 24h indirect calorimetry data in time. Pearson correlation analyses were performed using all HF+flavonoids groups and HF group. NF was excluded for these correlation analyses, to avoid false positive strong correlations due to NF. *P*-values smaller than 0.05 were considered significantly different.



## 5.3 RESULTS

### Flavonoid quantification in diets and serum

Flavonoids in the diets were measured at the start of the experiment and were between 87%-99% of the theoretical amounts. After one week at room temperature in the cages, contents of flavonoids in the diets did not change; and varied between 90 and 100% of the theoretical flavonoid amounts (Supplementary Table S5.2).

After 12 weeks on the HF+quercetin diet (HF+Q), the sum of quercetin and isorhamnetin in serum was 6.5 ± 1.4 μM [7]. After the HF+hesperetin diet (HF+H), hesperetin was detectable in half of the measured serum samples (with a detection limit of 150 nM), for which the mean concentration was 0.5 ± 0.8 μM. Epicatechin, and apigenin, were not detectable in serum of HF+epicatechin (HF+E) and HF+apigenin (HF+Ap) mice (with detection limits of 300 nM).

### Body weight, metabolisable energy intake and feed efficiency

Body weight was significantly increased due to HF compared to NF feeding during the whole intervention period, with a cumulative body weight gain of HF mice being four times higher than the weight gain of NF mice (Figure 5.1). Supplementation of HF with any of the flavonoids (HF+flavonoids) reduced the body weight gain significantly, as analyzed by curve fitting analysis (Figure 5.1a). Cumulative body weight gain over 12 weeks was for HF+Q mice 29% lower ($p<0.001$) [7] and for HF+H mice 21% lower ($p<0.05$) than for HF mice (Figure 5.1b). The cumulative metabolisable energy intake over 12 weeks was not significantly different for all HF+flavonoid groups compared to

HF, but it was significant different for HF vs NF (Figure 5.1c). As a result, feed efficiency was significant lower for HF+Q, HF+H and NF compared to HF (Figure 5.1d).

## Indirect calorimetry and activity

Respiratory exchange ratio (RER) and EE were not significantly different between any HF+flavonoid group and HF in week 11, the 24h patterns are shown in Figure 5.2a and

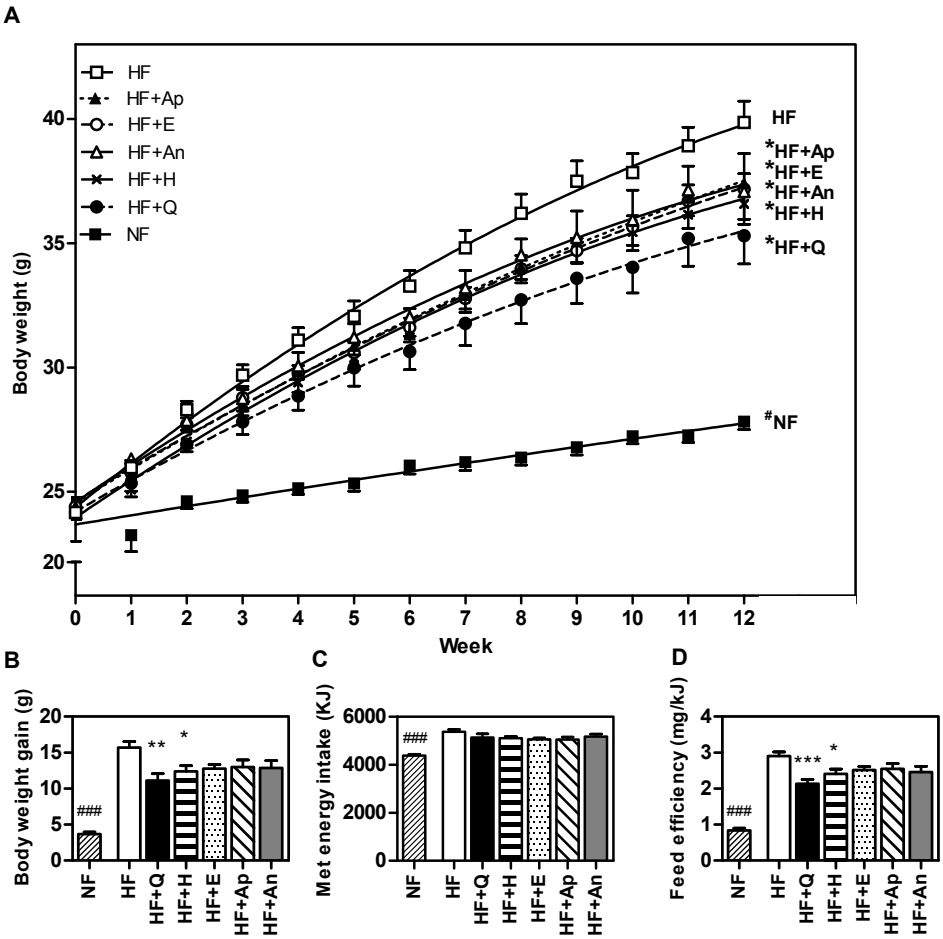


**Figure 5.1.** Flavonoids reduce HF-induced body weight gain and feed efficiency. Body weight of the mice during 12 weeks on a normal-fat diet (NF, 10 en% fat) or a high-fat diet (HF, 40%) with or without supplementation of the flavonoids quercetin (HF+Q), hesperetin (HF+H), epicatechin (HF+E), apigenin (HF+Ap) or anthocyanins (HF+An) (A). Total body weight gain over 12 weeks (B). Total metabolisable energy intake over 12 weeks (C). Total food efficiency, which is the ratio of body weight gain over metabolisable energy intake (D). Data are presented as mean ± SEM. * indicates a significant difference of HF+flavonoid to HF ($p<0.05$), **$p<0.01$, ***$p<0.001$, # indicates a significant difference of HF to NF ($p<0.05$), ###$p<0.001$.

5.2b. Mean 12h light and dark phase RER and EE values are in Supplementary Table S5.3. Mean RER values varied from 0.84-0.87, which implies that 48.3%-58.5% of the energy comes from glucose oxidation and 51.7%-41.5% from fat oxidation. HF mice had a significant lower RER compared to NF, which is caused by the lower carbohydrate and higher fat levels present in HF. EE was not significant different between HF and NF mice. HF mice were significantly less active during the dark phase compared to NF mice. No differences were observed for activity of HF+flavonoids groups compared to HF during the dark phase as well as the light phase (Figure 5.2c). RER, EE and activity for weeks 1 and 5 were all comparable to those of week 11 (Supplementary Figure S5.1).



## Motor performance

HF mice performed significantly poorer on the rotarod than NF mice, likely due to their higher body weight. Performance on rotarod was, however, not significantly different between any HF+flavonoid group and HF (Supplementary Figure S5.2). There were also no significant differences between the HF+flavonoid groups and HF in the measured gait parameters (Supplementary Table S5.4), however some parameters showed significant differences between HF and NF, also likely related to the large body weight differences.

## Serum lipids

HF significantly induced higher levels of 'MUFA and PUFA', 'PUFA', '18:2 fatty acids', '18:1 and 16:1 fatty acids' and 'omega 3 fatty acids' in serum compared to NF. No significant differences between HF and HF+flavonoid were observed in the serum lipid levels; however, some of the flavonoids showed a small trend towards the levels found for NF (Figure 5.3a).

## Hepatic lipid accumulation

HF-feeding significantly induced hepatic lipid accumulation as is apparent from an increase in liver weight (Figure 5.3b), area of lipids (Figure 5.3c) and lipid droplet number (Figure 5.3d) compared to values for NF-feeding. Liver weight was not affected by the flavonoid supplementations to the diets. Only supplementation with quercetin (HF+Q) significantly reduced the HF-induced lipid accumulation. As compared to HF, quercetin lowered the area of lipids by 71%, and the lipid droplet number by 31%. Representative pictures of hepatic lipid stainings are presented in Figure 5.3e.

## Hepatic gene expression

Hepatic expression levels of genes involved in fatty acid omega-oxidation were studied based on our previous observations that quercetin supplementation to a mild high-fat (30en%) diet-induced this pathway in liver [6]. HF feeding compared to NF revealed significant induction of omega-oxidation by regulation of *Acot3, Cyp4a10*, and *Cyp4a14.* However, no significant regulation of these genes, nor *Por* was observed in any of the HF+flavonoid groups compared to HF (Table 5.1). Subsequently, also other

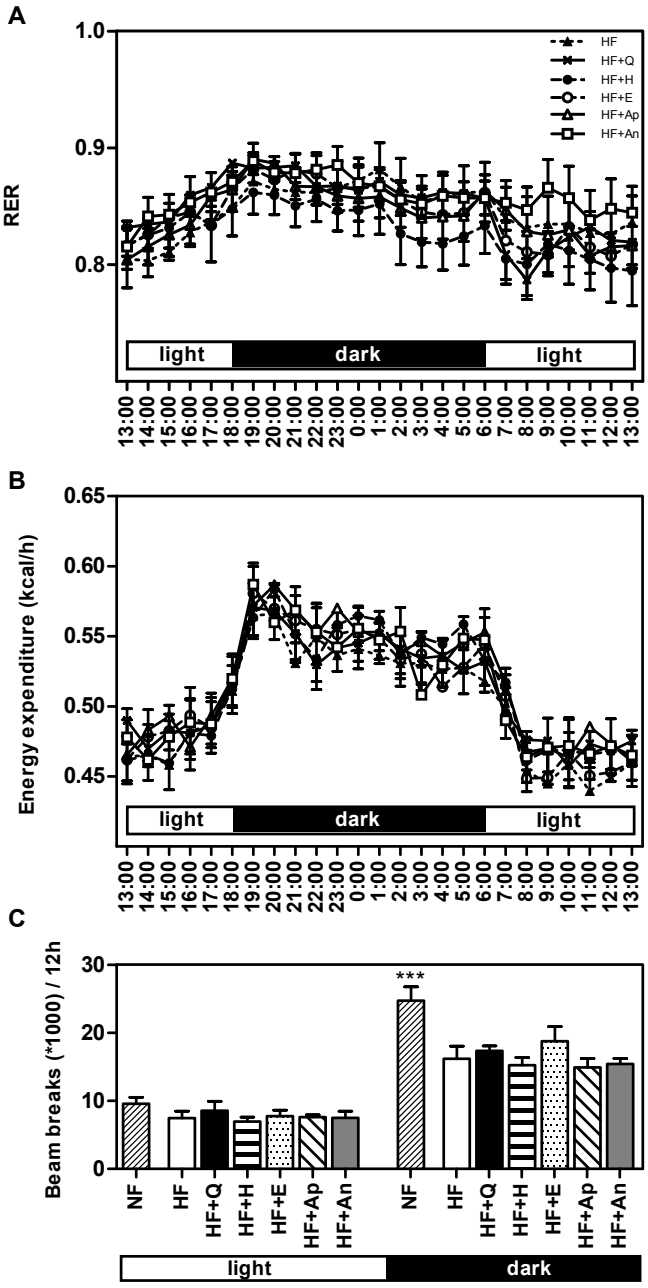


**Figure 5.2 Whole body RER, EE, and activity levels were not affected by flavonoid supplementation.** Indirect calorimetry measurements of 24h RER (A) and EE (B) in week 11. Activity measured during indirect calorimetry measurements for light (12 h) and dark (12h) period (C). Data are presented as mean ± SEM. ## indicates a significant difference of HF to NF ($p<0.001$). RER; respiratory exchange ratio, EE; energy expenditure, NF; normal fat diet, HF; high-fat diet; HF supplemented with quercetin; HF+Q, hesperetin; HF+H, epicatechin; HF+E, apigenin; HF+Ap, anthocyanins; HF+An.

genes involved in lipid metabolism were studied. *Acacb* and *Ppargc1a* were significantly regulated by HF vs NF. However, *Acacb*, *Fasn*, *Cpt1a*, *Ppara*, and *Ppargc1a* were not significantly different for any of the HF+flavonoid diets compared to HF (Table 5.1).

*Cyp2b9*, a target gene of the transcription factor CAR, was strongly up regulated by HF vs NF. Interestingly, this gene was significantly lower expressed upon supplementation of HF with all of the flavonoids (FC between -5.9 and -9.1, Table 5.1). *Cyp2b9* expression levels for all groups are shown in Figure 5.3f.



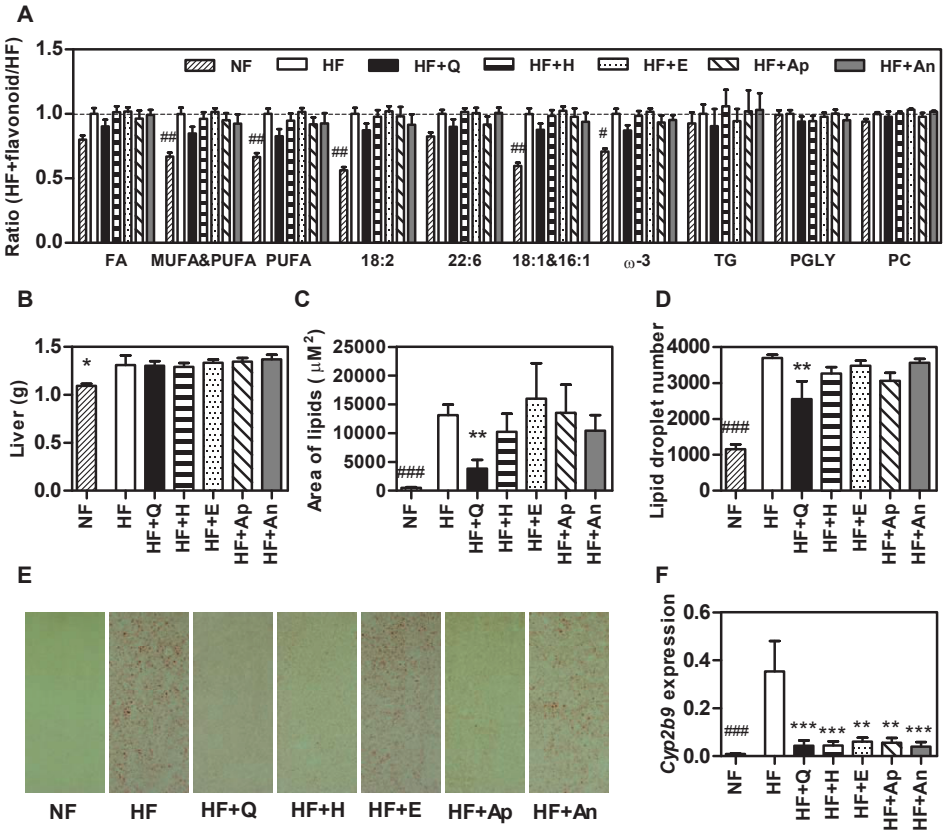


**Figure 5.3 Effects of flavonoids on HF-induced effects on serum lipids, liver weight and hepatic lipid accumulation.** Serum lipid fractions are shown as ratio of HF+flavonoid or NF over the average value of the HF-control (A). Liver weight at the end of the dietary intervention of 12 weeks (B). Quantification of total area of lipids per picture (C) and mean lipid droplet number per picture (D) in liver. Representative pictures of stained hepatic lipids by Oil red O (E). Hepatic Cyp2b9 expression (F). Data are presented as mean ± SEM. * indicates a significant difference of HF+flavonoid to HF, # indicates a significant difference of HF to NF ($p<0.05$), $^{\#\#}p<0.01$. PUFA; poly unsaturated fatty acids, MUFA; mono unsaturated fatty acids, FA; fatty acids, TG; triglycerides, PGLY; phosphoglycerides, PC; phosphatidylcholine, EC; esterified cholesterol, TC; total cholesterol, NF; normal fat diet, HF; high-fat diet; HF+Q, HF supplemented with quercetin; HF+Q, hesperetin; HF+H, epicatechin; HF+E, apigenin; HF+Ap, anthocyanins; HF+An.

**Table 5.1** Hepatic gene expression (RT-qPCR).

| | Gene Symbol | HF | HF+Q | HF+H | HF+E | HF+Ap | HF+An |
|---|---|---|---|---|---|---|---|
| | | vs NF | | | vs HF | | |
| Lipid Omega-Oxidation | Acot3 | 2.67$^{\#\#}$ | 1.07 | -1.07 | 1.07 | 1.16 | 1.05 |
| | Cyp4a10 | 2.06$^{\#\#}$ | -1.09 | 1.15 | 1.05 | -1.12 | 1.05 |
| | Cyp4a14 | 2.39$^{\#\#\#}$ | 1.11 | 1.23 | 1.09 | 1.06 | 1.04 |
| | Por | 1.34 | 1.08 | 1.14 | 1.12 | 1.07 | 1.02 |
| Lipid Metabolism | Acacb | -1.76$^{\#\#}$ | 1.07 | 1.01 | 1.11 | -1.10 | 1.12 |
| | Fasn | -1.10 | 1.10 | 1.05 | 1.17 | -1.05 | 1.02 |
| | Cpt1a | 1.25 | -1.06 | 1.05 | -1.02 | -1.12 | -1.01 |
| | Ppara | 1.29 | -1.07 | 1.09 | 1.02 | -1.09 | 1.01 |
| | Ppargc1a | -1.51$^{\#\#}$ | -1.06 | 1.05 | 1.10 | 1.18 | -1.09 |
| CAR target | Cyp2b9 | 40.60$^{\#\#\#}$ | -8.22*** | -8.02*** | -5.85** | -6.33** | -9.05*** |

Data are presented as mean fold change of HF vs NF and HF+flavonoids vs HF. $^{\#\#}$ indicates significant difference of HF to NF ($p<0.01$), $^{\#\#\#}p<0.001$, ** indicates a significant difference of HF+flavonoid to HF ($p<0.01$), ***$P<0.001$. NF; normal fat diet, HF; high-fat diet; HF supplemented with quercetin; HF+Q, hesperetin; HF+H, epicatechin; HF+E, apigenin; HF+Ap, anthocyanins; HF+An.

## Gene expression in white adipose tissue

Based on the above described results, it was decided to study gene expression in epiWAT only for NF, HF, HF+Q and HF+H groups. Genes were selected by their functions related to lipid metabolism. This showed significant regulation of *Fasn*, *Cpt1α*, *Ppargc1α*, *Pnpla2*, and *Lep* due to HF compared to NF feeding. *Lep* gene expression showed significant down regulation for HF+Q and HF+H mice compared to HF. No significant regulation by quercetin or hesperetin was observed for the other genes (Table 5.2).

## White adipose tissue and leptin

HF feeding significantly induced relative mesWAT and epiWAT weights compared to NF feeding. Supplementation with the flavonoids prevented this induction of relative mesWAT weight, which was significantly lower for HF+quercetin, HF+hesperetin and HF+anthocyanins compared to HF (Figure 5.4a). Relative epiWAT was only significantly decreased for HF+quercetin vs HF (Figure 5.4b). A trend for decreased relative WAT weights was seen for most of the HF+flavonoid diets compared to HF, in line with their reduced body weights.

Likewise, serum leptin levels, which are known to be correlated to total adipose tissue mass, showed significant higher values for HF mice compared to NF mice. Quercetin, hesperetin and anthocyanins supplementation to HF significantly lowered serum leptin levels compared to HF (Figure 5.4c), which also corresponds to the gene expression results of *Lep*.

**Table 5.2** Gene expression in white adipose tissue.

| Gene Symbol | Function | HF | HF+Q | HF+H |
|---|---|---|---|---|
| | | vs NF | vs HF | |
| Fasn | Fatty acids synthesis | -4.19### | 1.05 | -1.18 |
| Cpt1α | Fatty acid beta-oxidation | 1.83### | -1.32 | -1.22 |
| Ppargc1α | mitochondrial biogenesis | -2.13### | 1.10 | -1.10 |
| Lipe | Lipolysis | -1.10 | 1.02 | -1.05 |
| Pnpla2 | Lipolysis | -1.66### | 1.27 | 1.14 |
| Lep | Leptin | 5.80### | -1.70*** | -1.52** |
| Pparg | Adipocyte differentiation | -1.48 | -1.00 | -1.14 |
| Clmp | Adipocyte differentiation | 1.25 | -1.13 | -1.20 |

Data are presented as mean fold change of HF vs NF and HF+flavonoids vs HF. ### indicates significant difference of HF to NF (p<0.001),** indicates a significant difference of HF+flavonoid to HF (p<0.01), ***P<0.001. NF; normal-fat diet, HF; high-fat diet, HF+Q; HF supplemented with quercetin, HF+H; hesperetin.



Adipocyte size (Figure 5.4d) and macrophage infiltration (Figure 5.4e) in epiWAT were determined, and representative pictures of adipocyte stainings are shown in figure 5.4f. Mean adipocyte size was significantly higher for HF mice vs NF mice. Remarkably, supplementation of flavonoids to the HF diet did not significantly affect the HF-induced increased adipocyte size, indicating that the lower observed WAT weights were not due to smaller adipocytes; consequently, the number of adipocytes should be decreased.

MAC-2 staining as a marker of macrophage infiltration of epiWAT revealed no indication for inflammation [25] for all groups.

### Correlations

Pearson correlations between all parameters measured showed significant correlations between body weight gain and all parameters for HF and all HF+flavonoid groups (Figure 5.5a), except for the indirect calorimetry measurements (RER and EE). Strongest correlation was found between body weight gain and leptin (r=0.94, p<.0001, Figure 5.5b). Interestingly, *Cyp2b9* expression showed also a strong correlation (p<0.001) with body weight gain (Figure 5.5c), metabolisable energy intake, relative weights of epiWAT and mesWAT, and with serum leptin levels. The correlation plots shown in Figure 5.5b and 5.5c indicate quercetin as most potent flavonoid to modify the HF-induced effects in the direction of NF effects.

## 5.4 DISCUSSION

As expected, HF (40 en% fat) induced adiposity, as seen by body weight gain, more visceral adipose tissue and hepatic lipid accumulation, compared to NF (10 en% fat). We examined

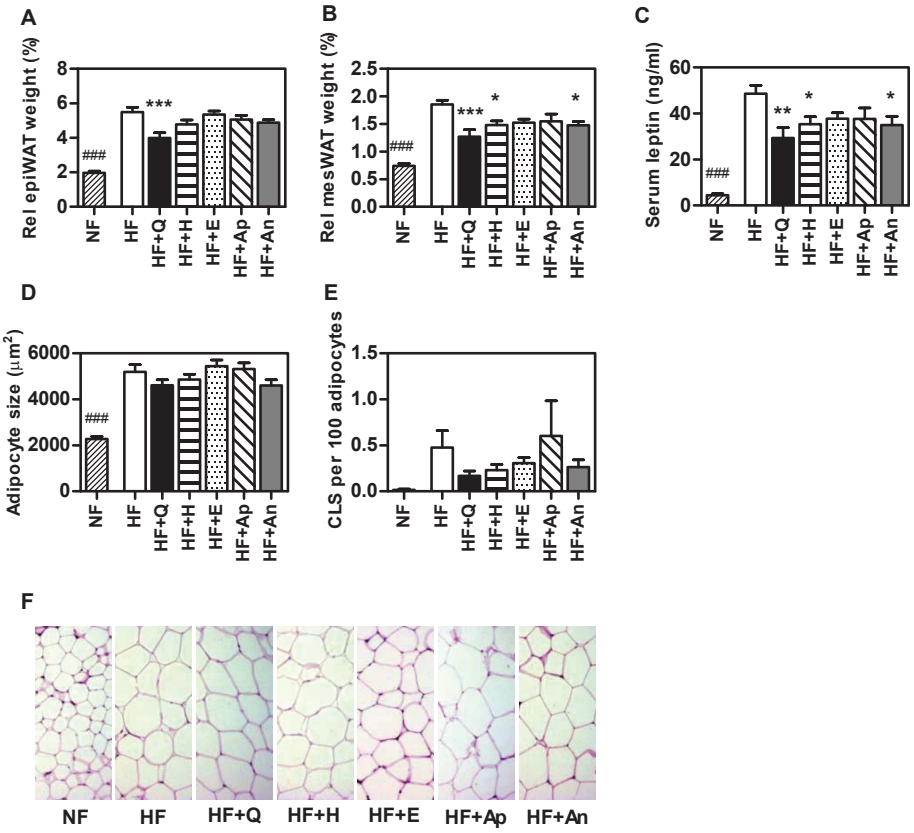


**Figure 5.4 Effects of flavonoids on HF-induced effects on white adipose tissue weights, serum leptin levels, adipocyte size and crown like structures in epiWAT.** Mesenteric white adipose tissue (mesWAT, A) and epididymal tissue (epiWAT, B) relative weight (gram/gram body weight) at the end of the dietary intervention. Serum leptin levels (C). Quantification of adipocyte sizes (D) and crown like structures (CLS) by macrophage staining (E) in epiWAT. Representative pictures of adipocyte stainings (F). Data are presented as mean ± SEM. * indicates a significant difference of HF+flavonoid to HF ($p<0.05$), **$p<0.01$, ***$p<0.001$, ### indicates a significant difference of HF to NF ($p<0.001$). NF; normal fat diet, HF; high-fat diet; HF supplemented with quercetin; HF+Q, hesperetin; HF+H, epicatechin; HF+E, apigenin; HF+Ap, anthocyanins; HF+An.

whether this unfavourable change could be prevented by individual supplementation of HF with five different flavonoids: quercetin, hesperetin, epicatechin, apigenin, or anthocyanins. All flavonoids reduced HF-induced body weight gain and hepatic *Cyp2b9* expression. Quercetin was the most potent flavonoid in beneficially affecting the HF-disturbed whole body energy balance and lipid handling. In addition to quercetin, also hesperetin and anthocyanins decreased relative mesWAT weights and serum leptin levels. None of the flavonoids affected energy intake, substrate use, and energy expenditure.

The reduced body weight gain by all flavonoids was not caused by reduced intake of metabolisable energy. As an example, for HF+quercetin fed mice metabolisable

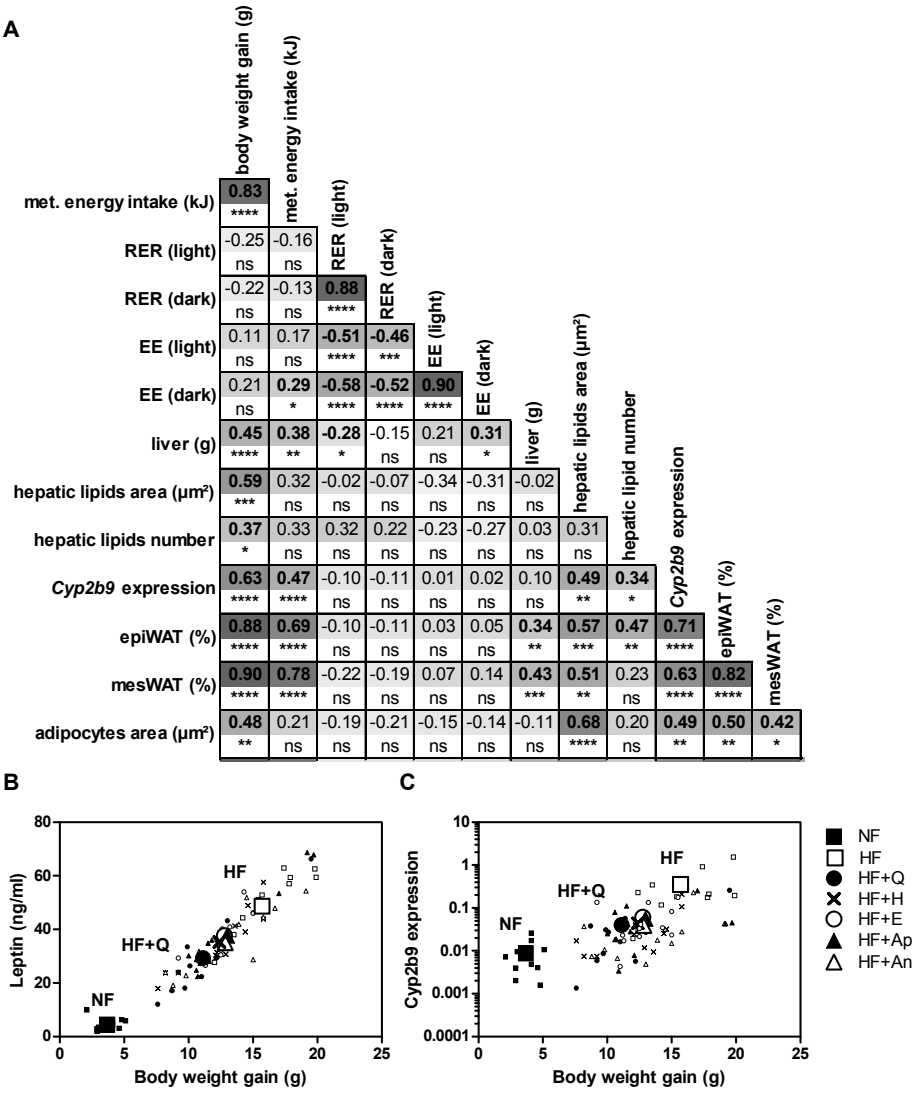


**Figure 5.5 Correlations between measured parameters.** Pearson correlations between body weight gain, metabolisable energy intake, RER, EE, liver weight, hepatic lipid accumulation, *Cyp2b9* expression, weight and area of WAT, and serum leptin levels were analysed for HF and all HF+flavonoids groups (A). Pearson r values are presented, with underneath the significance of the correlation; *p<0.05, **p<0.01, ***P<0.001, ****P<0.0001. Correlation plot of BW gain to leptin for HF, HF+flavonoids and NF (B). Correlation plot of BW gain to cyp2b9 expression (log2) for HF, HF+flavonoids and NF (C). Large symbols indicate the mean of the value per group, small symbols indicate the individual mice.

intake was marginally lower than for HF mice, but this difference can only explain a lower body weight of ~0.5 gram, based on the observed feed efficiency, instead of the observed reduction of 4.6 gram after 12 weeks. Also activity and energy expenditure

measurements in weeks 1, 5, and 11 of the intervention showed no differences between the HF+flavonoid groups and HF, and could therefore not explain the differences in final body weight. These results correspond with previous data which showed that changes in energy expenditure could not explain weight differences due to quercetin supplementation in mice [26,27]. Overall, energy intake, energy demand or energy loss, were not different between the HF+flavonoid groups and the HF control group and therefore cannot explain the flavonoid-induced body weight differences.

The flavonoid-induced body weight differences could neither be explained by an increase in lipid oxidation, as suggested earlier [6,28]. Our indirect calorimetry measurements showed no indication for differences in substrate use between the different HF+flavonoid groups and HF. There was no lowering of the RER value, which would indicate increased fatty acid versus glucose oxidation. This confirms an earlier observation for quercetin supplementation showing an absence of effects on RER values [26]. Also serum lipid fractions were not significantly changed by the flavonoids; only some lipid fractions showed a small tendency to decrease into the direction of values observed for NF mice. Furthermore, except *Cyp2b9,* all studied lipid metabolism genes, in liver and WAT for any of the flavonoid supplementations showed no significant differences in expression levels compared to HF. This indicates that the flavonoids did not induce measurable changes in lipid metabolism, which therefore cannot serve as an explanation for the observed body weight differences.

Since individual parameters alone cannot explain the effect of the flavonoids on HF-induced increases in body weight, this effect may possibly be explained by several smaller changes that cumulatively contribute to the total body weight balance. Besides reduction of body weight gain, also lowering of serum leptin levels and relative mesenteric adipose tissue weights were found by supplementation of quercetin, epicatechin and anthocyanins. We therefore analysed to which extent the various parameters measured were correlated to each other. Body weight gain, WAT weights, and leptin levels were strongly correlated (Figure 5.5c, $R>0.85$, $p<0.001$). Also, significant correlations were found for metabolisable energy intake, serum leptin levels, WAT percentages, adipocytes area, hepatic lipid accumulation, and hepatic *Cyp2b9* expression with many of the measured parameters, including body weight gain. In contrast, energy expenditure or RER were not correlated with body weight gain. Overall, these correlation analyses confirm that energy expenditure and lipid oxidation alone cannot explain the body weight gain lowering effect of the flavonoids. These correlations imply that several effects occur, which are related to the body weight gain differences, and these effects could possibly cumulatively contribute to the weight lowering effects.

It is of interest to note that whole body energy balance was studied throughout the experiment at several time points. Total energy intake, which was corrected for faecal losses, activity, and energy expenditure were taken into account, providing a comprehensive picture of whole body energy balance, a major strength of this study. In addition, liver as well as white adipose tissue were investigated, which gives an extensive overview in comparing the effects of the different flavonoids.

Additionally, the effects of flavonoid supplementations on the motor and gait performances was studied, because flavonoids are also suggested to have neuroprotective actions [29], which could lead to improved motor performance. No improvement was observed, however, also no indication for adverse effects on these performance parameters was obtained.

Furthermore, we measured the presence of the flavonoids in the diets and in serum, which showed absorption of quercetin and hesperetin. The other flavonoids were not detectable in serum, which could be ascribed to their pharmacokinetics known to include a relatively fast rate of elimination [15,30,31].

Interestingly, hepatic *Cyp2b9* transcript levels were strongly down regulated by all flavonoids compared to HF ($p<0.01$). *Cyp2b9* and C*yp2b10* are the major mice homologs of human CYP2B6. These P450 enzymes are involved in metabolism of exogenous and endogenous compounds, such as steroid hormones, prostaglandins, and fatty acids. Furthermore, *Cyp2b9* is known to be regulated by the transcription factor CAR [32,33], which we previously proposed as a possible target of quercetin [6,7]. Importantly, HF increased *Cyp2b9* expression 40-fold compared to NF. This strong up regulation of *Cyp2b9* by HF suggests that the amount of lipids in the diet influenced the transcription level of *Cyp2b9*. In a HF dietary context, flavonoids down regulated *Cyp2b9* with fold changes ranging from 6 to 9. This decreased expression of *Cyp2b9* in the various HF+flavonoid groups as compared to the HF group positively correlated with reduced body weight gain, mesenteric WAT weight, serum leptin levels, and hepatic lipid accumulation. These results suggest a relation of the effects of flavonoids on *Cyp2b9* with lipid homeostasis, which could be possibly regulated via CAR. Overall, the reduced *Cyp2b9* transcript levels by all flavonoids indicate a relation between *Cyp2b9* and the preventive effects of the flavonoids on the HF-induced effects. This suggests that *Cyp2b9* expression can be a marker in a possible common mode of action of the flavonoids. It is tempting to speculate that the newly observed common effects of the flavonoids on hepatic Cyp2b9 expression and adiposity are mechanistically related, but this requires further investigation.

Of all studied flavonoids, quercetin showed the strongest lowering effects on HF-induced parameters. Quercetin not only affected most parameters, but also showed the most prominent effects. This is supported by correlation analysis (Figure 5.5a). It is particularly illustrated by the correlation plot of leptin vs. body weight gain (Figure 5.5b) and of Cyp2b9 vs body weight gain (Figure 5.5c), which indicate that quercetin better prevented the HF-induced effects resulting in values closer to those observed for mice fed NF.

Furthermore, the strong correlation of leptin and body weight gain for all HF+flavonoid groups suggests that leptin can be used as a sensitive marker for the effects on adiposity. This is especially of interest given that leptin, known to regulate body weight gain, can be measured easily and rapidly in circulation. This positions leptin as a potential practical and useful marker to quantify flavonoid effects on HF-induced adiposity, to be used in animal as well as human studies.

In conclusion, a direct comparison of metabolic effects of quercetin, hesperetin, epicatechin, apigenin and anthocyanins, indicated that all flavonoids beneficially affected HF-induced disturbance of whole body energy balance and lipid handling, with serum leptin levels as a sensitive marker. This confirms the suggested potential of these flavonoids in lowering CVD risk factors. Furthermore, the reduction of hepatic *Cyp2b9* transcript levels was shown for all flavonoids. Overall, quercetin appeared to be the most potent flavonoid in preventing HF-induced effects.

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**Table S5.1** Sequences of the primers used for RT-qPCR.

| Gene Symbol | Forward primer (5'-->3') | Reverse primer (5'-->3') | Anealing temp (°C) |
|---|---|---|---|
| Acot3 | GCTGTGACCTACCTGCTCAGTCA | ATATAGAGCCATTGATGATGACAGCGG | 60 |
| B2m | CCCCACTGAGACTGATACATACGC | AGAAACTGGATTTGTAATTAAGCAGGTTC | 62 |
| Clmp | CAGTGTGAGTCTGCCTCTG | CAGTCACCCGTACCACAC | 58 |
| Cpt1a | AAAGATCAATCGGACCCTAGACA | CAGCGAGTAGCGCATAGTCA | 57 |
| Csad | CCATGTGGATGCTGCCTGGGG | AAGAGCGGAGCACTGCAGCC | 56.7 |
| Cyp2b9 | ACCACAAGCGCCACCCTCCA | TGGTAGCCGGTGTGAGCCGAT | 55.7 |
| Cyp4a10 | TCTACCCACCTGTCCCAGGC | ACACCTCTGGATTTGGCCACA | 58 |
| Cyp4a14 | TTCTTTCGCCTGCGGAATGC | CACTCCATCTGTGTGCTCGTGA | 62 |
| Fabp5 | GTGGCGCCTGATGGAAAGCC | TCCACGATCATCTTCCCATCCTTCA | 58 |
| Fasn | AGTTAGAGCAGGACAAGCCCAAG | GTGCAGAGCTGTGCTCCTGA | 55 |
| Hao2 | TGTTGAAGGCACTAGCCCTTGGAG | AGGTCTGGACTGATCTCAGCAACTG | 58 |
| Hprt1 | TGACACTGGTAAAACAATGCAAACTTTG | GAGGTCCTTTTCACCAGCAAGCT | 62 |
| Lep | GGCTTTGGTCCTATCTGTCTTATGTTC | CCCTCTGCTTGGCGGATACC | 60 |
| Lipe | TGGAACTAAGTGGACGCAAGCC | TCAAGGTATCTGTGCCCAGTAAGC | 58 |
| Pnpla2 | ACCACCCTTTCCAACATGCTACC | GCTACCCGTCTGCTCTTTCATCC | 58 |
| Pparg | ACCACTCGCATTCCTTTGAC | AAGGCACTTCTGAAACCGAC | 63.5 |
| Ppargc1a | CCCTGCCATTGTTAAGACC | TGCTGCTGTTCCTGTTTTC | 60 |
| Por | CGAGGGCAAGGAGCTGTACC | CACAGGTGGTCGATGGGTGG | 62 |
| Tfrc | CCTTGCACTCTTTGGACATGCTCATC | AACCCTGATGACTGAGATGGCGG | 60 |

Primers were designed using the NCBI Primer-Blast (NCBI Web site) or based on literature for *Ppargc1a*[1] , *Clmp*[2]. *Acot3*, acyl-CoA thioesterase 3; *B2m*, Beta-2 microglobulin; *Clmp*, CXADR-like membrane protein; *Cpt1a*, Carnitine palmitoyltransferase 1A; *Csad*, Cysteine sulfinic acid decarboxylase; *Cyp2b9*, Cytochrome P450 family 2b9; *Cyp4a10*, Cytochrome P450 family 4a10; *Cyp4a14*, cytochrome P450 family 4a14; *Fabp5*, Fatty acid binding protein 5 epidermal; *Fasn*, Fatty acid synthase; *Hao2*, Hydroxyacid oxidase 2; *Hprt1*, hypoxanthine phophoribosyltransferase 1; *Lep*, Leptin; *Lipe*, Lipase, hormone sensitive; *Pnpla2*, Patatin-like phospholipase domain; *Pparg*, Peroxisome proliferator activated receptor gamma; *Ppargc1a*, Peroxisome proliferator activated receptor; *Por*, P450 (cytochrome) oxidoreductase; *Tfrc*, transferrin receptor.

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**Table S5.2** Flavonoid quantification in diets at the start of the experiment stored at -20 °C and after one week at room temperature (RT).

| | Flavonoid start levels (%) | Flavonoid levels after one week at RT (%) |
|---|---|---|
| Quercetin | 88 | 92 |
| Hesperetin | 97 | 93 |
| Epicatechin | 99 | 100 |
| Apigenin | 92 | 95 |
| Anthocyanins | 87 | 90 |

Data are presented as percentage of the theoretical amount of flavonoids added to the diets.



**Table S5.3** Gait analysis with catwalk in week 10.

| Parameter | NF | HF | HF+Q | HF+H | HF+E | HF+Ap | HF+An |
|---|---|---|---|---|---|---|---|
| Run Duration (s) | 2.89 | 3.35 | 3.28 | 3.09 | 3.21 | 3.09 | 3.06 |
| RF Stand (s) | 0.17 | 0.20 | 0.19 | 0.19 | 0.19 | 0.19 | 0.19 |
| RF StandIndex | -4.79 | -3.84 | -3.88 | -4.08 | -3.97 | -4.15 | -4.12 |
| RF MaxContactAt (%) | 35.80 | 35.83 | 34.95 | 36.73 | 35.16 | 35.78 | 37.83 |
| RF MaxContactArea ($cm^2$) | 0.22 | 0.25 | 0.24 | 0.25 | 0.25 | 0.24 | 0.24 |
| RF MaxContactMaxIntensity | 139.78 | 145.70 | 142.61 | 145.27 | 143.50 | 143.00 | 147.11 |
| RF MaxContactIntensity | 71.09 | 72.95 | 71.45 | 72.41 | 72.81 | 71.69 | 72.94 |
| RF PrintLength (cm) | 0.84 | 0.87 | 0.84 | 0.84 | 0.86 | 0.84 | 0.86 |
| RF PrintWidth (cm) | 0.75 | 0.78 | 0.76 | 0.76 | 0.79 | 0.76 | 0.75 |
| RF PrintArea ($cm^2$) | 0.30 | 0.33 | 0.32 | 0.32 | 0.32 | 0.31 | 0.32 |
| RF MaxIntensityAt (%) | 55.19 | 61.04 | 57.58 | 61.06 | 59.83 | 60.23 | 61.34 |
| RF MaxIntensity | 148.73 | 157.95 | 153.53 | 157.14 | 155.60 | 155.13 | 159.49 |
| RF MinIntensity | 36.52 | 36.34 | 36.49 | 36.38 | 36.48 | 36.37 | 36.44 |
| RF Intensity | 74.70 | 77.34 | 75.61 | 76.92 | 77.01 | 75.86 | 76.82 |
| RF IntensityOfThe15MostIntensePixels | 114.00 | 120.52 | 116.98 | 119.92 | 119.54 | 116.59 | 120.82 |
| RF Swing (s) | 0.13 | 0.14 | 0.14 | 0.14 | 0.14 | 0.14 | 0.14 |
| RF SwingSpeed (cm/s) | 53.67 | 51.58 | 49.04 | 48.65 | 49.74 | 48.66 | 47.79 |
| RF StrideLength (cm) | 6.88 | 6.79 | 6.69 | 6.59 | 6.72 | 6.40 | 6.55 |
| RF StepCycle (s) | 0.30 | 0.33 | 0.34 | 0.33 | 0.33 | 0.32 | 0.33 |
| RF DutyCycle (%) | 55.15 | 58.46 | 56.65 | 56.64 | 56.97 | 57.45 | 56.50 |
| RF SingleStance (s) | 0.13 | 0.14 | 0.14 | 0.14 | 0.14 | 0.14 | 0.14 |
| RF InitialDualStance (s) | 0.02 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.02 |
| RF TerminalDualStance (s) | 0.02 | 0.03 | 0.02 | 0.03 | 0.02 | 0.03 | 0.03 |
| RH Stand (s) | $0.14^{###}$ | 0.18 | 0.17 | 0.17 | 0.17 | 0.17 | 0.17 |
| RH StandIndex | $-8.72^{###}$ | -6.37 | -6.72 | -6.78 | -6.60 | -7.02 | -6.62 |

**Continuing of Supplementary Table S5.3**

| Parameter | NF | HF | HF+Q | HF+H | HF+E | HF+Ap | HF+An |
|---|---|---|---|---|---|---|---|
| RH MaxContactAt (%) | 28.28 | 27.02 | 26.75 | 25.78 | 27.12 | 28.10 | 27.40 |
| RH MaxContactArea (cm²) | 0.20# | 0.24 | 0.25 | 0.25 | 0.25 | 0.24 | 0.25 |
| RH MaxContactMaxIntensity | 162.92 | 172.67 | 173.94 | 170.76 | 169.42 | 171.17 | 168.14 |
| RH MaxContactIntensity | 79.27 | 82.09 | 82.87 | 82.49 | 80.98 | 80.97 | 81.57 |
| RH PrintLength (cm) | 0.79# | 0.86 | 0.88 | 0.83 | 0.87 | 0.86 | 0.86 |
| RH PrintWidth (cm) | 0.68 | 0.75 | 0.74 | 0.72 | 0.76 | 0.75 | 0.76 |
| RH PrintArea (cm²) | 0.26## | 0.32 | 0.33 | 0.31 | 0.32 | 0.31 | 0.32 |
| RH MaxIntensityAt (%) | 45.71 | 52.42 | 52.44 | 48.88 | 52.16 | 52.45 | 52.23 |
| RH MaxIntensity | 172.38# | 182.99 | 184.92 | 179.76 | 179.63 | 180.92 | 178.84 |
| RH MinIntensity | 39.45 | 38.31 | 38.82 | 39.22 | 39.35 | 38.40 | 38.99 |
| RH Intensity | 83.99 | 87.22 | 87.60 | 87.73 | 86.24 | 85.92 | 86.83 |
| RH IntensityOfThe15MostIntensePixels | 132.83 | 146.12 | 147.84 | 144.97 | 144.10 | 143.35 | 144.04 |
| RH Swing (s) | 0.13 | 0.14 | 0.14 | 0.13 | 0.13 | 0.14 | 0.13 |
| RH SwingSpeed (cm/s) | 46.73 | 46.56 | 44.61 | 44.30 | 46.58 | 43.98 | 44.80 |
| RH StrideLength (cm) | 6.10 | 6.24 | 6.00 | 5.89 | 5.91 | 5.94 | 5.95 |
| RH StepCycle (s) | 0.27 | 0.31 | 0.30 | 0.30 | 0.29 | 0.30 | 0.30 |
| RH DutyCycle (%) | 49.77## | 54.71 | 53.73 | 53.87 | 54.90 | 53.21 | 53.80 |
| RH SingleStance (s) | 0.12 | 0.13 | 0.13 | 0.13 | 0.12 | 0.12 | 0.12 |
| RH InitialDualStance (s) | 0.01 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |
| RH TerminalDualStance (s) | 0.01 | 0.03 | 0.03 | 0.03 | 0.02 | 0.02 | 0.03 |
| LF Stand (s) | 0.17 | 0.19 | 0.19 | 0.19 | 0.19 | 0.19 | 0.19 |
| LF StandIndex | -4.48 | -4.16 | -3.89 | -3.94 | -3.87 | -4.02 | -3.82 |
| LF MaxContactAt (%) | 34.41 | 34.55 | 34.73 | 35.89 | 35.32 | 34.45 | 34.01 |
| LF MaxContactArea (cm²) | 0.22## | 0.26 | 0.24 | 0.25 | 0.24 | 0.24 | 0.24 |
| LF MaxContactMaxIntensity | 136.84 | 145.75 | 142.53 | 146.92 | 145.99 | 143.82 | 144.91 |
| LF MaxContactIntensity | 70.40 | 72.39 | 71.61 | 72.87 | 72.52 | 71.50 | 72.00 |
| LF PrintLength (cm) | 0.82 | 0.86 | 0.83 | 0.86 | 0.84 | 0.84 | 0.84 |
| LF PrintWidth (cm) | 0.74 | 0.76 | 0.76 | 0.76 | 0.75 | 0.76 | 0.79 |
| LF PrintArea (cm²) | 0.30 | 0.33 | 0.31 | 0.33 | 0.31 | 0.31 | 0.31 |
| LF MaxIntensityAt (%) | 57.58 | 61.34 | 59.85 | 61.03 | 59.17 | 60.41 | 60.56 |
| LF MaxIntensity | 148.27 | 158.10 | 155.26 | 158.92 | 157.39 | 156.20 | 156.83 |
| LF MinIntensity | 36.69 | 36.28 | 36.36 | 36.55 | 36.36 | 36.37 | 36.40 |
| LF Intensity | 74.54 | 76.73 | 75.80 | 77.22 | 76.66 | 75.82 | 75.88 |
| LF IntensityOfThe15MostIntensePixels | 113.01 | 120.18 | 117.42 | 120.85 | 119.28 | 117.27 | 118.14 |
| LF Swing (s) | 0.14 | 0.14 | 0.14 | 0.14 | 0.14 | 0.14 | 0.14 |
| LF SwingSpeed (cm/s) | 52.78 | 49.44 | 48.25 | 49.67 | 49.72 | 47.88 | 48.89 |
| LF StrideLength (cm) | 6.84 | 6.80 | 6.63 | 6.54 | 6.71 | 6.44 | 6.55 |

**Continuing of Supplementary Table S5.3**

| Parameter | NF | HF | HF+Q | HF+H | HF+E | HF+Ap | HF+An |
|---|---|---|---|---|---|---|---|
| LF StepCycle (s) | 0.30 | 0.34 | 0.33 | 0.33 | 0.33 | 0.33 | 0.33 |
| LF DutyCycle (%) | 54.48 | 56.90 | 56.21 | 57.46 | 57.02 | 56.51 | 57.30 |
| LF SingleStance (s) | 0.13 | 0.14 | 0.14 | 0.14 | 0.14 | 0.14 | 0.14 |
| LF InitialDualStance (s) | 0.02 | 0.03 | 0.02 | 0.03 | 0.02 | 0.03 | 0.03 |
| LF TerminalDualStance (s) | 0.02 | 0.03 | 0.02 | 0.03 | 0.03 | 0.03 | 0.02 |
| LH Stand (s) | 0.14## | 0.17 | 0.16 | 0.17 | 0.17 | 0.17 | 0.17 |
| LH StandIndex | -9.49## | -7.13 | -6.69 | -7.43 | -6.65 | -6.67 | -6.48 |
| LH MaxContactAt (%) | 29.10 | 28.82 | 27.01 | 27.45 | 26.04 | 26.46 | 26.87 |
| LH MaxContactArea (cm²) | 0.21## | 0.24 | 0.25 | 0.27 | 0.26 | 0.24 | 0.23 |
| LH MaxContactMaxIntensity | 167.66 | 174.38 | 171.56 | 178.64 | 174.68 | 171.47 | 165.12 |
| LH MaxContactIntensity | 82.46 | 82.72 | 82.00 | 84.02 | 82.49 | 82.22 | 79.47 |
| LH PrintLength (cm) | 0.81 | 0.84 | 0.86 | 0.87 | 0.87 | 0.83 | 0.85 |
| LH PrintWidth (cm) | 0.67 | 0.74 | 0.76 | 0.76 | 0.77 | 0.74 | 0.75 |
| LH PrintArea (cm²) | 0.26# | 0.31 | 0.32 | 0.34 | 0.33 | 0.31 | 0.31 |
| LH MaxIntensityAt (%) | 47.27 | 51.38 | 52.68 | 48.95 | 53.20 | 49.70 | 51.83 |
| LH MaxIntensity | 176.53 | 183.79 | 181.55 | 186.84 | 184.83 | 180.92 | 176.39 |
| LH MinIntensity | 39.74 | 38.16 | 38.25 | 38.72 | 38.48 | 39.37 | 38.63 |
| LH Intensity | 87.05 | 87.91 | 86.84 | 89.33 | 87.40 | 87.30 | 85.20 |
| LH IntensityOfThe15MostIntensePixels | 137.75 | 147.96 | 145.69 | 151.88 | 148.60 | 144.29 | 140.91 |
| LH Swing (s) | 0.14 | 0.14 | 0.13 | 0.14 | 0.14 | 0.13 | 0.13 |
| LH SwingSpeed (cm/s) | 44.07 | 45.62 | 44.41 | 44.62 | 44.90 | 44.18 | 45.78 |
| LH StrideLength (cm) | 6.13 | 6.17 | 5.93 | 6.04 | 6.12 | 5.68 | 5.82 |
| LH StepCycle (s) | 0.27 | 0.30 | 0.30 | 0.31 | 0.30 | 0.29 | 0.29 |
| LH DutyCycle (%) | 48.48## | 53.62 | 53.46 | 53.39 | 53.26 | 54.13 | 54.77 |
| LH SingleStance (s) | 0.12 | 0.12 | 0.12 | 0.13 | 0.12 | 0.12 | 0.12 |
| LH InitialDualStance (s) | 0.01 | 0.03 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |
| LH TerminalDualStance (s) | 0.01 | 0.02 | 0.02 | 0.02 | 0.03 | 0.03 | 0.02 |
| StepSequence NumberOfPatterns | 29.42 | 30.92 | 30.83 | 31.83 | 30.83 | 32.08 | 31.75 |
| StepSequence CA (%) | 23.15 | 18.25 | 22.07 | 27.74 | 17.81 | 24.65 | 27.37 |
| StepSequence CB (%) | 19.25 | 25.14 | 28.57 | 19.55 | 26.36 | 22.58 | 18.02 |
| StepSequence AA (%) | 10.19 | 9.05 | 9.85 | 11.63 | 11.74 | 9.85 | 10.80 |
| StepSequence AB (%) | 42.88 | 46.23 | 37.14 | 38.97 | 39.71 | 37.94 | 40.98 |
| StepSequence RA (%) | 1.87 | 1.04 | 1.08 | 1.06 | 1.35 | 3.19 | 2.04 |
| StepSequence RB (%) | 2.65# | 0.28 | 1.29 | 1.05 | 3.05* | 1.79 | 0.78 |
| StepSequence RegularityIndex (%) | 92.64 | 92.92 | 92.30 | 92.33 | 93.47 | 92.12 | 93.12 |
| BOS FrontPaws (cm) | 1.20 | 1.21 | 1.20 | 1.22 | 1.27 | 1.20 | 1.26 |
| BOS HindPaws (cm) | 2.66 | 2.96 | 2.76 | 2.78 | 2.85 | 2.87 | 2.85 |

**Continuing of Supplementary Table S5.3**

| Parameter | NF | HF | HF+Q | HF+H | HF+E | HF+Ap | HF+An |
|---|---|---|---|---|---|---|---|
| Average Speed | 22.49 | 20.03 | 19.91 | 20.06 | 19.87 | 19.33 | 19.65 |
| Maximum Variation (%) | 20.89 | 21.46 | 23.27 | 21.19 | 22.21 | 23.74 | 21.25 |
| Cadence | 13.65 | 12.21 | 12.33 | 12.37 | 12.41 | 12.58 | 12.46 |
| NumberOfSteps | 148.00 | 152.00 | 152.58 | 158.75 | 151.25 | 159.08 | 156.17 |
| PrintPositions RightPaws (cm) | 1.23 | 1.12 | 1.25 | 1.23 | 1.29 | 1.13 | 1.33 |
| PrintPositions LeftPaws (cm) | 1.12 | 1.27 | 1.21 | 1.16 | 1.14 | 1.53 | 1.46 |
| PhaseDispersions RF->LH | 16.41 | 24.67 | 8.35 | 14.74 | 8.71 | 10.40 | 15.65 |
| PhaseDispersions LF->RH | 9.45 | 24.40 | 16.64 | 14.98 | 15.33 | 7.66 | 8.77 |
| PhaseDispersions LH->RH | 38.70 | 39.85 | 38.88 | 38.11 | 37.97 | 38.85 | 38.08 |
| PhaseDispersions LF->RF | 50.03 | 49.02 | 50.01 | 50.56 | 49.12 | 49.44 | 51.21 |
| PhaseDispersions RF->RH | 47.29 | 50.66 | 47.73 | 48.17 | 47.48 | 49.22 | 48.01 |
| PhaseDispersions LF->LH | 46.96 | 49.44 | 47.42 | 48.99 | 49.75 | 45.95 | 47.57 |
| Couplings RF->LH | 21.35 | 28.60 | 13.79 | 20.54 | 12.91 | 16.22 | 21.53 |
| Couplings LF->RH | 14.31 | 29.96 | 22.47 | 19.69 | 22.48 | 13.57 | 13.31 |
| Couplings LH->RF | 17.81 | 26.75 | 24.39 | 25.42 | 31.06 | 15.32 | 16.92 |
| Couplings RH->LF | 25.87 | 27.48 | 25.00 | 24.20 | 25.03 | 17.47 | 8.38 |
| Couplings LH->RH | 45.65 | 45.28 | 44.69 | 46.24 | 43.22 | 45.07 | 45.02 |
| Couplings LF->RF | 49.57 | 48.64 | 49.85 | 50.75 | 48.89 | 48.83 | 50.78 |
| Couplings RH->LH | 44.48 | 45.79 | 44.79 | 45.57 | 46.97 | 43.38 | 43.69 |
| Couplings RF->LH | 50.08 | 51.17 | 49.80 | 49.11 | 50.84 | 50.35 | 49.00 |
| Couplings RF->RH | 49.59 | 50.93 | 48.84 | 49.86 | 48.66 | 50.44 | 49.52 |
| Couplings LF->LH | 49.87 | 50.14 | 49.20 | 50.51 | 50.70 | 46.71 | 48.43 |
| Couplings RH->RF | 44.51 | 45.74 | 47.83 | 46.55 | 47.07 | 45.18 | 45.87 |
| Couplings LH->LF | 45.63 | 47.10 | 46.74 | 45.81 | 46.26 | 47.65 | 46.82 |
| Support Zero (%) | 0.15 | 0.06 | 0.05 | 0.02 | 0.03 | 0.03 | 0.02 |
| Support Single (%) | 5.90$^{\#\#\#}$ | 2.02 | 2.94 | 1.90 | 2.64 | 2.78 | 2.21 |
| Support Diagonal (%) | 73.54 | 69.45 | 71.44 | 70.70 | 70.24 | 69.06 | 71.57 |
| Support Girdle (%) | 3.44$^{\#}$ | 2.08 | 2.47 | 2.23 | 1.43 | 2.67 | 1.65 |
| Support Lateral (%) | 2.09 | 1.19 | 1.14 | 1.33 | 1.48 | 1.61 | 1.02 |
| Support Three (%) | 13.48$^{\#\#}$ | 21.97 | 19.10 | 20.25 | 20.53 | 19.90 | 20.02 |
| Support Four (%) | 1.39 | 3.23 | 2.87 | 3.56 | 3.65 | 3.94 | 3.52 |

Parameters were not significantly different between HF+flavonoids groups and HF. Some parameters were significantly different between HF and NF, which are indicates with $^{\#}(p<0.05)$, $^{\#\#}(p<0.01)$, $^{\#\#\#}(p<0.001)$. RF; right front paw, RH; right hind paw, LF; left front paw, LH; left hind paw, BOS; base of support.

**Table S5.4** 24-hour indirect calorimetry measurements of RER and EE in week 11.

| Indirect calorimetry | | NF | HF | HF+Q | HF+H | HF+E | HF+Ap | HF+An |
|---|---|---|---|---|---|---|---|---|
| RER | dark | 1.00# ± 0.07 | 0.87 ± 0.05 | 0.84 ± 0.07 | 0.87 ± 0.07 | 0.86 ± 0.05 | 0.87 ± 0.07 | 0.87 ± 0.05 |
| | light | 0.94# ± 0.08 | 0.83 ± 0.07 | 0.82 ± 0.08 | 0.85 ± 0.06 | 0.83 ± 0.06 | 0.85 ± 0.06 | 0.83 ± 0.07 |
| EE (kcal/h) | dark | 0.53 ± 0.06 | 0.55 ± 0.06 | 0.54 ± 0.06 | 0.56 ± 0.06 | 0.54 ± 0.07 | 0.56 ± 0.06 | 0.55 ± 0.06 |
| | light | 0.44 ± 0.06 | 0.48 ± 0.06 | 0.48 ± 0.05 | 0.48 ± 0.06 | 0.47 ± 0.07 | 0.48 ± 0.06 | 0.48 ± 0.06 |

Indirect calorimetric data are shown for both 12-hour dark and 12-hour light phase. Data are presented as mean ± SD (n=9). # indicates significant difference of HF to NF ($p<0.05$). RER; respiratory exchange ratio, EE; Energy Expenditure, NF; normal-fat diet, HF; high-fat diet, HF+Q; HF supplemented with quercetin, HF+H; HF supplemented with hesperetin, HF+E; HF supplemented with epicatechin, HF+Ap; HF supplemented with apigenin, HF+An; HF supplemented with anthocyanins.

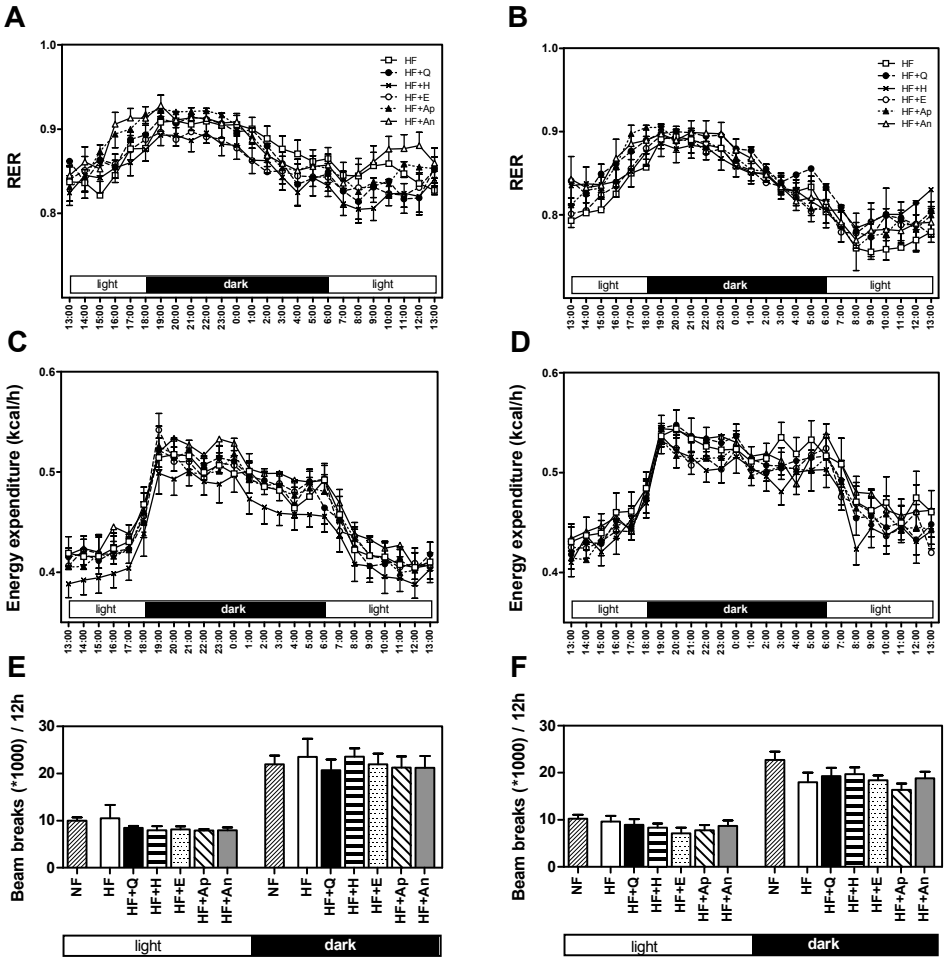


**Figure S5.1 Whole body RER, energy expenditure, and activity levels were not affected by flavonoid supplementation in week 1 and 5.** Indirect calorimetry measurements of 24 hour RER in week 1 (A) and in week 5 (B) and energy expenditure in week 1 (C) and in week 5 (D). Activity measured during indirect calorimetry measurements for light (12 h) and dark (12h) period in week 1 (E) and in week 5 (f). Data are presented as mean ± SEM. # indicates a significant difference of NF to HF. NF, normal fat diet; HF, high-fat diet; HF+Q, HF supplemented with quercetin; HF+H, HF supplemented with hesperetin; HF+E, HF supplemented with epicatechin; HF+Ap, HF supplemented with apigenin; HF+An, HF supplemented with anthocyanins; RER, respiration exchange ratio.

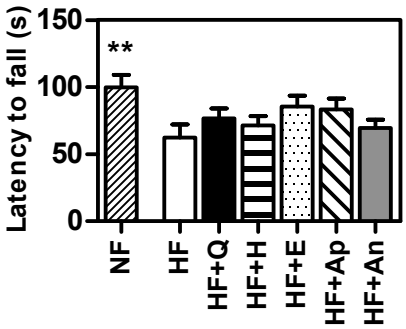


**Figure S5.2.** Rotarod measurements in week 9. Data is presented as latency to fall in seconds.

# CHAPTER 6

## QUERCETIN TESTS NEGATIVE FOR GENOTOXICITY IN TRANSCRIPTOME ANALYSES OF LIVER AND SMALL INTESTINE OF MICE

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## ABSTRACT

Given the positive results of quercetin in *in vitro* genotoxicity studies, *in vivo* genotoxicity of this dietary flavonoid was studied in liver and small intestine, where highest exposure to quercetin is expected. This is especially of interest considering high intake by widely available food supplements.

Quercetin (0.33%) supplemented to a high-fat diet was administered to mice during 12 weeks. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels revealed no indications for hepatotoxicity. General microarray pathway analysis of liver and small intestinal tissue samples showed no regulation of genotoxicity related pathways. In addition, analysis of DNA damage pathways in these tissues did also did point at genotoxicity. Furthermore, comparison with a published classifier set of transcripts for identifying genotoxic compounds did not reveal any similarities with the regulation of this classifier set of transcripts by quercetin. Available microarray datasets of known genotoxic compounds, 2-acetylaminofluorene (2-AAF) and aflatoxin B1 (AFB1) in mice were taken along as positive controls for comparison, and indeed showed genotoxic properties (regulation of genotoxic related genes) in the analyses. This transcriptomic study showed that supplementation with quercetin at ~350 mg/kg bw/day for 12 weeks in mice gave no indications of quercetin-induced genotoxicity in liver and small intestine.

## 6.1 INTRODUCTION

Flavonoids present in foods of plant origin like fruits, vegetables, and tea, are thought to play a role in the beneficial effects of these foods [1]. Apples, onion and tea are major dietary sources of one of these flavonoids, quercetin, and in addition quercetin is widely available as a dietary supplement in Western countries. However, the safety of quercetin has been under discussion for several decades, since also possible adverse effects of quercetin, including genotoxicity, have been reported [2-4].

Genotoxicity results in DNA damage and when not repaired, this will result in lasting mutations, which can ultimately lead to cancer. Quercetin tested positive in various *in vitro* genotoxicity tests. For example genotoxic properties of quercetin were shown in bacterial systems including the Ames test, with and without metabolic activation [4-8]. This genotoxicity has been related to the quinone-quinone methide chemistry of quercetin [5]. Furthermore, in several cultured human and rodent cells, formation of micronuclei, DNA single strand breaks and chromosomal aberrations were observed after quercetin exposure [7,9-12]. It was also shown that quercetin can generate DNA adducts in different cell types *in vitro* [13].



However, genotoxicity was not shown in rodents after oral quercetin administration measured by among others micronuclei, chromosomal aberrations and unscheduled DNA synthesis in bone marrow, liver, and gastric mucosa cells [14-18]. Several long-term (ranging from 64 weeks to lifespan) *in vivo* studies showed no carcinogenicity after quercetin exposure ranging from 0.2-10% (providing ~60-3500 mg/kg bw/day in rodents) [19-22]. Other *in vivo* studies have shown, however, an indication for higher incidence of tumours after quercetin exposure. In rats fed a 0.1% (providing ~50 mg/kg bw/day) quercetin diet for 58 weeks, tumours in intestine and bladder were found [3]. A 2-year study with 0.1-4% (providing ~50-2000 mg/kg bw/day) quercetin within the National Toxicology Program reported carcinogenicity in kidneys of male rats induced by the highest dose of quercetin [2]. These indications of genotoxicity *in vitro* and the non-conclusive results on carcinogenicity *in vivo*, have raised concerns regarding the safety of quercetin. The genotoxicity of quercetin, even without metabolic activation, may specifically be of concern for organs of first contact, especially upon intake of highly dosed food supplements. Supplements with intakes as high as 1000-1500 mg/day represent dose levels far beyond average dietary intake (20 mg/day). At these high dose levels detoxification of quercetin via metabolism may be saturated resulting in exposure of the intestine and possibly liver to un-metabolised quercetin. This implies that *in vivo* genotoxicity studies in these first pass tissues could provide further insight in the possible hazards of high dose quercetin supplements.

Nowadays, transcriptomic analysis has been shown to be an informative tool to predict genotoxicity [23,24]. Therefore, the aim of the present study was to characterise the potential genotoxicity of quercetin in the small intestine as well as in the liver of mice by transcriptomic analysis, in order to provide new additional information to evaluate the safety of quercetin.

## 6.2 MATERIAL & METHODS

### Animals and treatment

The experiment was performed according to the Dutch Animal Experimentation Act (1996) and the experimental protocol was approved by the Animal Welfare Committee of Wageningen University, Wageningen, The Netherlands (DEC 2011079). In brief, 24 male C57BL/6JOlaHsd mice (Harlan Laboratories, Horst, The Netherlands) were individually housed and maintained under environmentally controlled conditions (temperature 21°C, 12 h/12 h light-dark cycle, 55 ± 15 % humidity), with *ad-libitum* access to food and water. At arrival, the mice were 9 weeks old and after adaptation for 3 weeks the mice (n=12) received a standardized high-fat diet (40en% fat; [25]) without or with supplementation of 0.33% *(w/w)* quercetin (~325 mg/kg bw/day) (Sigma, Zwijndrecht, The Netherlands). Effects on body weight and lipid and energy metabolism have been reported before [26]. One quercetin fed mouse was excluded from all analyses, because a nasal abscess developed in week 6. After 12 weeks of intervention, all mice were fasted for 2-4 hours during the light phase and anesthetized by inhalation of 5% isoflurane using $O_2$ as a carrier. Blood was sampled via orbital extraction in collect serum tubes (Greiner Bio-one, Longwood, USA), kept on ice for max 2 hours, and centrifuged for 10 min at 3000g at 4 °C to obtain serum, aliquoted, and stored at -80°C. After blood collection, mice were killed by cervical dislocation, and liver was dissected and weighted, and small intestinal scrapings were snap frozen in liquid nitrogen and stored at -80°C.

### ALT and AST measurements

Possible hepatotoxicity was analysed by measuring serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels using enzymatic assay kits (Bioo Scientific Corporation, Austin, USA; MaxDiscoveryTM ALT and AST Enzymatic Assay, resp.), according to the manufacturer's instructions. Five µl of fresh serum was used per well and samples were measured in duplicate and averaged. Students' *t*-test was used to compare the two normal distributed groups (GraphPad Prism version 5.03, San Diego, CA, USA).

### Microarrays

RNA from liver and small intestinal scrapings was isolated using RNeasy columns (Qiagen, Venlo, The Netherlands) and used for microarray analysis. For global transcriptome analysis liver and small intestinal samples of individual mice and 8x60K Agilent whole-mouse genome microarrays (G4852A, Agilent Technologies Inc., Santa Clara, CA) were used according to the manufacturer's protocol with a few modifications as described previously [27]. cDNA was synthesized for each animal from 200 ng RNA. Normalisation and data analysis were performed as published [28] using Feature Extraction version 10.7.3.1 (Agilent Technologies). Based on visual inspection

three arrays of liver were excluded in which hybridization was not homogenous, and no arrays of small intestine were excluded. Microarray data have been deposited in NCBI Gene Expression Omnibus (GEO) under accession number GSE51343 (liver) and GSE63227 (small intestine).

### Microarray data analysis

First, Student's *t*-tests were used with false discovery rate (FDR) adjustment for multiple testing correction according to Benjamini-Hochberg [29]. Of the 34,373 and 31,718 probes being expressed in liver and small intestine respectively, no significantly differently expressed genes between the quercetin and the control group were found (FDR $p<0.05$).

Follow-up analyses were performed based on a *t*-test without FDR adjustment, to prevent to discard data which could possibly indicate genotoxicity by using too stringent criteria ($p<0.05$). Fold changes were expressed as ratio of intervention group versus control group. General pathway analysis was performed using MetaCore (Thomson Reuters, US). Furthermore, regulated genes ($p<0.05$) present in 'DNA damage' specific marked pathways in Metacore were studied.

For comparison with known genotoxic compounds, published microarray data (NCBI GEO, accession number GSE43977) of genotoxic compounds 2-acetylaminofluorene (2-AAF) and aflatoxin B1 (AFB1) in C57BL/6J mice were taken along. These specific gene sets were selected based on availability of microarray data of studies in mice with known genotoxic compounds given via the diet for at least 7 days and a sufficient number of arrays per group ($n>6$). These mice received 2-AAF (300 ppm, ~40 mg/kg bw/day) and AFB1 (1ppm, ~0.15 mg/kg bw/day) via feed for 7 days, afterwards microarrays were performed with RNA from liver tissue [23]. The doses were identified as suitable sub toxic doses to identify gene expression patterns [23]. In addition, these doses were at least 5 times higher than the benchmark dose ($BMD_{10}$), the dose that induces 10% extra tumour incidence above background levels [30].

Moreover, regulation of a published classifier set of transcripts, reported to predict the genotoxic potential of compounds [23], was used to investigate the possible genotoxicity of quercetin.

## 6.3 RESULTS AND DISCUSSION

### Hepatotoxicity

General hepatotoxicity markers being ALT and AST levels in serum revealed a significantly lower level of ALT in serum of quercetin fed mice and no significant differences for AST between quercetin and control mice (Figure 6.1). Higher levels of ALT and AST are indications for hepatotoxicity, but all observed values in quercetin mice were within the normal range for ALT and AST levels [31]. Overall, this indicates that the tested dose of quercetin did not cause hepatotoxicity.

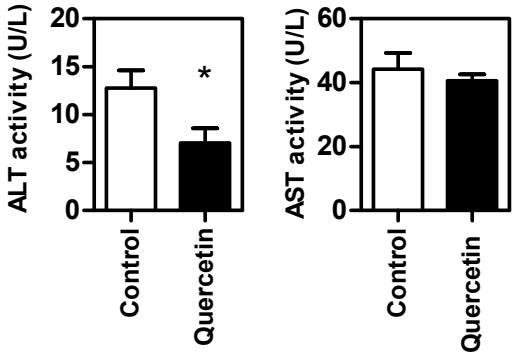


**Figure 6.1.** ALT and AST activity in serum of mice exposed to ~350 mg/kg bw/day quercetin for 12 weeks as compared to controls. Data are presented as mean ± SEM. Alanine aminotransferase; ALT, aspartate aminotransferase; AST.



## General microarray analysis

Significantly expressed genes ($p<0.05$) resulted in 1,648 regulated probes in liver and 1,230 in small intestine. Previous pathway analysis in Metacore with these regulated transcripts in liver revealed no pathways regulated by quercetin (FDR<0.01) [26]. For the small intestine, pathway analysis revealed several pathways regulated by quercetin (FDR<0.01), but these regulated pathways were not involved in or related to possible genotoxicity or DNA damage (Supplementary Table 1). For comparison, the data obtained with the positive genotoxic controls 2-AAF and AFB1 revealed regulated pathways involved in DNA damage in mice [23]. Therefore, for both liver and small intestine, we concluded that general pathway analysis of the transcriptomic data revealed no genotoxic effects of quercetin in liver and small intestine of mice after 12 weeks exposure.

## DNA damage pathways

Subsequently, the gene expression pattern was studied in more detail focussing on genotoxicity of quercetin in liver or small intestine, to confirm our conclusion of the general pathway analysis. A comparison was made with hepatic microarray data of the known genotoxic compounds 2-AAF and AFB1 [23]. Transcripts in the 11 pathways related to DNA damage in the pathway analysis program Metacore were investigated. 2-AAF regulated 32 different transcripts and AFB1 regulated 40 transcripts in these DNA damage pathways (Figure 6.2). For quercetin in liver, only eight regulated transcripts were found in these DNA damage pathways, and nine in small intestine (Figure 6.2). Interestingly, all four overlapping transcripts for quercetin in liver responded in the opposite direction as was seen for the genotoxic compounds. Two overlapping transcripts in the small intestine responded in the same direction as the genotoxic compounds.

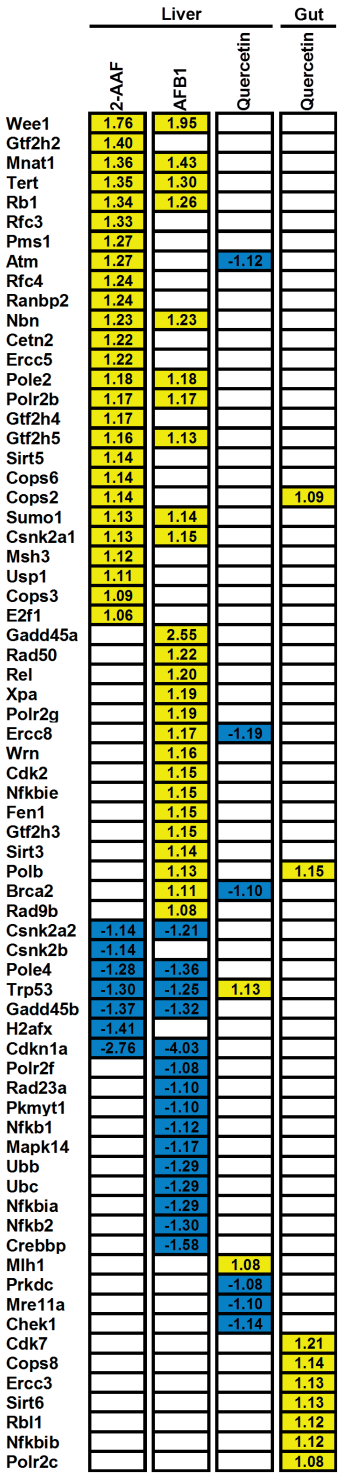


**Figure 6.2 Regulated transcripts by genotoxins (2-AAF and AFB1) versus quercetin present in 'DNA damage' pathways.** Gene expression of quercetin is studied in liver and small intestine. Gene expression in liver of the known genotoxic compounds 2-AAF and AFB1 is taken along for comparison. Fold changes are presented as gene expression of intervention diet over control diet. Significant up-regulation is indicated in yellow, down regulation in blue ($p<0.05$) and non-significant regulation in white ($p>0.05$). 2-AAF; acetylaminofluorene, AFB1; aflatoxin B1.

A small subset of four transcripts was only regulated by quercetin in the liver and seven transcripts in the small intestine. These transcripts were not regulated by the known genotoxic compounds. In liver, all four of these transcripts were related to DNA damage response, but they were both up and down regulated and involved in different DNA repair mechanisms, like DNA mismatch repair, DNA double strand break repair, and checkpoint mediated cell cycle arrest. This therefore provided no clear indication of regulation of one mechanism into the same direction; furthermore, this can also be a protective action of quercetin to DNA damage. The seven transcripts specifically regulated by quercetin in the small intestine were all up-regulated. Up-regulation of *Cops8*, *Ercc3*, and *Sirt6* is associated with a protective response against DNA damage [32-34]. Inactivation of *Rbl1* is related to tumorigenesis [35]. Therefore, the up-regulation of these four transcripts suggested a protective response of quercetin. There are no reports in the literature on genotoxic or carcinogenic responses related to regulation of the genes *Nfkbib* and *Pol2r*, while up-regulation of *Cdk7* is associated with carcinogenesis [32]. It should also be taken in consideration that 5% of the transcripts will be regulated by chance, since a statistical threshold at p-value of 0.05 was used.

Also given the fact that the number of regulated transcripts by quercetin in the DNA damage pathways is rather low and the gene expression in these pathways is mostly the opposite of the gene expression of the genotoxic 2-AAF and AFB1, this analysis suggests that there was no clear indication for genotoxicity of quercetin in liver and small intestine.

## Classifier set of transcripts to identify genotoxic compounds

Next, a published classifier set of hepatic transcripts to identify genotoxic compounds [23] was used to compare expression patterns induced by 2-AAF and AFB1 to the pattern induced by quercetin (Figure 6.3). Most of the transcripts were not regulated by quercetin in liver or small intestine. Only two transcripts were significantly regulated, however, these were regulated in the opposite direction compared to 2-AAF and AFB1. This further supports that quercetin did not exert genotoxic effects in liver and small intestine in this study.

### *Carcinogenicity of quercetin in vitro and in vivo*

The only two studies that previously showed genotoxicity of quercetin in rodents [2,3] have been criticized. Re-evaluations of the study of the National Toxicology Program which showed an increase of kidney tumours only in male F344/N rats [2] led to the conclusions that the observed tumours were specific for the rat strain used and probably related to α2u-globulin nephropathy, which occurs only in male rats [36,37]. The other *in vivo* study [3] was also criticized [38] because the observed carcinogenicity could possibly be ascribed to the use of a specific rat strain and a whole grain diet in the study, which is however not fully understood. As mentioned in the introduction, the other long term studies with even higher doses of quercetin showed no carcinogenicity of quercetin [19-22]. Even

| | Liver | | | Gut |
|---|---|---|---|---|
| | 2-AAF | AFB1 | Quercetin | Quercetin |
| 4931408D14Rik | 2.03 | 1.98 | nd | nd |
| Tiam2 | 2.17 | 2.48 | 1.01 | nd |
| Nr4a1 | 2.18 | 1.56 | 1.04 | -1.19 |
| Il1b | 2.17 | 2.01 | -1.03 | -1.01 |
| Cyp1a2 | 1.18 | 1.99 | 1.13 | nd |
| 9630026M06Rik | 1.67 | 1.52 | nd | nd |
| Gys2 | 1.90 | 1.61 | -1.17 | nd |
| Id2 | 2.03 | 1.83 | 1.05 | -1.05 |
| Nr1d2 | 1.62 | 1.71 | -1.05 | -1.12 |
| Phf17 | 1.50 | 1.60 | -1.06 | -1.09 |
| Il1a | 1.40 | 1.41 | -1.06 | 1.06 |
| 4930406D14Rik | -1.28 | -1.50 | nd | nd |
| Creld2 | -2.16 | -2.25 | 1.39 | 1.18 |
| Nedd4l | -1.97 | -1.70 | 1.09 | -1.01 |
| Ihpk2 | -2.65 | -1.89 | nd | nd |
| 2310076L09Rik | -2.09 | -1.95 | nd | nd |
| Zbtb16 | -2.73 | -2.96 | 1.13 | 1.06 |
| Slc45a3 | -3.46 | -3.38 | 1.10 | 1.04 |

**Figure 6.3 Regulated transcripts of the genotoxicity classifier set by 2-AAF and AFB1 [23] versus by quercetin.** Gene expression of quercetin is studied in liver and small intestine. Gene expression in liver of the known genotoxic compounds 2-AAF and AFB1 is taken along for comparison. Fold changes are presented as gene expression of intervention diet over control diet. Significant up-regulation is indicated in yellow, down regulation in blue ($p<0.05$), non-significant regulation in white ($p>0.05$), and not determined regulation with nd 2-AAF; acetylaminofluorene, AFB1; aflatoxin B1.

so, no genotoxicity was observed after oral quercetin administration in rodents [14-18]. Thus overall, carcinogenic and genotoxic properties of quercetin have not been conclusively shown *in vivo*.

In contrast, the genotoxicity of quercetin has been demonstrated *in vitro* in multiple studies. The interpretation of these *in vitro* studies is uncertain, because the exposure of cells with quercetin *in vitro* differs from what really happens in the body. Apart from the intestinal cells, other cells in the body are exposed to much lower concentrations of quercetin, because metabolism in the intestinal wall and liver will modify quercetin resulting in low systemic availability of quercetin itself (Kroon et al. 2004). The quercetin metabolites or conjugates, e.g. glucuronides and sulfates, circulating in the blood are chemically distinct from their parent compound and their biological effects are likely to be different from that of the native compound [39]. Furthermore, under cell culture conditions, flavonoids are rapidly oxidized resulting in oxidation products and $H_2O_2$ [40], which, when not properly controlled, can also explain the observed DNA damage.

This discrepancy between *in vitro* and *in vivo* exposure, as is also seen for other flavonoid mediated effects [41], may explain why we and most other authors did not found *in vivo* genotoxic effects.

In this study the dose of quercetin was ~350 mg/kg bw/day for 12 weeks, which is about 1,000 times higher than human dietary intake and 15 times higher than human supplementary intake of quercetin.

However, our results should also be seen in the light of the recent studies in which was shown in rats that quercetin exposure (0.03% quercetin diet, ~40 mg/kg bw/day) during pregnancy had effects on the foetus as quercetin can cross the placenta and can accumulate in the foetus. This could increase the risk on rearrangements in a gene which can be linked to childhood leukaemia. It was concluded that guidelines for using quercetin supplements during pregnancy are needed [42,43].

## Conclusions

In the present transcriptomics study, supplementation with quercetin at ~350 mg/kg bw/day for 12 weeks gave no indications of quercetin-induced genotoxicity in liver and small intestine of mice. The small intestine is of special interest, because upon oral intake it is exposed to the highest amount of quercetin. We conclude that there is no reason for concern of genotoxicity in liver and small intestine after dietary intake of quercetin.



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**Table S1.** Top 10 of regulated transcriptomics pathways in small intestine by 0.33 % quercetin supplementation to a high-fat diet in mice.

| Maps | # regulated genes | # total genes | p-value | FDR |
|---|---|---|---|---|
| Immune response_T cell receptor signaling pathway | 11 | 53 | 6.4E-07 | 4.5E-04 |
| Immune response_Function of MEF2 in T lymphocytes | 10 | 51 | 3.6E-06 | 1.3E-03 |
| Development_VEGF signaling via VEGFR2 - generic cascades | 12 | 84 | 1.2E-05 | 2.9E-03 |
| Immune response_Antigen presentation by MHC class I | 7 | 28 | 2.0E-05 | 2.9E-03 |
| Immune response_NFAT in immune response | 9 | 51 | 2.7E-05 | 2.9E-03 |
| Immune response_TCR and CD28 co-stimulation in activation of NF-kB | 8 | 40 | 3.0E-05 | 2.9E-03 |
| Signal transduction_Activation of PKC via G-Protein coupled receptor | 9 | 52 | 3.2E-05 | 2.9E-03 |
| Cell adhesion_Gap junctions | 7 | 30 | 3.3E-05 | 2.9E-03 |
| LRRK2 and immune function in Parkinson's disease | 6 | 22 | 4.7E-05 | 3.7E-03 |
| CFTR folding and maturation (normal and CF) | 6 | 24 | 8.1E-05 | 5.7E-03 |

# CHAPTER 7

## GENERAL DISCUSSION

# 7.1 MAIN FINDINGS AND OUTLINE

## Main findings

The aim of this thesis was to identify mechanisms of beneficial health effects of flavonoids related to lipid metabolism and to characterise if high intake of these compounds would pose a risk for possible genotoxic effects. Elevated serum lipid levels, which were one of the important markers in our research, are known risk factors for cardiovascular diseases (CVD). Circulating and hepatic lipids are usually determined with fast and simple peroxidase-based assays. However, we have observed that flavonoids interfere in these assays, most likely due to inhibition of the peroxidase of these assays, as described in chapter 2. This means that the observed lipid lowering effects of flavonoids could have been overestimated in research when such peroxidase-based methods have been used, and therefore we have used alternative methods to adequately investigate lipid lowering effects of flavonoids. In chapter 3 we described that quercetin intake increased hepatic lipid omega-oxidation and lowered corresponding circulating lipid levels. In this study, a mild high-fat (MHF) background diet was used. We have suggested a possible role of the transcription factor constitutive androstane receptor (*CAR)* in these effects. Subsequently, high-fat (HF) diet-induced serum and hepatic lipids and body weight gain were found to be reduced by quercetin, which is described in chapter 4. The differences in the observed effects seen in chapter 3 and 4 suggested that the background diet that was used modified the effects induced by quercetin. In chapter 5 the effects of quercetin were compared to other flavonoids from different flavonoid subclasses. Overall, quercetin was the most potent flavonoid in reducing the HF diet-induced effects, but all studied flavonoids showed effects on HF diet-induced body weight gain. Furthermore, decreased expression of hepatic *Cyp2b9*, a known target gene of *CAR*, was found for all studied flavonoids. Given that possible beneficial effects of flavonoids may encourage higher flavonoid intakes, safety aspects have to be considered. Quercetin, the flavonoid shown to be the most active in our studies, has been linked to genotoxicity. Therefore, the genotoxic properties of quercetin were studied based on transcriptome profiling in small intestine and liver as described in chapter 6. This transcriptomic analysis showed no indication of genotoxicity of quercetin in either tissue at dose levels that were far above normal dietary intake.

## Outline of the discussion

The following sections will discuss a number of factors that might potentially influence observed health effects of flavonoids. Positive health effects are found in several epidemiological studies, however confirmation in intervention trials with flavonoid containing food supplements is scarce, and also discrepancies between animal studies and between human studies are found. This chapter will discuss our studies in relation to each other and in relation to other studies to gain insight in differences in outcomes that have been observed. The first paragraph describes differences between the *in*

*vitro* and *in vivo* situation (paragraph 7.2). The importance of choosing the right methods for studying flavonoid-mediated effects is discussed in paragraph 7.3. The dietary context (paragraph 7.4), and the doses used could also affect flavonoid-mediated effects (paragraph 7.5). Next, some additional factors that may affect the ultimate results of studies characterising health effects of flavonoids, like gender and microbiota (paragraph 7.6). The implications of these factors for studying flavonoid-mediated effect in humans are subsequently discussed in paragraph 7.7. Finally, this chapter will present the main conclusions of this thesis (paragraph 7.8).

## 7.2 *IN VITRO* VERSUS *IN VIVO*

When considering experimental data on flavonoid-mediated effects it is important to realise that the *in vitro* situation may differ from the *in vivo* situation. For example, genotoxic effects of quercetin have been often found *in vitro* [1-4], while these genotoxic effects were not observed *in vivo* [5-7]. In chapter 6 we have shown with transcriptome analyses that genotoxicity of quercetin was absent in liver and small intestine. Similarly, for example for flavonoid-mediated antioxidant effects, it was shown that although flavonoids are strong antioxidants *in vitro*, these effects could not explain disease prevention *in vivo*, given the low *in vivo* concentrations [8].

The interpretation of *in vitro* studies can be questionable when cells are being exposed to high concentrations of flavonoid aglycones, which differs from what is encountered in the body. With the possible exception of intestinal cells, other cells in the body are only exposed to very low concentrations of flavonoid aglycones. Metabolism in the intestinal epithelial cells and liver will modify the flavonoids entering the circulation [9]. The metabolites formed, conjugates e.g. glucuronides and sulfates, circulating in the blood, are chemically distinct from their parent aglycones. The biological effects of these conjugates are very likely to be different from that of the native aglycones [10].

Furthermore, under cell culture conditions, flavonoids are rapidly oxidized resulting in oxidation products and $H_2O_2$, especially when not properly controlled in the experiment [11]. These oxidation products can affect cells and that may wrongly suggest functional effects on health or damage parameters.

In general, several other conditions are usually not taken into account *in vitro;* for example the crosstalk between organs, and influences of hormones. Cell culture conditions are physiologically different from *in vivo* conditions, for example the use of high glucose concentrations in cell culture medium can influence energy metabolism of the cells [12]. Cell culturing may also result in loss of functions, with the loss of p450 enzyme activity in the human hepatic cell line HepG2 as an example [13].

Therefore, several factors make that the *in vitro* situation differs from the *in vivo* situation. This discrepancy may explain why sometimes contradictory beneficial health effects or genotoxic effects of flavonoids are reported. In this thesis, the underlying mechanisms of flavonoid-mediated health effects is investigated, mainly in mouse

models. Further research could be performed *in vitro*, by selecting a model where the identified flavonoid targets are present. These experiments have to be properly controlled for auto-oxidation of flavonoids and should be performed including the naturally occurring metabolites of flavonoids.

## 7.3 ANALYTICAL METHODS

The methods used to analyse flavonoid-mediated effects can also influence the observed effects. First of all interference of flavonoids in enzyme-based assays will be discussed in this paragraph. Subsequently, the use of transcriptomics in flavonoid research will be discussed.

### Enzyme-based assays

Enzyme-based assays are generally accepted and used in research, including flavonoid research. In chapter 2, flavonoids were shown to interfere with enzyme-based assays for the determination of triglycerides (TG) and free fatty acids (FFA). These assays are based on peroxidases that convert intermediate products into a coloured product. The absorbance of these coloured products is subsequently measured and related to the amount of TG and FFA. However, several flavonoids are known to be able to inhibit peroxidase activity [14-16], resulting in apparent lower detected levels of the primary product by using these peroxidase-based assays.

In chapter 3 of the present thesis, serum TG and FFA levels of mice on a 0.33% quercetin diet for 12 weeks were measured with enzyme-based assays and two independent analytical methods. The results obtained showed that the lipid lowering effect in quercetin-fed mice detected with the enzyme-based assays were clearly stronger than the effects measured with gas chromatography and $^{1}$H-NMR (Figure 7.1). This confirmed the interference of flavonoids with these assays (chapter 2). Furthermore, these lipid measurements in serum of an *in vivo* experiment, a matrix that logically contains mainly metabolised quercetin, showed that this interference can play a role under physiologically relevant conditions.

Besides the TG and FFA assays, there are many more assays where peroxidases are used for detection [17,18]. Horseradish peroxidase is commonly used in assays such as ELISA, Western blots and immunohistochemistry. In these assays, the target binds to the primary antibody, which binds to the secondary antibody which is linked to horseradish peroxidase, and the peroxidase will convert a substrate for final detection into a coloured product. The reason that peroxidases are often used in these assays is because a variety of substrates is available [18]. Importantly, this means that with the use of peroxidase-based ELISAs and possibly with more methods used in flavonoid research vigilance is needed and alternative enzymes should be used in these assays or alternative methods should be chosen to prevent overestimation of the lowering effects by flavonoids.

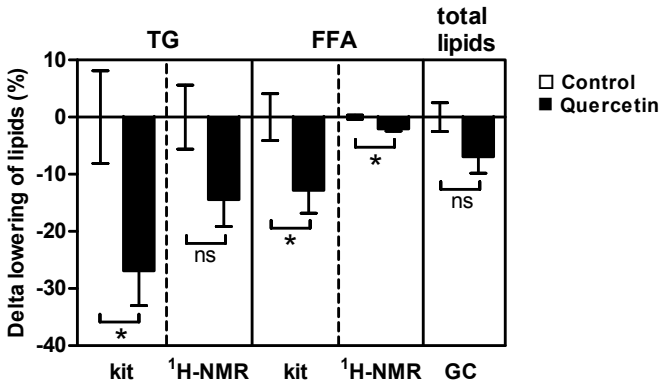


**Figure 7.1.** Serum lipid lowering measured in the same animals with enzyme-based assays (kits) versus other analytical methods. Values are presented as the delta of the lowering of serum lipids in percentage of the levels found for quercetin (black) vs. control (white) mice, mean ± SEM (n=12). Asterisks indicates statistical difference, *p<0.05. TG; triglycerides, FFA; free fatty acids, $^{1}$H-NMR; $^{1}$H-nuclear magnetic resonance, GC; gas chromatography.



These assays are also often used for hepatic lipid quantification, therefore, in this thesis hepatic lipids were quantified with an alternative method; Oil Red O staining. For serum lipid profiling, gas chromatography was used. Furthermore, we also have implemented a $^{1}$H-NMR method for serum lipid profiling. We have adjusted this method, which was known for human samples, for the use of much smaller volumes available from mouse experiments.

## Transcriptomics

Transcriptomic tools are nowadays widely used to study whole genome gene expression. With transcriptome profiling it is possible to search unsupervised for changes that may identify as yet unknown underlying mechanisms, because it is not needed to select targets or processes beforehand [19]. Since a clear hypothesis for a mechanism of action for flavonoid-mediated health effects was initially not available, this technique and type of analysis was used in chapters 3 and 4.

The flavonoids induced relatively small effects on gene expression in our studies. In chapter 3 we have performed a correction for multiple testing. We did not use this correction in chapter 4, because the observed hepatic gene regulation was much smaller. This demands vigilance in the interpretation of the data. While differential expression of single genes can be coincidental, validity of the findings can be supported by functional coherence between regulated genes. This is the case in chapter 4, where all three known hepatic *Cyp2b* genes were down-regulated by quercetin. Furthermore, identified targets were confirmed by an independent technique (RT-qPCR).

In chapter 5, in contrast to chapters 3 and 4, we have performed transcriptome analysis in a focused manner to identify potential genotoxic effects of quercetin. In

essence, transcriptome profiling was used to assess whether quercetin induced a genotoxicity gene expression profile.

Altogether, the results in this thesis show that the microarray technique can be used to identify flavonoid-mediated mechanistic effects, which could be related to biological relevant physiological effects. In addition, transcriptome profiling can be used to develop or assess novel biomarkers or biomarker profiles [19,20], analogous to the genotoxicity profiling utilised in chapter 5.

## 7.4 DIETARY CONTEXT

The dietary context used in experiments studying flavonoid-mediated effects, can also influence the observed effects of flavonoids. Both chapter 3 and chapter 4 describe a dietary intervention in mice supplemented with the same dose of quercetin and with a similar set-up of the experiment. There was only one important difference between these studies; the background diet used was different. The difference of the diet was in fat energy % as well as in fat composition. In chapter 3, we report that quercetin in a MHF diet (30 en% fat) increased hepatic lipid omega-oxidation and lowered corresponding circulating lipid levels, while body weight gain, hepatic lipid accumulation and serum leptin levels were not significantly different. In chapter 4, quercetin reduced HF (40 en%) diet-induced body weight gain, hepatic and serum lipids, and serum leptin levels, while no effects on omega-oxidation were found. The diet thus seemed to have an effect on the induced mechanism of action of quercetin and on the diet-induced body weight gain, hepatic lipid accumulation and leptin levels. These differences and the possible dietary effects on bioavailability and genotoxic properties of flavonoids will be discussed below.



### Dietary context and the underlying mechanism of flavonoid-mediated effects

In chapter 3, increased hepatic lipid omega-oxidation is proposed as the underlying mechanism for the lowered serum lipid levels induced by quercetin. We have performed a similar study at 28°C. this study was not included in the preceding chapters of this thesis (unpublished data). The housing temperature 28°C is within the thermoneutral zone of mice. At thermoneutrality the energy requirements are the lowest, facilitating induction of diet-induced obesity in mice [21,22]. In both studies exactly the same background diet, mouse strain, age, sex, individual housing and food accessibility were used, but the temperature was either 21 °C (the study reported in this thesis) or 28°C (unpublished data). Interestingly, the gene expression showed the same pattern of regulation of genes related to lipid omega-oxidation, the effects were smaller when the mice were housed at thermoneutral temperature, but showed the same trend of up-regulation of genes related to omega-oxidation (Table 7.1).

**Table 7.1** Hepatic gene expression of genes related with lipid omega-oxidation and CAR in three different quercetin studies in mice.

| | Gene symbol | Gene name | Chapter 3 30en% MHF diet 21 °C | | Unpublished data 30en% MHF diet 28 °C | | Chapter 4 40en% HF diet 21 °C | |
|---|---|---|---|---|---|---|---|---|
| | | | FC | p | FC | p | FC | p |
| Omega-oxidation | Acot3 | Acyl-CoA thioesterase 3 | 2.38 | <0.0001 | 1.98 | 0.03 | -1.27 | 0.22 |
| | Car | Constitutive androstane receptor | 1.37 | <0.0001 | 1.22 | 0.13 | -1.12 | 0.035 |
| | Cyp4a10 | Cytochrome P450, family 4a10 | 2.98 | <0.0001 | 1.33 | 0.35 | 1.03 | 0.77 |
| | Cyp4a14 | Cytochrome P450, family 4a14 | 6.68 | <0.0001 | 1.96 | 0.027 | 1.11 | 0.70 |
| | Por | P450 (cytochrome) oxidoreductase | 1.97 | <0.0001 | 1.45 | 0.17 | 1.02 | 0.86 |
| CAR targets | Cyp2b9 | Cytochrome P450, family 2b9 | 1.88 | 0.0028 | n.a. | n.a. | -2.3 | 0.0009 |
| | Cyp2b10 | Cytochrome P450, family 2b10 | n.a. | n.a. | n.a. | n.a. | -2.63 | 0.0028 |
| | Cyp2b13 | Cytochrome P450, family 2b13 | n.a. | n.a. | n.a. | n.a. | -2.39 | 0.0006 |
| | Fabp5 | Fatty acid binding protien 5, epidermal | -1.43 | 0.0038 | n.a. | n.a. | 1.59 | 0.0075 |

Fold Changes are depicted as the expression values of quercetin over control animals. Gene expression data of the experiments as described in chapter 3 and 4 were determined by microarray, the unpublished gene expression was determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). MHF; mild high-fat, HF; high-fat, FC; Fold Change, p; p-value, n.a.; not assessed.

The serum lipid profiling showed lowered levels of palmitic acid (16:0) and oleic acid (18:1(n-9)) for the quercetin versus control group at 21°C, and the experiment at 28°C showed the same trend (Figure 7.2). Palmitic acid (16:0) and oleic acid (18:1(n-9) are fatty acids, which specifically can be oxidised via omega-oxidation. The effects on gene expression level and on serum lipids in the experiment where the mice were housed at thermoneutral temperature were smaller, but followed the same trend as found in the experiment with housing at room temperature. This is in agreement with other data that suggest that thermoneutrality diminishes diet-induced metabolic differences [23]. Nevertheless, these data indicate that with the same background diet, the same underlying effects were induced by quercetin.

In chapter 3 we have suggested that the transcription factor CAR could have an important role in the quercetin-mediated regulation of lipid omega-oxidation. The omega-oxidation related *Cyp4a* genes, are also known to be targets of peroxisome proliferator-activated receptor alpha (PPARA). However, the connection of the differentially expressed genes, the up-regulation of *Car* and the known influence of CAR on lipid metabolism [24,25], suggested a relation of the transcription factor CAR with omega-oxidation in our experiment. Interestingly, in chapters 4 and 5 we have shown that HF-induced expression of *Cyp2b9* was lowered by quercetin and other flavonoids. *Cyp2b9* is a known target gene of CAR and not of PPARA.



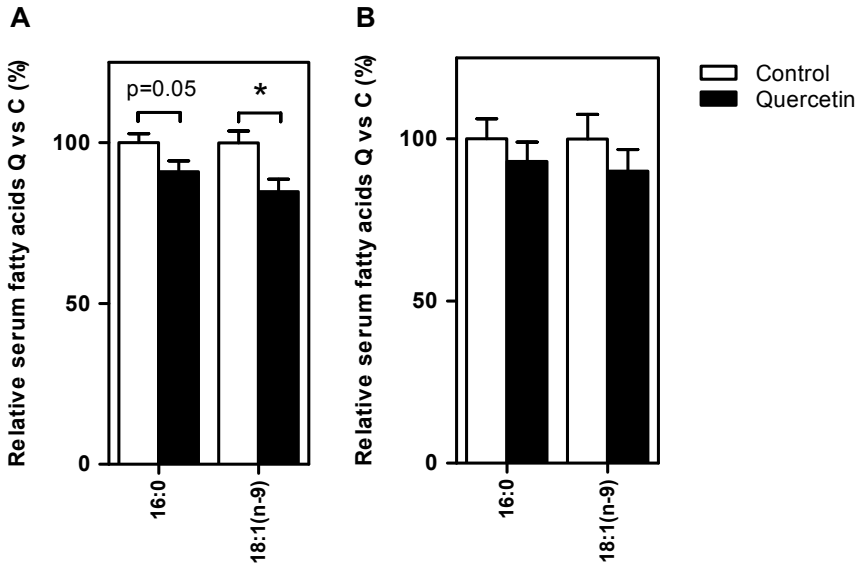


**Figure 7.2 Relative serum palmitic (16:0) and oleic acid (18:1(n-9)) originating from total lipids.** Serum lipids of the study described in chapter 3, 30 en% MHF diet housing at 21°C (A) and unpublished data of a study with the same setup and 30en% MHF diet, but animals were housed at 28°C (B). Data is presented as mean ± SEM (n=12). Asterisks indicate a significant difference between the control (C, white) and the quercetin (Q, black) group, *p<0.05.

In chapter 3 where the MHF diet was used, an increased expression of omega-oxidation by quercetin supplementation was shown, while using a HF diet (chapter 4), no regulation of omega-oxidation was found (Table 7.1). The difference might be explained by the fact that HF diets induced omega-oxidation [26], which possibly masks further stimulation by quercetin.

The regulation of *Car* and its *Cyp2b* target genes are all oppositely regulated in the experiment with the MHF diet compared to the experiment with the HF diet (chapter 3 vs. chapter 4). This is also the case for *Fabp5,* encoding a protein that can bind and transport PUFAs, including linoleic acid, into the liver [27]. Linoleic acid was abundantly present in the HF diet (chapter 4), the amount was twofold higher than in the MHF diet (chapter 3). It was shown *in vitro* that linoleic acid can attenuate the activation of CAR and induction of *Cyp2b9* mRNA [28,29]. We have also studied the induction of *CYP2B6* (the human homolog of *Cyp2b10)* in human liver HepaRG cells by the known CAR inducer CITCO. We observed that the induction *CYP2B6* by CITCO (0.5 μM) was attenuated by addition of linoleic acid (100 μM) (Figure 7.3). Together this suggests that the background diet, and particularly the linoleic acid content, modulated the hepatic gene expression response to quercetin.



Next to reduced body weight gain, all flavonoids showed reduced expression of hepatic *Cyp2b9* (chapter 5). It has also been shown that many flavonoids can induce activation of CAR [30,31]. This similar regulation by all flavonoids suggests a common mode of action of the flavonoids studied, and suggests that the results as shown in chapter 3 and 4 could possibly be similar for other flavonoids. Overall, CAR seems to play a role in flavonoid-mediated effects on lipid metabolism.

## Dietary context and flavonoid-mediated effects on hepatic lipid accumulation and body weight gain

In the previous section, it is discussed that the energy % of fat in the diet and/or the fat composition could affect the molecular effects of quercetin. The physiological

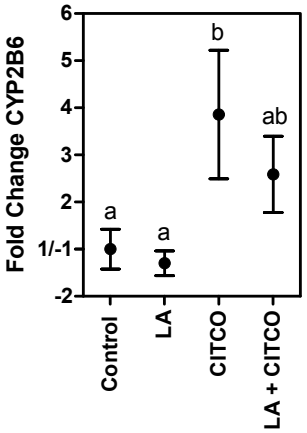


**Figure 7.3 Gene expression of CYP2B6 in HepaRG cells after exposure to linoleic acid (LA) (100 μM) and CITCO (0.5 μM).** Fold Changes are depicted as the expression values of exposed cells over control cells. Results are presented as mean ± SEM of three independent experiments (n=3). One-way ANOVA followed by a Bonferonni's post hoc test was used to compare the different exposures. Different letters indicate a significant difference, $p<0.05$.

effects found, e.g. body weight gain and hepatic lipid accumulation may also be modulated by the diet. In the MHF experiment we did not find any effect of quercetin on body weight gain or hepatic lipid accumulation (chapter 3), while in the HF-experiment quercetin reduced the HF diet-induced hepatic lipid accumulation and body weight gain (chapter 4). In table 7 experiments in rodents are listed where quercetin was added to the diet. There are differences in the dose, duration and the background diet used. Strikingly, in most cases when a diet was used that induced body weight gain and/or hepatic lipid accumulation, this effect was reduced by quercetin supplementation. Next to our own observation [32], two other studies using a 40 en% HF diet in mice with quercetin supplementation of 0.025-0.05% reduced diet-induced body weight gain and hepatic lipid accumulation after supplementation during respectively 9 and 20 weeks. [33,34]. Also the hepatic lipid accumulation induced with high cholesterol or methionine and choline deficient (MCD) diets was decreased by quercetin supplementation [35,36]. Only one study that used a HF diet of 45 en% for 8 weeks did not show effects of quercetin on diet-induced body weight gain and hepatic lipid accumulation [37]. The other studies presented in table 7.2 used diets that did not induce abundant body weight gain and/or hepatic lipid accumulation and indeed no effects of quercetin were observed on body weight gain or hepatic lipids. This indicates that quercetin may prevent body weight gain and hepatic lipid accumulation only under adverse dietary conditions which induce adiposity, but not with relatively healthy normal diets.



**Table 7.2** Rodent studies using quercetin supplementation in different diets.

| Quercetin in diet (%) | Diet (en% fat) | Weeks | Animals | Body weight | Hepatic lipids | Ref |
|---|---|---|---|---|---|---|
| 0.025 | High-fat (40en%) | 9 | C57BL/6J mice | ↓ | ↓ | 33 |
| 0.05 | High-fat (40 en%) | 4, 8, 20 | C57BL/6J mice | ↓ (wk 20) | ↓ (wk 20) | 34 |
| 0.33 | High-fat(40en%) | 12 | C57BL/6JOlaHsd mice | ↓ | ↓ | Chapter 4 / 32 |
| ~1 | High cholesterol | 8 | Swiss albino mice | ND | ↓ | 36 |
| ~0.04, 0.2 | unknown | 10 | Lean and obese Zucker rats | ↓ (0.2%) | ND | 38 |
| 0.005 | MCD | 2, 4 | C57BL/6J mice | ↔ | ↓ | 35 |
| 0.05 | Normal (16.7 en%) | 4, 8, 20 | C57BL/6J mice | ↔ | ↔ | 34 |
| 0.005 | Normal | 2, 4 | C57BL/6J mice | ↔ | ↔ | 35 |
| 0.33 | Mild High-fat (30 en%) | 12 | C57BL/6JOlaHsd mice | ↔ | ↔ | Chapter 3 / 39 |
| 0.1, 1 | Normal (11.5en%) | 10, 41 | Fisher 344 rats | ↔ | ND | 40 |
| 0.8 | High-fat (45 en%) | 3, 8 | C57BL/6J mice | ↔ | ND | 37 |
| 0.03 | Mild High-fat (35en%) | 4 | Wistar rats | ↔ | ND | 41 |
| 1 | Normal (20en%) | 2 | ICR mice | ↔ | ND | 42 |

MCD; methionine and choline deficient, ND; not determined.

## Dietary effects on leptin

In chapter 5, a strong correlation between leptin and body weight gain was observed. Leptin is secreted by white adipose tissue and is a peptide hormone which regulates body weight gain. Serum leptin levels were significantly reduced by quercetin as shown in the HF-experiment (chapter 5), while serum leptin levels in the MHF-study (chapter 3), where the diet did not induce abundant adiposity, were not significantly different (data not shown). This corresponds with the found differences in body weight gain (chapters 4 and 5), and the absence of body weight differences (chapter 3) between quercetin and control mice. Significant reductions in body weight gain and serum leptin levels were also shown for mice fed with a diet with hesperetin and anthocyanins supplementation (chapter 5). Effects on serum leptin levels were also found by others when studying the effect of anthocyanins on body weight gain. Supplementation with anthocyanins for 12 weeks significantly reduced HF diet-induced body weight gain, hepatic lipid accumulation, and serum leptin levels [43,44]. HF diet-induced body weight gain and serum leptin levels were also reduced by blueberry anthocyanins in mice after 10 weeks [45], while black raspberry anthocyanins showed no effects on HF diet-induced body weight gain and serum leptin levels [46]. Quercetin also induced reduced body weight gain and serum leptin levels by monosodium glutamate induced obesity in rats [47]. It was also shown that quercetin had no effects on body weight and serum leptin levels after 4 weeks of quercetin supplementation to a MHF diet [41]. Together these results reveal that when flavonoid-mediated effects on body weight gain and adiposity were found, serum leptin levels were even more sensitively affected. The effects of flavonoids on diet-induced adiposity seemed to be related to serum leptin levels. Leptin can be easily and rapidly measured in plasma or serum, and can therefore be used as an easy and useful early biomarker in humans for the effects of flavonoids on adiposity.

## Dietary effects on bioavailability of flavonoids

For flavonoids it is known that bioavailability can also be influenced by dietary factors. Dietary fibres are suggested to decrease flavonoid absorption several folds [48], while dietary lipids are shown to increase the bioavailability of flavonoids. For example, in humans bioavailability of quercetin was increased by a fat-rich breakfast compared to a fat-free breakfast, the area under the curve for 24 h plasma concentrations was increased between 19-43% [49]. Dietary proteins, carbohydrates and micronutrients are also suggested to have inhibitory or enhancing effects on the bioavailability of flavonoids [48]. Therefore, the dietary context can influence the bioavailability of flavonoids, which can thereby influence the flavonoid-mediated effects.

## Effects of dietary flavonoids on genotoxicity

The dietary flavonoids can also influence the bioavailability of genotoxic compounds, thereby influencing the genotoxic effects. The influence of flavonoids on the

bioavailability of genotoxic compounds can be via effects on transport proteins in the intestinal tract. Mostly this will result in a reduced absorption of carcinogenic compounds [50]. However, for example, the trans cellular transport of the pro-carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) was increased by addition of flavonoids in Caco-2 monolayers [51].

Furthermore, flavonoids can also affect metabolism, via interaction with phase 1 and phase 2 enzymes [52]. Flavonoids are known to inhibit cytochrome P450 (CYPs) enzymes involved in phase 1 metabolism. For example the flavonoid naringenin, mainly present in grapefruit juice, is a known inhibitor of CYP3A4. This phase 2 enzyme is, among others, involved in the biotransformation of the carcinogen aflatoxin B1 (AFB1) into its genotoxic epoxide metabolite [53].

Direct effects on phase 1 enzyme sulfotransferaxe (SULT) activity with the flavonoid nevadensin is for example also observed, this enzyme is involved in the bio-activation of allylalkoxybenzenes to carcinogens [54,55].

Together this indicates that flavonoids can affect absorption and metabolism of genotoxic compounds, and this can result in detoxification or bioactivation of genotoxic compounds.



## 7.5 DOSES

The doses used in the experiments could also be of influence on the flavonoid-mediated effects. First of all, this section will discuss the flavonoid doses used in our experiments compared to human intake. Then, the effects of the dose on the efficacy, bioavailability and genotoxicity of flavonoids will be presented.

### Flavonoid doses used as compared to human intake

The dose of quercetin in the studies described in this thesis was 0.33% (*w/w*) of the diet, because previous findings showed that this was in the efficacy range for quercetin (between 0.1% and 1%) [40]. The other flavonoids were added in equimolar amounts to the diet, which means supplementation of 0.29 to 0.5% (*w/w*) to the diets. The doses used were also within the range which is normally used in rodent flavonoid interventions as shown in table 1.2 of the general introduction and table 7.2 of this chapter. The doses of the individual flavonoids in the mice experiments provided ~350 to 500 mg/kg bw/day for 12 weeks. In humans, the total estimated intake of flavonoids via food is between 1.5 and 15 mg/kg bw/day [56]. Thus, the intake of the individual flavonoids that we have used in our mice interventions is between 200 and 1000 times higher than the estimated human dietary intake and about 15-100 times higher than the estimated human intake resulting from the use of flavonoid supplements. Thus, the doses used in the experiments were much higher than the average human dietary or supplementary intake, and it is of interest to elaborate if lower doses can also be effective.

## Dose and efficacy of flavonoids

Interestingly, in the experiment described in chapter 5, next to the HF-diet and the HF-diet with anthocyanins supplementation (HF+An10, 0.5%), we took along another group, which received a ten times lower dose of anthocyanins (HF+An1, 0.05%, unpublished data). This ten times lower dose of anthocyanins was shown to result in at least the same effect on HF diet-induced body weight gain, mesenteric white adipose tissue mass and hepatic lipid accumulation (Figure 7.4). The higher dose of anthocyanins did not directly result in stronger effects, this could probably be caused by saturation of uptake of flavonoids in some organs. Some other studies also did not find a dose-response relation when studying the efficacy of flavonoids. In a study in rats where three doses (10, 20 or 30 mg/kg bw/day) of hesperetin were given in combination with 1,2-dimethylhydrazine for induction of colon carcinogenesis, all doses of hesperetin showed significant reduction of colon cancer indications. However, the effect was suppressed formation of more indicators of colon cancer [58,59]. For the polyphenol resveratrol, it was also shown that of the three doses used (6, 30 and 60 mg/kg bw/day), the intermediate dose showed the highest potency for anti-obesity, and this dose showed significant effects on more parameters than the other doses [60]. In this experiment, tissue distribution was also studied for these three doses of resveratrol. In liver, a dose-dependent amount of resveratrol metabolites was found. But in adipose tissue, the metabolites increased only between the two lowest doses, and were similar for the middle and highest dose. In muscle, also similar amounts were detected for the middle and highest dose. It was suggested that there is a maximum in the capacity of certain tissues for metabolite incorporation, which cannot be exceeded by supplementation with higher doses [61].

Most likely there will be an optimal beneficial intake for flavonoids, this concept was also described for bioactive food components in general [62]. Based on the data presented in Figure 7.4 it is concluded that the beneficial health effects on hepatic lipid metabolism as observed in our mice experiments, could probably be translated to humans by using doses which can be obtained with flavonoid supplements. However, even by taking this optimal beneficial intake concept into account, these beneficial health effects could possibly not translated to humans by dietary intake of flavonoids. For further research it is important to determine the optimal effective dose of flavonoids.

## Dose and genotoxicity

The traditional dose-response relation, that a higher dose will be more effective, can also be expected for genotoxic effects of flavonoids. Indeed, dose-dependent effects on carcinogenicity of quercetin were shown in rodents: when different doses were used (providing ~50-2000 mg/kg bw/day), the highest doses showed more tumour formation [63]. Furthermore, DNA adduct formation was shown to be dose-dependent for known genotoxic compounds [64], which suggests that stronger genotoxic effects can be expected at higher doses of flavonoids, as long as the bioavailability of the flavonoids is also dose-dependent.

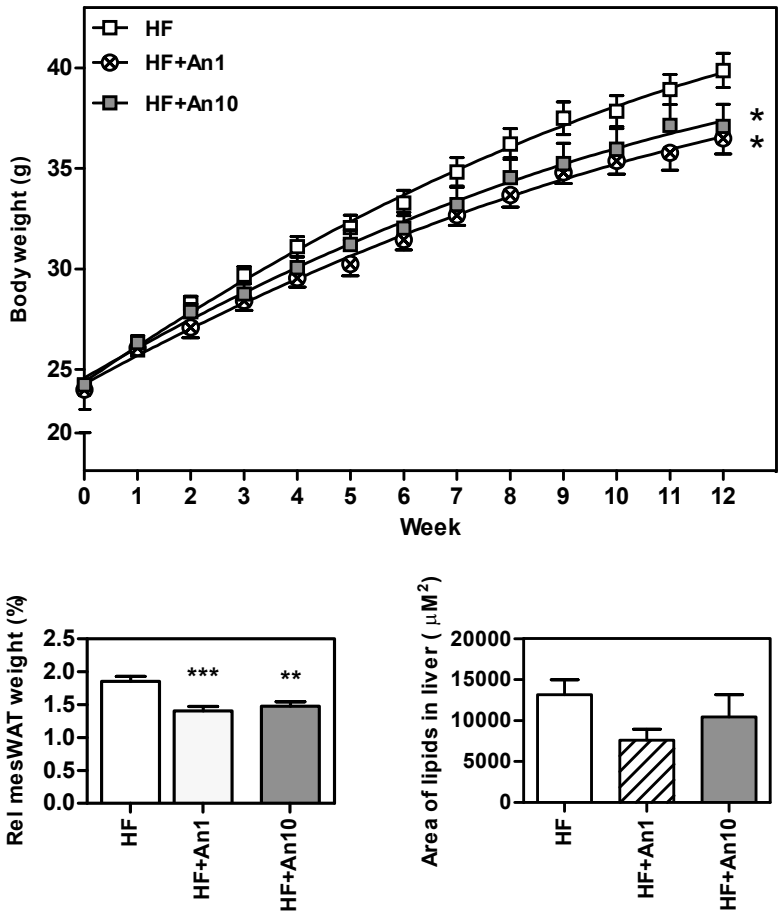


**Figure 7.4 Effects of two different doses of anthocyanins on body weight gain, relative mesWAT weight and lipid accumulation in liver.** Anthocyanins were added to a HF diet with 0.033% (HF+An1) and 0.33% (HF+An10). Curve fitting was used to analyze body weight gain using Proast software [57]. One-way ANOVA with a Dunnett's post hoc test was used the compare the relative mesWAT weight and area of hepatic lipids between the HF+An groups and the HF control group. Data is presented as mean ± SEM (n=8-12), asterisks indicate significant differences, *p<0.05, **P<0.01, ***p<0.001. MesWAT, mesenteric white adipose tissue.

The dose of quercetin used, in the experiment where we have studied the genotoxicity, was ~350 mg/kg bw/day for 12 weeks, which is about 1,000 times the human dietary intake of quercetin. This gave no indications of quercetin-induced genotoxicity in liver and small intestine of mice. Genotoxicity was also not found in rodents after oral quercetin administration measured by among others micronuclei, chromosomal aberrations and unscheduled DNA synthesis in bone marrow, liver, and gastric mucosa cells [5-7,65,66]. Furthermore, several other long-term (ranging from 64 weeks to lifespan) *in vivo* studies showed no carcinogenicity after quercetin exposure

providing ~60-3500 mg/kg bw/day in rodents [67-70]. Hence, there is no reason for concern over genotoxicity in liver and intestine after dietary intake of quercetin.

The quercetin doses used in this mouse experiment are 15 times higher than human supplementary intake of quercetin. For non-genotoxic substances a safety factor of 100 is normally used to correct for inter-species differences and human variability. Of note, recent studies in rats showed that a quercetin exposure of ~40 mg/kg bw/day during pregnancy can affect the foetus, as quercetin can cross the placenta and accumulates in the foetus. These studies also reported that the rearrangements in a gene which can be linked to childhood leukaemia was increased [71,72]. Therefore, the safety of high supplementary intake of flavonoids should be further studied, and possibly specific guidelines for using quercetin supplements during pregnancy are needed.

## 7.6 OTHER POTENTIAL FACTORS INFLUENCING EFFICACY OF FLAVONOIDS



In this paragraph two more factors will be discussed which could potentially influence flavonoid-mediated effects, namely gender and colonic microbiota.

### Gender

The rodent studies of the present thesis and other studies investigating effects of quercetin as mentioned in table 7.2, were performed only with male mice. Data in female rodents are lacking, while the effects could be different between males and females. It is for example shown for the dietary compound beta-carotene that it can induce opposite effects in gene expression for male and female mice [73]. Also the flavonoids genistein and quercetin-3-O-rutinoside (rutin) have shown sex-dependent differences in effects in kidney and on serum markers of cardiovascular health, respectively [74,75]. Furthermore, sensitivity for activation of CAR and CYP4A, involved in the suggested mechanisms of action of quercetin in chapter 3, is known to be sex-dependent [76,77]. For further research it is important to also study effects in females and to analyse if sex dependent differences in flavonoid mechanisms of action exist.

### Colonic microbiota

Flavonoids are extensively metabolised by bacteria in the colon. Individual differences in microbiota could have an influence on the extent and kind of metabolism of flavonoids that predominantly occurs. This will influence the amount and composition of the colonic metabolites, which also could have effects on health outcomes [78]. Excessive amounts of flavonoids entering the colon could also inhibit the growth of colonic microbiota, and thus influencing the metabolite profile in the colon and subsequently in the circulation[79]. The exact implications of colonic microbiota for the effects of flavonoids are not known and need to be further investigated [80].

## 7.7 IMPLICATIONS FOR STUDYING FLAVONOID-MEDIATED EFFECTS IN HUMANS

For the translation of the flavonoid-mediated effects found in animals to humans several factors should be taken into account. Many of the factors mentioned above have specific implications when performing flavonoid research in humans and contribute to the explanation of discrepancies that are found between human and animal studies [81]. Factors which have specific implications for studying flavonoid-mediated effects in humans compared to animals are discussed below.

### Mouse as a model

Identifying mechanisms of actions in specific organs, e.g. liver, is not easily done in humans, therefore the mouse is widely used as a model for studying diet-induced obesity, insulin resistance, cardiovascular diseases, and hepatic lipid accumulation [82-84]. The effects of flavonoids on lipid metabolism as shown in this thesis, have been studied before in a mouse model [85-87]. However, for the translation to humans, these specific effects on lipid metabolism should be confirmed in humans. Apart from physiological differences between humans and mice in lipid metabolism, the following factors should be considered.



### Flavonoid metabolism

The metabolism of flavonoids can also be different between mice and humans. It is described that various mono-, di- and tri-conjugates are found for quercetin in the circulation of rodents as well as in humans [88], suggesting an overall comparable pattern of conjugates formed after flavonoid metabolism in mice and humans. However, the amount of specific conjugates of quercetin for example can be different between mice and humans [89-95] and also the biological effects of conjugates can be different [10,89]. Therefore, possible differences in the major flavonoid conjugates present in the systemic circulation could affect the observed physiological effects.

### Dietary context

The dietary context can modulate the molecular and physiological effects of flavonoids, as discussed above. In human intervention studies, mostly no controlled defined background diet is used. Moreover, in observational studies flavonoid intake is estimated on the basis of general dietary intake [96-100], not corrected for the differences in dietary background intake between subjects. This can contribute to the explanation why more abundant effects are found in animal studies than in human interventions. Therefore, it is important to consider the dietary context in detail in flavonoid research. Ideally a fully-controlled dietary background should be used in further research, alternatively the dietary intake could be measured, which can be used for correction for possible confounding effects of the dietary context.

### Purity of flavonoids

Human intervention studies that showed beneficial effects on functional markers for CVD, are almost exclusively based on administration of flavonoid-rich foods, such as cocoa, tea, berries or wine [96-100]. These flavonoid-rich foods or extracts also contain many other substances that may have an effect, such as for example theobromine and caffeine. For instance, caffeine was present in many human intervention studies with green tea flavan-3-ol containing extracts. Effects on body weight were only seen in green tea extracts that also contained caffeine [101]. Therefore, it is important that pure individual flavonoids will be studied, since now there is only very limited information on effects of individual flavonoids in humans. In rodents, pure flavonoids have been studied more often, and these studies could give important clues for human research.

Taken together, for the design of effective flavonoid studies in humans, we have to control or correct for several factors like dietary context, dose, purity of flavonoids, flavonoid bioavailability and flavonoid metabolism. In addition, it may be advisable to characterize participants with respect to microbiota and genetic constitution (for example polymorphisms in metabolizing enzymes). This will lead to more reliable and relevant experiments, which contribute to the unravelling of the potential beneficial effects on lipid metabolism of flavonoids.



## 7.8 CONCLUSIONS

In this thesis several effects of flavonoids in mice are described. Interference of flavonoids in peroxidase-based assays was demonstrated, which will overestimate the effects of flavonoids. Therefore, vigilance is needed in using these and other peroxidase-based assays in flavonoid research. The flavonoid quercetin showed effects on hepatic lipid metabolism and on whole body energy balance. The mechanistic and physiological effects of quercetin appeared to be modulated by the fat content and fat composition of the dietary background. The effects on hepatic lipid metabolism seemed to be related or influenced by the transcription factor CAR. All five studied flavonoids, quercetin, hesperetin, epicatechin, apigenin and anthocyanins showed reduction of HF diet-induced body weight gain, with quercetin showing the strongest effects in preventing adiposity. No genotoxic effects of quercetin were found in our transcriptomic analysis in small intestine and liver of mice.

Our studies revealed beneficial health effects of flavonoids in mice on hepatic lipid metabolism, which could probably be translated to humans by using doses which can be obtained with flavonoid supplements, but possibly not by dietary intake of flavonoids. Individual differences in consumed diet, colonic microbiota, and gender, also contributing to differences in flavonoid bioavailability and metabolism, probably hampered observations of flavonoid effects on lipid metabolism in human experiments. Fully-controlled experiments using pure flavonoids, with fixed background conditions in

animals and humans, could contribute to better understanding of underlying mechanisms of the health effects of flavonoids on lipid metabolism, and these studies would help to better evaluate flavonoids as potential functional healthy dietary compounds.

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# SUMMARY

Consumption of foods containing flavonoids is associated with a reduced risk of cardiovascular diseases (CVD), possibly by lipid-lowering effects. On the other hand, for one of these flavonoids, quercetin, also genotoxicity was shown especially in *in vitro* bioassays. Therefore, the first aim of this thesis was to identify mechanisms underlying potential beneficial health effects of flavonoids. The focus was on hepatic lipid metabolism and circulating lipids and a molecular and physiological approach was used. Secondly, we aimed to study the potential *in vivo* genotoxic effects of quercetin by transcriptome analyses in liver and small intestine, since these represent the tissues of first contact exposed to relatively high levels upon oral intake of flavonoids.

Circulating lipids are important CVD-related risk markers, which are in general determined with commercially available enzyme-based assays. However, the usual enzyme in these assays, peroxidase, has previously been reported to be inhibited by flavonoids. Therefore, we have studied in **chapter 2** whether these assays can adequately be used in flavonoid research. We observed that various flavonoid aglycones interfere with peroxidase used in triglycerides (TG) and free fatty acids (FFA) enzymatic assays, reporting incorrect lower TG and FFA levels than actually present. Furthermore, addition of metabolites such as isorhamnetin or quercetin-3-O-glucuronide, the major metabolite of quercetin in human and rat plasma, to murine serum also resulted in a significant reduction of the detected TG levels, while a trend was seen towards reduced FFA levels. It can be concluded that when applying these biochemical assays, vigilance is needed and alternative analytical methods assessing FFA or TG levels should preferably be applied for studying the biological effects of flavonoids on TG and FFA levels.

In **chapter 3** mechanistic and physiological effects of quercetin on hepatic lipid metabolism were studied. C57BL/6JOlaHsd male adult mice received a mild high-fat (30 en%) diet without or with supplementation of 0.33% (w/w) quercetin for 12 weeks. Gas chromatography and $^{1}$H-NMR were used to quantitatively measure serum lipid profiles. Whole genome microarray analysis of liver tissue was used to identify potential mechanisms underlying altered circulating lipid levels by quercetin supplementation. Body weight, energy intake and hepatic lipid accumulation did not differ significantly between the quercetin and the control group. In serum of quercetin-fed mice, TG levels were decreased by 14% ($p<0.001$) and total poly unsaturated fatty acids (PUFA) levels were increased by 13% ($p<0.01$). Levels of palmitic acid, oleic acid, and linoleic acid were all decreased by 9-15% ($p<0.05$) in quercetin-fed mice. Both palmitic acid and oleic acid can be oxidized by omega-oxidation. Gene expression profiling showed indeed that quercetin increased hepatic lipid metabolism, especially omega-oxidation. At the gene level, this was reflected by the up-regulation of cytochrome P450 (Cyp) *4a10*, *Cyp4a14*, *Cyp4a31* and *Acyl-CoA thioesterase* 3 (*Acot3*). Two relevant regulators,

cytochrome P450 oxidoreductase (*Por*, rate limiting for cytochrome P450 activities) and the transcription factor constitutive androstane receptor (*Car*; official symbol *Nr1i3*) were also up- regulated in the quercetin-fed mice. We concluded that quercetin intake increased hepatic lipid omega-oxidation and lowered corresponding circulating lipid levels, which may contribute to potential beneficial effects of quercetin on CVD.

Subsequently, in **chapter 4** effects of quercetin supplementation were studied in mice given a high-fat (40 en%) background diet. The set-up of the experiment was the same as in chapter 3, with the exception of the background diet that was used, which was different in fat content and composition. This high-fat diet-induced body weight gain, and serum and hepatic lipid accumulation, which are all known risk factors for CVD. The aim of this study was to investigate the effects and underlying molecular mechanisms of the effects of the flavonoid quercetin on hepatic lipid metabolism in mice given this high-fat diet background. C57BL/6JOlaHsd male adult mice received the high-fat diet without or with supplementation of 0.33% (w/w) quercetin for 12 weeks. Body weight gain was 29% lower in quercetin fed mice versus control mice ($p<0.01$), while the energy intake was not significantly different. Quercetin supplementation lowered high-fat diet-induced hepatic lipid accumulation to 29% of the amount present in the control mice ($p<0.01$). $^{1}$H-NMR serum lipid profiling revealed that the supplementation also significantly lowered high-fat diet-induced increases in serum lipid levels. Global gene expression profiling of liver showed that cytochrome P450 2b (*Cyp2b*) genes, key target genes of the transcription factor *Car*, were down-regulated. However, the induction of omega-oxidation observed by quercetin supplementation to a mild high-fat (30en%) diet (chapter 3), was not observed this time with the high-fat (40en%) diet. Cumulatively, quercetin decreased high-fat diet-induced body weight gain, hepatic lipid accumulation and serum lipid levels. This was accompanied by regulation of cytochrome P450 2b genes in liver, which are considered to be under transcriptional control of CAR. The quercetin effects are likely dependent on the fat content and composition of the diet.

In **chapter 5** we investigated whether flavonoids from other flavonoid subclasses can exert the same effects as we observed for quercetin. Effects of quercetin, hesperetin, epicatechin, apigenin and anthocyanins, in C57BL/6JOlaHsd male adult mice fed a high-fat diet for 12 weeks were compared, relative to a normal-fat diet. High-fat diet-induced body weight gain was significantly lowered by all flavonoids (17-29%), but most by quercetin. Quercetin significantly lowered high-fat diet-induced hepatic lipid accumulation (by 71%). High-fat diet-induced increases of mesenteric adipose tissue weight and serum leptin levels were significantly lowered by quercetin, hesperetin, and anthocyanins. Adipocyte cell size and adipose tissue inflammation were not affected.

The effects on body weight and adiposity could not be explained by individual significant differences in energy intake, energy expenditure, nor by differences in activity. Lipid metabolism was not changed as measured by indirect calorimetry or expression of known lipid metabolic genes in liver and white adipose tissue. Hepatic expression of *Cyp2b9* was

strongly down-regulated by all flavonoids. Overall, all five flavonoids lowered parameters of high-fat diet-induced adiposity, with quercetin being most effective.

Next to the beneficial health effects of flavonoids, the safety of flavonoids is under discussion, mainly because of potential genotoxic effects found for quercetin *in vitro*. Therefore, in **chapter 6** the *in vivo* genotoxicity of this flavonoid was studied by transcriptome analyses in two tissues, small intestine and liver, where the highest exposure to quercetin is expected. This is especially of interest in view of high intake by widely available food supplements. Quercetin (0.33%) supplemented to a high-fat diet was administered to C57BL/6JOlaHsd male adult mice during 12 weeks. Serum alanine aminotransferase and aspartate aminotransferase levels revealed no indications for hepatotoxicity. General microarray pathway analysis of liver and small intestinal tissue samples showed no regulation of genotoxicity related pathways. In addition, analysis of DNA damage pathways in these tissues did also not point at genotoxicity. Furthermore, comparison with a published classifier set of transcripts for identifying genotoxic compounds did not reveal any similarities in the regulation of these classifier set by quercetin. Available microarray datasets of known genotoxic liver carcinogens, 2-acetylaminofluorene and aflatoxin B1 in mice were taken along as positive controls for comparison, and indeed showed genotoxic properties (regulation of genotoxic related genes) in the analyses. This transcriptomic analysis showed that supplementation with quercetin at ~350 mg/kg bw/day for 12 weeks did not induce genotoxicity in liver and small intestine.

In conclusion, we have shown *in vivo* efficacy of flavonoids reflected by effects on metabolic health parameters, including hepatic lipid metabolism. These effects on hepatic lipid metabolism seemed to be related or influenced by the transcription factor CAR. The dietary contexts appeared to modify the health effects. The five studied flavonoids in general showed the same effects, with quercetin being the most effective. No genotoxicity of quercetin was found by transcriptome analyses in liver and small intestine. Overall, we have obtained indications for beneficial health effects of flavonoids in mice, which makes it interesting to study if these effects can be extrapolated to humans to further explore their potential as functional compounds of dietary flavonoid intake.

# SAMENVATTING

Het consumeren van voedingsmiddelen rijk aan flavonoïden wordt geassocieerd met een verminderd risico op hart- en vaatziekten, mogelijk als gevolg van een verlaagd lipide-gehalte in het bloed. Echter, voor één van deze flavonoïden, quercetine, geven *in vitro* proeven aanwijzingen voor mogelijke genotoxiciteit. Het eerste doel van dit proefschrift was het identificeren van mechanismen die ten grondslag liggen aan gezondheids-bevorderende effecten van flavonoïden. Hierbij lag de focus op het vetmetabolisme in de lever en het lipide-gehalte in het bloed. Het tweede doel was om de mogelijke genotoxische effecten van quercetine in de lever en dunne darm te onderzoeken. Deze twee weefsels werden gekozen omdat deze als eerste blootgesteld worden aan relatief hoge niveaus flavonoïden bij consumptie van voedingsmiddelen of supplementen rijk aan flavonoïden.

Lipide-gehalten in het bloed zijn belangrijke risico-indicatoren voor hart- en vaatziekten. Lipiden in het bloed worden over het algemeen bepaald met behulp van commercieel beschikbare enzymatische assays. Deze assays zijn meestal gebaseerd op het gebruik van het enzym peroxidase. Echter, het is bekend dat het enzym peroxidase kan worden geremd door flavonoïden. Daarom hebben we in **hoofdstuk 2** bestudeerd of deze assays op een adequate manier gebruikt kunnen worden in onderzoek met flavonoïden. We laten zien dat verschillende flavonoïden interfereren met peroxidase in triglyceriden- en vrije vetzuur-assays. Dit resulteerde in incorrecte, lagere gemeten waarden van triglyceriden en vrije vetzuren dan de daadwerkelijk aanwezige waarden. Verder resulteerde het toevoegen van metabolieten van quercetine aan muizenserum ook in een significante, maar incorrecte schijnbare afname van de gemeten waarden van triglyceriden. Deze afname werd ook waargenomen in de gemeten waarden van de vrije vetzuren, hoewel dit effect niet significant was. Daarom is voorzichtigheid geboden wanneer deze op peroxidase-gebaseerde assays worden gebruikt bij het bestuderen van de effecten van flavonoïden. Alternatieve methoden, zoals 1H-NMR, LC-MS of gas-chromatografie worden daarom aanbevolen voor het bestuderen van de effecten van flavonoïden op triglyceriden en vrije vetzuren in het bloed.

In **hoofdstuk 3** hebben we de mechanistische en fysiologische effecten van quercetine op vetmetabolisme bestudeerd. Volwassen C57BL/6JOlaHsd muizen kregen een dieet met een mild hoog vetgehalte (30 en%) met of zonder toevoeging van 0.33% (w/w) quercetine gedurende een periode van 12 weken. Lichaamsgewicht en energie-inname waren niet verschillend tussen de quercetine en de controle groep. In het bloed werden lipide-profielen bepaald door middel van gas-chromatografie en $^{1}$H-NMR. In het bloed van de muizen die het dieet verrijkt met quercetine kregen, was het triglyceride niveau 14% lager ($p<0.001$) en het gehalte meervoudig onverzadigde vetzuren was toegenomen met 13% ($p<0.01$) ten opzichte van de controle groep. Verder waren in het bloed van de muizen die het quercetine-dieet kregen, de niveaus van de 16:0, 18:1(n-9) en 18:2(n-6) vetzuren 9-15%

lager ($p<0.05$). Mogelijke onderliggende mechanismen voor de afname van de lipide-gehalten in het bloed werden onderzocht m.b.v. genoom brede genexpressie analyse. We vonden op-regulatie van de genen *Cyp4a10*, *Cyp4a14*, *Cyp4a31* en *Acot3*. Deze genen zijn betrokken bij omega-oxidatie van vetzuren, een proces waarbij de vetzuren 16:0 en 18:1(n-9) kunnen worden geoxideerd. Twee andere relevante genen, *Por* en *Car,* waren ook op-gereguleerd in de muizen die waren blootgesteld aan quercetine. Inname van quercetine verhoogde in deze studie de vetzuur omega-oxidatie en verlaagde het gehalte van specifieke vetzuren (16:0 en 18:1(n-9). Deze effecten zouden ten grondslag kunnen liggen aan mogelijk preventieve effecten van quercetine op hart- en vaatziekten.

In **hoofdstuk 4** hebben we de effecten van quercetine bestudeerd in een bredere context door het toe te voegen aan een dieet met een hoger vetgehalte (40 en%). De opzet van dit experiment was gelijk aan het experiment uit hoofdstuk 3, met uitzondering van het achtergrond dieet dat hier werd gebruikt. Dit dieet met hoog vetgehalte veroorzaakte een toename van het lichaamsgewicht, een toegenomen vervetting van de lever en een toename van het lipide-gehalte in het bloed. Deze effecten zijn risicofactoren voor hart- en vaatziekten. Het doel van deze studie was om de effecten van quercetine te onderzoeken op het vetmetabolisme in de lever en de onderliggende mechanismen die hieraan ten grondslag liggen in muizen die een dieet met een hoog vetgehalte kregen. Volwassen C57BL/6JOlaHsd muizen kregen het dieet met of zonder toevoeging van 0.33% (w/w) quercetine gedurende een periode van 12 weken. Na deze 12 weken was het lichaamsgewicht 29% minder toegenomen in de muizen waar quercetine was toegevoegd aan het dieet. Omdat de energie-inname tussen de groepen gelijk was, kan het verschil in energie-inname niet de verklaring zijn voor het verschil in gewichtstoename. De vervetting van de lever was ook afgenomen met 29% in vergelijking met de mate van leververvetting in de controle muizen. De bepaling van het lipide-profiel m.b.v. $^{1}$H-NMR liet ook zien dat quercetine de door het vet geïnduceerde serum lipide-gehaltes verminderde. Microarray analyse van leverweefsel liet een op-regulatie zien van *Cyp2b* genen, target genen van CAR. De inductie van omega-oxidatie zoals beschreven in hoofdstuk 3 waar quercetine werd toegevoegd aan een dieet met een mild hoog vetgehalte, zagen we niet terug in deze studie waarin gebruik gemaakt werd van een dieet met een hoog vetgehalte. Quercetine verminderde de door het hoog-vet-dieet geïnduceerde lichaamsgewichttoename, vervetting van de lever en de lipide-gehalten in het bloed. Dit ging gepaard met regulatie van *Cyp2b* genen in de lever. De door quercetine geïnduceerde effecten zijn waarschijnlijk afhankelijk van het vetgehalte en de samenstelling van het dieet.

In **hoofdstuk 5** hebben we onderzocht of flavonoïden van andere flavonoïde-subklassen dezelfde effecten hebben als de effecten die we hebben gevonden voor quercetine. De effecten van quercetine, hesperetine, epicatechine, apigenine en anthocyaninen werden vergeleken in muizen die 12 weken een dieet met een hoog vetgehalte (40 en%) kregen waaraan deze flavonoïden werden toegevoegd. Het

controle dieet veroorzaakte een toename in lichaamsgewicht. Door toevoeging van de verschillende flavonoïden, verminderde de toename in lichaamsgewicht met 17-29%, afhankelijk van de specifieke flavonoide. De grootste effecten werden gevonden voor quercetine. Quercetine verlaagde de door het dieet geïnduceerde vervetting van de lever met 71%. De hoeveelheid mesenterisch vetweefsel en de leptine-waarden in het bloed werden significant minder verhoogd in de groep met het dieet met quercetine, hesperetine of anthocyaninen. De gemiddelde grootte van de adipocyten en de mate van inflammatie in het vetweefsel bleven onveranderd. De effecten op lichaamsgewicht en adipositas kunnen niet worden verklaard door individuele significante verschillen in de energie inname, het energieverbruik, of de lichaamsactiviteit van de muizen. Het vetmetabolisme werd gemeten met behulp van indirecte calorimetrie en expressie van genen die betrokken zijn bij vetmetabolisme in lever en vetweefsel. Het vetmetabolisme bleek niet veranderd te zijn door toevoeging van de flavonoïden aan het dieet. Genexpressie van Cyp2b9 was sterk omlaag gereguleerd door alle flavonoïden. Parameters van dieet-geïnduceerde adipositas werden verlaagd door alle vijf de flavonoïden, waarbij quercetine de sterkste effecten liet zien.

Naast de gezondheids-bevorderende effecten van flavonoïden, staat de veiligheid van flavonoïden ook ter discussie, vooral door de mogelijk genotoxische effecten van quercetine die werden gevonden in *in vitro* experimenten. Daarom hebben we in **hoofdstuk 6** de *in vivo* genotoxiciteit van quercetine onderzocht door middel van genoom brede genexpressie analyses in weefsel afkomstig van de dunne darm en de lever van muizen blootgesteld aan quercetine. Dit zijn de organen die naar verwachting blootgesteld worden aan de relatief hoogste concentraties quercetine. Dit is relevant met het oog op breed beschikbare quercetine-supplementen, waarmee een hoge dosis quercetine kan worden ingenomen. Quercetine (0.33%, w/w) werd toegevoegd aan een dieet met een hoog vet gehalte, en werd gedurende 12 weken gegeven aan C57BL/6JOlaHsd muizen. Serum alanine aminotransferase en aspartaat aminotransferase waarden gaven geen indicatie voor leverschade. Genexpressie analyse in weefsel van zowel de lever als dunne darm toonde geen regulatie aan van processen die geassocieerd zijn met genotoxiciteit. Een analyse van processen gerelateerd aan DNA schade liet ook geen indicatie zien voor genotoxiciteit van quercetine. Verder liet een vergelijking met een bekende set van genen die gebruikt worden voor het identificeren van genotoxische stoffen zien dat er geen overeenkomt was met de door quercetine gereguleerde genen. Beschikbare lever microarray datasets van bekende genotoxische stoffen dienden als positieve controle en lieten inderdaad regulatie zien van genen die gerelateerd zijn aan genotoxiciteit. Hieruit concludeerden we dat toevoeging van quercetine aan het dieet (350 mg/kg bw per dag) van muizen gedurende 12 weken echter geen genotoxiciteit in de lever en dunne darm induceerde.

Concluderend hebben we laten zien dat quercetine *in vivo* effecten heeft op parameters die gerelateerd zijn aan metabole gezondheid, zoals vetmetabolisme in

de lever. Deze effecten op vetmetabolisme in de lever lijken gerelateerd te zijn aan de transcriptiefactor *CAR*. De dieet-context kan deze gezondheids-bevorderende effecten beïnvloeden. Over het algemeen lieten de vijf flavonoïden vergelijkbare effecten zien, waarbij quercetine het meest effectief was. Er werden geen aanwijzingen voor genotoxiciteit van quercetine gevonden in de lever en de dunne darm. We hebben duidelijke aanwijzingen voor gezondheids-bevorderende effecten laten zien van flavonoïden in muizen. Daarom is het interessant om in de toekomst te bestuderen of deze effecten geëxtrapoleerd kunnen worden naar de mens, om zo de potentie van flavonoïden als functionele voedingscomponenten verder te onderzoeken.

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# CURRICULUM VITAE

Elise F. Hoek-van den Hil was born on 21th July 1986 in Brakel, the Netherlands. In 2004 she completed pre-university education at 'De Lage Waard' in Papendrecht and started the Bsc Nutrition and Health at Wageningen University. During the MSc Nutrition and Health at Wageningen University, she did her MSc thesis at the division Toxicology of Wageningen University, where she worked on the intestinal sulfonation of the flavonoid hesperetin and the effect of hesperetin metabolites and hesperetin enantiomers on EpRE-mediated gene expression *in vitro*. She conducted her internship at the Hospital 'Ziekenhuis Gelderse Vallei' in Ede, where she worked on the development and improvement of a method for analysis of nutritional intake of patients. She graduated for the MSc Nutrition and Health with the specialisation 'Food Toxicology' in September 2009. Elise worked from October 2009 till January 2015 as a PhD student at Wageningen University on a VLAG project which was a collaboration between Human and Animal Physiology, the division Toxicology, and the research institute RIKILT. She worked in this project on the underlying mechanisms of flavonoid-mediated health effects, focussing on lipid metabolism and genotoxicity, as described in this thesis. She followed also the postgraduate education in toxicology, resulting in registration as toxicologist after PhD graduation. From December 2014, she works as Food Safety Scientist at Danone, Utrecht.

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**Hoek-van den Hil EF**, van Schothorst EM, van der Stelt I, Hollman PCM, Keijer J, Rietjens IMCM. Quercetin tests negative for genotoxicity in transcriptome analyses of liver and small intestine of mice. *Submitted*

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# OVERVIEW OF COMPLETED TRAINING ACTIVITIES

## Discipline specific activities

Course Organ Toxicology, PET, 2009
Course Toxicogenomics, PET, 2010
Course Mutagenesis & Carcinogenesis, PET 2011
Course Molecular toxicology, PET, 2011
Course Toxicological risk assessment, PET, 2012
Course Ecotoxicology, PET, 2013
NVT days, Nederlandse Vereniging Toxicology, 2010, *poster presentation*
Symposium bioenergetics, NCMLS New Frontiers, Nijmegen, 2010
Congress MITO food, Wageningen, 2011, *poster presentation*
Doctoral Workshop in Molecular Nutrition Tarragona, Spain, 2012, *oral presentation*
Landelijk voedingsgeneeskundig congres, Amsterdam, 2012, *poster presentation*
Nutritional Science days, NWO, 2013, *oral presentation*
Congress Polyphenols, Lisbon, Portugal, 2014, *oral presentation*
Nutritional Science days, NWO, 2014, *oral presentation*

## General courses

VLAG PhD week, VLAG, 2010
Mini Symposium How to write a world class paper, WUR, 2010
VLAG lecture information efficiency, VLAG, 2010
WIAS science day, WIAS, 2010
Presentation skills, coaching, 2012
Course Risk Communication, PET, 2012
RMC reviewer, WIAS, 2013
Course Career perspectives, WGS, 2014

## Optionals

Attending Scientific presentations, human and animal physiology and toxicology WUR, 2009-2015
MSc course proefdierkunde, article 9, WUR, 2010
Visit NIZO, Young AFSG, 2012
PhD trip Toxicology, Italy and Swiss, 2011

**Colophon**

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