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RESEARCH PAPER

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). Increased circulating lipid levels and hepatic lipid accumulation are known risk factors for CVD. The aim of this study 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 downregulated. 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.

Keywords Bioactive food component · Flavonoid · Gene expression profiling · Hepatic lipid accumulation · Lipid metabolism

Abbreviations

Acot3	Acyl-CoA thioesterase 3
B2m	Beta-2 microglobulin
Car	Constitutive androstane receptor
Csad	Cysteine sulphinic acid decarboxylase
CVD	Cardiovascular diseases
Cyp	Cytochrome P450
En%	Energy%
Fabp5	Fatty acid binding protein 5
FA	Fatty acids
Hao2	Hydroxyacid oxidase 2
HOMA-IR	Homoeostasis model assessment–insulin resistance
Hprt1	Hypoxanthine phosphoribosyltransferase 1
Por	Cytochrome P450 oxidoreductase
RT-qPCR	Real-time quantitative polymerase chain reaction

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Introduction

High consumption of fruits and vegetables is protective against cardiovascular diseases (CVD) (van't Veer et al. 2000). Flavonoids in fruits and vegetables are suggested to contribute to these protective effects (Mink et al. 2007). Moreover, epidemiological studies have shown that the intake of the flavonoid quercetin is associated with a reduction of CVD risk (Arts and Hollman 2005; Hertog et al. 1993; Hollman et al. 2010). 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 (Hoek-van den Hil et al. 2013). The cardiovascular protective properties of quercetin might therefore be explained by the lipid lowering effect of quercetin, since increased circulating levels of free fatty acids and triglycerides are known risk factors for CVD (Boden 2008; Harchaoui et al. 2009; Jouven et al. 2001). Furthermore, growing evidence shows that increased lipid accumulation in the liver also increased the risk of CVD (Scorletti et al. 2011; Targher et al. 2010).

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 (de Boer et al. 2006; Hoek-van den Hil et al. 2013; Jung et al. 2013; Kobori et al. 2011; Odbayar et al. 2006; Stewart et al. 2009; Wein et al. 2010). 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 (Hoek-van den Hil et al. 2013). 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 (Voigt et al. 2013). 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.

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 5 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 (Voigt et al. 2013) (Research Diets Services B.V., Wijk bij Duurstede, the Netherlands; supplementary table S1). During the intervention, the mice ($n = 12$) received high-fat diet (40 en% 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 (Hoek-van den Hil et al. 2013). Body weight and food intake of individual mice were weekly monitored. Faeces were 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 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), kept on ice for max 2 h, and centrifuged for 10 min at 3,000*g* 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 (Hoek-van den Hil et al. 2013). Before analysis, samples were hydrolysed by β-glucuronidase/sulphatase 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 (Hoek-van den Hil et al. 2012), alternative methods were used. Neutral lipids were stained in frozen liver sections ($n = 6$) with Oil red O (Sigma) and quantified as described (Hoek-van den Hil et al. 2013). 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 (Hoek-van den Hil et al. 2013).

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 (Sailer et al. 2013) 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 (Hoek-van den Hil et al. 2013) according to the MIQE guidelines (Bustin et al. 2009). Data were normalised against reference genes beta-2 microglobulin (*B2m*) and hypoxanthine phosphoribosyltransferase 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 S2.

Microarray analysis

For global transcriptome analysis, liver samples of individual mice and 8 × 60 K 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 (van Schothorst et al. 2007). cDNA was synthesised for each animal from 200 ng RNA. Normalisation and data analysis were performed as published (Pellis et al. 2003) using Feature Extraction version 10.7.3.1 (Agilent Technologies). Based on visual inspection, three arrays were excluded in which hybridization 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.

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 (Fig. 1a). Total body weight gain after 12 weeks was 29 % lower in the quercetin fed mice compared to the control mice ($p < 0.01$; Fig. 1b), while digestible (equals metabolisable) energy intake over 12 weeks was not significantly different (Fig. 1c). Consequently, the calculated food efficiency was 26 % lower for the quercetin fed mice ($p < 0.001$) (Fig. 1d). The weekly food intake and energy content of faeces used for the calculation is provided in supplementary figure S1.

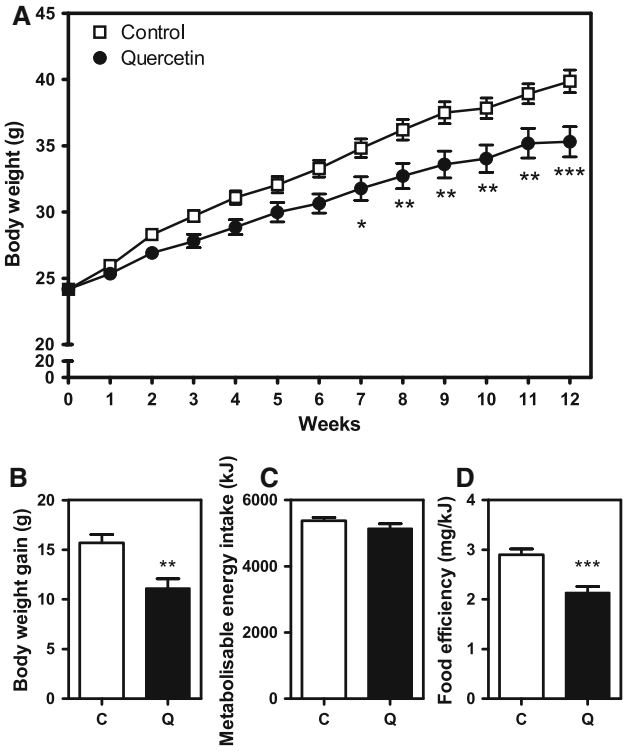

Fig. 1 Quercetin reduces body weight gain and food efficiency. Body weight of control ($n = 12$) and quercetin ($n = 11$) fed mice during 12 weeks on high-fat diet (**a**). Cumulative body weight gain over 12 weeks (**b**). Cumulative metabolisable energy intake over 12 weeks (**c**). Calculated total 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 (Hoek-van den Hil et al. 2012), we quantified various serum lipid fractions with ^{1}H NMR and hepatic lipid accumulation with histological Oil red O staining.

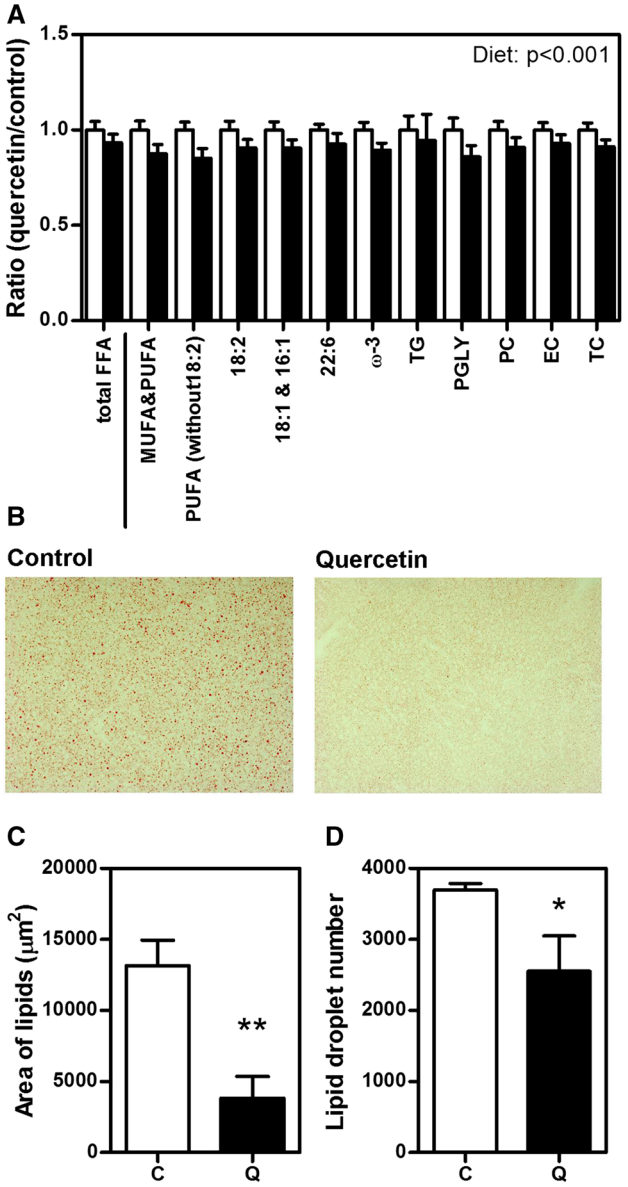

Fig. 2 Quercetin decreased high-fat diet induced serum lipid levels and hepatic lipid accumulation. **a** Serum lipid fractions are shown as ratio of quercetin fed mice ($n = 11$) over the average value of the control mice ($n = 12$). Two-way ANOVA; diet significant ($p < 0.01$). **b** Representative 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

Two-way ANOVA analysis revealed that quercetin supplementation has a significant lowering effect on the high-fat diet induced serum lipid levels (Fig. 2a).

Table 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

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 (Fig. 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 (Hoek-van den Hil et al. 2013), were studied. However, RT-qPCR analysis indicated no significant regulation of *Cyp4a14*, *Cyp4a10*, *Acot3*, nor *Por* (Table 1).

Subsequently, we profiled gene expression in the liver using whole genome microarrays, since we observed large differences in hepatic lipid accumulation between 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 (Fig. 3). Cysteine sulphinic acid decarboxylase (*Csad*), encoding the rate limiting enzyme in taurine biosynthesis, was upregulated 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 S3).

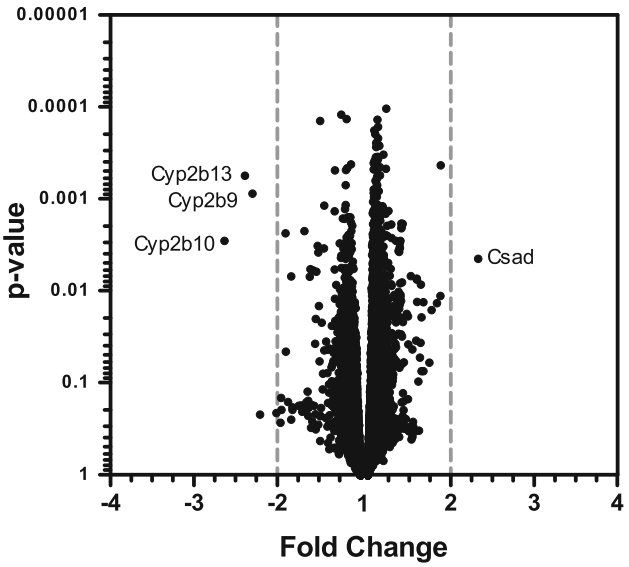

Fig. 3 Volcano plot of all expressed probes by global hepatic gene expression analysis. *P* values of all probes are plotted against the fold change of each probe, quercetin ($n = 10$) versus control ($n = 11$). *Dotted lines* indicate used cut-off value of an absolute fold change >2.0. Selected gene symbols are depicted in the figure; cytochrome P450 (Cyp) enzymes *Cyp2c9*, *Cyp2c10*, and *Cyp2c13*, and cysteine sulphinic acid decarboxylase (*Csad*)

Three cytochrome P450 enzyme encoding genes, *Cyp2b9*, *Cyp2b10*, and *Cyp2b13*, were all downregulated 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 (Hoek-van den Hil et al. 2013). The top regulated genes ($p < 0.01$ and absolute fold change > 1.5) and confirmation of these genes by RT-qPCR are shown in Table 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*).

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 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

Table 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 sulphinic 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

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 (Hoevenaars et al. 2013; Voigt et al. 2013). 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 (Jung et al. 2013; Kobori et al. 2011), 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 (de Boer et al. 2006; Odbayar et al. 2006; Stewart et al. 2009; Wein et al. 2010). Our previous data (30 en% fat diet) (Hoek-van den Hil et al. 2013) 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 (Hoek-van den Hil et al. 2013). These specific changes in serum triglyceride levels 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 (Hardwick 2008). Here, the high-fat diet induced a fatty liver, thus ω-oxidation and especially *Cyp4a* transcripts are expected to be upregulated, 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 homoeostasis (Sueyoshi et al. 1999). Importantly, these *Cyp2b* genes are known to be under control of CAR (Honkakoski et al. 1998; Sueyoshi et al. 1999). Thus, although differences in liver gene expression are seen in this study compared to our previous study (Hoek-van den Hil et al. 2013), 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 (Finn et al. 2009). 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 S1). *Fabp5*, the fourth gene among the top upregulated 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 (Ogawa et al. 2011). 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 (Li et al. 2006, 2007). 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 (Pearson et al. 2008).

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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Conflict of interest E.F. Hoek-van den Hil, E.M. van Schothorst, I. van der Stelt, H.J.M. Swarts, D. Venema, M. Sailer, J. Vervoort, P.C.H. Hollman, I.M.C.M. Rietjens, J. Keijer declare that they have no conflict of interest.

Ethical standard All institutional and national guidelines for the care and use of laboratory animals were followed.

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