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**Title:** Fatty acid metabolism and metabolic inflammation: two important players in the development of insulin resistance

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FCR γ-CHAIN⁻/⁻ MICE ARE PROTECTED AGAINST DIET-INDUCED OBESITY AND INSULIN RESISTANCE

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In preparation
ABSTRACT

Inflammation plays a key role in the pathogenesis of diet-induced insulin resistance and type 2 diabetes. Recent studies have suggested that immunoglobulins (Ig) released by B-lymphocytes during the development of obesity play a key role in this pathology, although their mechanism of action is still unknown. Ig activate cellular responses by cross linking membrane receptors specific for the Fc portion of the Ig molecule (FcR). In this study, we have investigated whether functional FcR play a role in the development of diet-induced adipose tissue inflammation and diet-induced insulin resistance. To this end, FcR γ-chain−/− mice that are characterized by a decreased FcR-mediated activation by IgG and IgE antibodies, were fed a high fat diet (HFD). FcR γ-chain−/− mice gained less weight on HFD which was neither caused by decreased food intake nor by increased energy expenditure. However, a decreased postprandial response to a lipid load was observed, which suggests altered lipid absorption in the intestine of FcR γ-chain−/− mice. Basal glucose and insulin levels were decreased in FcR γ-chain−/− mice compared to WT controls and hyperinsulinemic euglycemic clamp analysis revealed decreased peripheral insulin resistance in FcR γ-chain−/− mice. The adipose tissue of FcR γ-chain−/− mice expressed lower levels of inflammatory genes and contained no crown-like structures. Taken together, these results demonstrate that the FcR γ-chain plays a key role in HFD induced obesity, insulin resistance and adipose tissue inflammation and are in line with a key role for Ig-mediated responses in this pathology.
Obesity and its related metabolic disorders such as insulin resistance and type 2 diabetes are associated with low-grade systemic inflammation, characterized by increased local and systemic production of pro-inflammatory cytokines and adipokines. Macrophage infiltration of expanding adipose tissue is one of the causes leading to increased cytokine production. These macrophages are classified as ‘classically’ activated macrophages or M1 macrophages that have increased expression of the pro-inflammatory cytokines IL-6, TNF-α and MCP-1. Specific pro-inflammatory subsets of T-lymphocytes have also been found to be enriched in adipose tissue from the obese. It has been suggested that T_h1 lymphocytes help recruit macrophages into adipose tissue and stimulate the M1 macrophage inflammatory activation state.

Until recently, the role of B-lymphocytes and Ig in the development of adipose tissue inflammation and insulin resistance was poorly characterized. Duffaut et al. found an early accumulation of B-lymphocytes in adipose tissue when mice were subjected to a high fat diet (HFD). In Pima Indians, with an inherited susceptibility for diabetes type 2, gamma globulin levels were positively correlated with BMI and predicted type 2 diabetes, which suggest that activation of the humoral immune system is involved in the development of insulin resistance. Moreover, Winer et al. have unequivocally shown that B-lymphocytes and antibodies play a key role in obesity associated insulin resistance. They showed that mice, which do not have mature B-lymphocytes, are protected against obesity-associated insulin resistance and in adoptive transfer experiments they demonstrated that IgG antibodies induce insulin resistance in the presence of a HFD. The underlying mechanisms of the antibody effector pathways are currently still unknown. In this manuscript, we have used FcR γ-chain-/- mice to investigate whether functional FcR could play a role in the development of diet-induced inflammation of adipose tissue and diet-induced insulin resistance. FcR γ-chain-/- mice do not express the signal transducing γ-chain of FcγRI and III and FcεRI and therefore these mice have a diminished activation of IgG and IgE antibody effector pathways. Here we show that mice deficient for FcR γ-chain are protected against diet-induced obesity, diet-induced adipose tissue inflammation and diet-induced insulin resistance, indicating that functional activating FcRs might play a role in the development of the metabolic syndrome.

Animals
The generation of FcR γ-chain deficient mice in 100% C57BL/6 background has been described previously. FcR γ-chain-/- were bred at the Leiden University Medical Center, Leiden, The Netherlands. Wild-type (WT) control mice (C57Bl6/J background) were
purchased from Charles River (Maastricht, The Netherlands). Mice used in experiments were males, housed under standard conditions with free access to water and food. Mice were fed a high fat diet (HFD) for 11 weeks (45% energy derived from lard fat; D12451, Research Diet Services, Wijk bij Duurstede, The Netherlands) to induce obesity. Body weight was measured regularly during the diet intervention. All experiments were approved by the animal ethics committee of Leiden University Medical Center.

**Plasma parameters**

To determine plasma metabolite levels during week 11 of HFD feeding, tail vein blood was collected after an overnight fast, into capillary tubes coated with paraoxon (Sigma, St. Louis, MO) to prevent ongoing *in vitro* lipolysis. The tubes were placed on ice and centrifuged. Plasma triglyceride (TG), total cholesterol (TC), free fatty acid (FA), plasma phospholipids (PL), keton bodies, glucose and insulin levels were determined using commercially available kits (11488872 and 236691, Roche Molecular Biochemicals, Indianapolis, IN, NEFA-C and phospholipids B Wako Chemicals GmbH, Neuss, Germany, ab83390, Abcam, Cambrigde, UK, Intruchemie, Delfzijl, The Netherlands and Crystal Chem Inc., IL, USA, respectively).

**Determination of adipocyte size and preadipocyte differentiation capacity**

Adipose tissue from the gonadal (unilateral), subcutaneous (unilateral) and visceral region were removed from the mice after 11 weeks of HFD and kept in PBS. The tissue was minced and digested in 0.5g/l collagenase in HEPES buffer (pH 7.4) with 20 g/l of dialyzed bovine serum albumin (BSA, fraction V, Sigma, ST Louis, USA) for 1 h at 37°C. The disaggregated adipose tissue was filtered through a nylon mesh with a pore size of 236 μm. For the isolation of mature adipocytes, cells were obtained from the surface of the filtrate and washed several times. Cell size was determined using an imaging processing technique which automatically determines fat cell sizes from microscopic pictures of isolated adipocytes (~1000 cells/fat tissue sample). From the measured cells the adipocyte size distribution, adipocyte number and mean adipocyte diameter were determined. Subsequently the volume-weighted mean adipocyte diameter was calculated, which is a measure of the mean adipocyte diameter corrected for the amount of fat that an adipocyte can store [12]. For the isolation of preadipocytes, the infranatant of the adipose tissue filtrate was centrifuged at 200 x g for 10 min at room temperature and treated with erythrocyte lysis buffer. The cells were washed 2 times, re-suspended in DMEM/NUT.MIX.F12 medium and supplemented with 10% fetal calf serum (FCS) and 100 μg/ml penicillin-streptomycin and incubated into 96-well plates (12 wells/adipose tissue region) at a density of 40 000 cells/ml and kept at 37°C, in 5% CO₂. The cells were expanded until confluency after which adipocyte differentiation was induced using an adipogenic medium containing DMEM/F12 with dexamethasone 0.1μM, 3-isobutyl-1-methylxanthine 25μM, insulin 17 nM, indomethacin 60μM, 10% FCS and 100 μg/ml penicillin-streptomycin. After 3 days, medium was changed to maintenance medium containing insulin 17 nM and 10%FCS. After 5 more days the cells were lipid filled and intra cellular lipid accumulation was determined using a fluorometer after incubating the cells with Nile Red.
RNA isolation and qPCR analysis

RNA from gonadal fat of mice was isolated after 11 weeks of HFD feeding using the Nucleospin RNA /Protein kit (Macherey-Nagel, Düren, Germany) according to the instructions of the manufacturer. The quality of each mRNA sample was examined by lab-on-a-chip technology using Experion Stdsens analysis kit (Biorad, Hercules, CA). 600 ng of total RNA was reverse-transcribed with iScript cDNA synthesis kit (Bio-Rad) and obtained cDNA was purified with Nucleospin Extract II kit (Macherey-Nagel). Real-Time PCR was carried out on the IQ5 PCR machine (Biorad) using the Sensimix SYBR Green RT-PCR mix (Quantace, London, UK). mRNA levels were normalized to mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (Gapdh) or cyclophilin (cyclo). Primer sequences are listed in table 1.

Immunohistochemistry

Immunohistochemistry was performed on formalin fixed and paraffin embedded sections of gondal adipose tissue of mice fed a HFD for 11 weeks. For detection of macrophages/monocytes, an F4/80+ antibody (1:150) (Serotec) was used. Visualization of the complex was done using diaminobenzidene for 10 minutes. Negative and positive controls were included. Haematoxylin and Eosin (HE) stainings of sections were done using a standard protocol.

Hyperinsulinemic Euglycemic Clamp analysis

During week 11, hyperinsulinemic euglycemic clamps were performed as described earlier 13. Briefly, after an overnight fast, animals were anesthetized and an infusion needle was placed in the tail vein. Basal glucose parameters were determined during a 60-min period, by infusion of D-[^3-^3]H]glucose to achieve steady-state levels. A bolus of insulin (3 mU) was given and a hyperinsulinemic euglycemic clamp was started with a continuous infusion of insulin (5 mU/h) and D-[^3-^3]H]glucose and a variable infusion of 12.5% D-glucose (in PBS) to maintain blood glucose levels at euglycemic levels. Blood samples were taken every

Table 1. Primers used for quantitative real-time PCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>F4/80</td>
<td>CTTTGCTATGGGCTTCCAGCTCA</td>
<td>GCAAGGAGCACAGAGTTATCGTGG</td>
</tr>
<tr>
<td>CD68</td>
<td>ATCCCCCCTGCTCTCTCTCTCA</td>
<td>TTGCATTTCCACGACGAGAAG</td>
</tr>
<tr>
<td>MCP-1</td>
<td>GCATCTGCCCTAGGTCTCTCCA</td>
<td>TTCACGTGCACACTGCTGTCACCTCA</td>
</tr>
<tr>
<td>IL-6</td>
<td>ACCACGCTCTCCCTCCTCTCA</td>
<td>CTCATTTCCACGATTTCCAG</td>
</tr>
<tr>
<td>IL-10</td>
<td>GACACACATCTGCTAACCAGACTC</td>
<td>ATCAGTTCACCTGCTCCAG</td>
</tr>
<tr>
<td>ACOX1</td>
<td>TATGGATCATCAGCCAGAAAGG</td>
<td>ACAGAGCCAGGGGTGCATC</td>
</tr>
<tr>
<td>CPT1a</td>
<td>GAGACTCCACGCGCATAGCA</td>
<td>ATGGGTTGGGATGTGAG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGCCACCACCAACTGCTTAGC</td>
<td>GCATGGACTGTGTCATGAG</td>
</tr>
<tr>
<td>CYCLO</td>
<td>CAAATGCTGGACCAAAACACA</td>
<td>GCCATGCCAGCATCCACTGTC</td>
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F4/80, marker for macrophages; CD68, marker for macrophages; MCP-1, monocyte chemotactrant protein-1; IL-6, interleukin-6; IL-10, interleukin-10; ACOX1, acyl-coenzyme A oxidase 1; CPT1a, carnitine palmitoyltransferase 1a; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CYCLO, cyclophilin
5-10 min from the tip of the tail to monitor plasma glucose levels (AccuCheck). Seventy, eighty and ninety minutes (steady-state) after the start of the clamp, blood samples (70 μl) were taken for determination of plasma glucose, insulin and FA concentrations using commercially available kits (Instruchemie, Crystal Chem Inc., and Wako Pure Chemical Industries). Turnover rates of glucose (μmol/min/kg) were calculated during the basal period and in steady-state clamp conditions as the rate of tracer infusion (dpm/min) divided by the plasma-specific activity of ³H-glucose (dpm/μmol). All metabolic parameters were expressed per kilogram of bodyweight. The hepatic glucose production (HGP) is calculated from the rate of disappearance (Rd) and glucose infusion rate (GIR) by the following equation: Rd = HGP + GIR. The Rd is measured from Steele’s equation in steady state using the tracer infusion rate (Vin) and plasma-specific activity (SA) of ³H-glucose (dpm/μmol) by the following formula: Rd = Vin/SA.

**Indirect calorimetry**

During week 5 and 10 of HFD feeding groups of 8 mice were subjected to individual indirect calorimetry measurements using a Comprehensive Laboratory Animal Monitoring System (Columbus Instruments, Columbus Ohio, US). Cages were made of clear plexiglass (30 x 10 x 9 cm (l x b x h)). A period of 24 hours prior to the start of the experiment allowed the acclimatization of the animals to the cages and the single housing. Experimental analysis started at 09:00 h and continued for 36 hours. Analyzed parameters included real time food and water intake, as well as meal size, frequency and duration. Oxygen consumption (VO₂) and carbon dioxide production rates (VCO₂) were measured at intervals of 7 minutes. Respiratory exchange ratio (RER) as a measure for metabolic substrate choice was calculated as the ratio between VCO₂ and VO₂. Carbohydrate (CHO) and fat (FA) oxidation rates were calculated using the following formulas:¹⁴:

\[
\text{CHO} = \frac{(4.585 \times VCO₂) - (3.226 \times VO₂)}{4} / 1000
\]

\[
\text{FA} = \frac{(1.695 \times VO₂) - (1.701 \times VCO₂)}{9} / 1000
\]

Total energy expenditure was calculated from the sum of CHO and FA oxidation. Activity was monitored as 2-dimensional infrared beam breaks.

**Liver lipids**

Lipids were extracted from livers of WT and FcR γ-chain⁻/⁻ fed a HFD for 11 weeks according to a modified protocol from Bligh and Dyer.¹⁵ Briefly, small liver pieces were homogenized in ice-cold methanol. After centrifugation, lipids were extracted by addition of 1800 μL chloroform: methanol (3:1 v/v) to 45 μL homogenate. The chloroform phase was dried and dissolved in 2% Triton X-100. Hepatic TG, TC and PL concentrations were measured using commercial kits as described earlier. Liver lipids were expressed per mg protein, which was determined using the BCA protein assay kit (Pierce).
Postprandial response
To investigate the handling of postprandial TG, mice were fed a HFD for 7 weeks. Mice were fasted 4 h (from 8.00 am to 12.00 pm) prior to the start of the experiment. Mice received an intragastric load of 200 μl olive oil (Carbonell extra virgin, Cordoba, Spain). Prior to the bolus and 1, 2, 4, and 8 h after bolus administration blood samples were obtained via tail vein bleeding for determination of plasma TG and FA as described above.

Fecal TG content
Fecal TG content was determined in feces collected during week 6 of HFD feeding. TG was extracted according to a modified protocol from Bligh and Dyer. In short, 100 mg of crushed feces was homogenized in methanol:chloroform (2:1). 600 μl chloroform was added for TG extraction and samples were centrifuged. This procedure was repeated twice to maximize TG extraction. After centrifugation, the chloroform layer was collected and chloroform was evaporated. The remaining TG pellet was dissolved in 2% Triton-x-100 (Sigma). TG content was determined using a commercially available kit as described earlier.

Statistical analysis
Data are presented as means ± SD. Statistical differences were calculated using the unpaired T-test (SPSS 16, SPSS Inc, Chicago, IL). A P-value < 0.05 was regarded statistically significant.

5.3 RESULTS
FcR γ-chain−/− mice are protected against HFD-induced obesity
To induce obesity, male FcR γ-chain−/− and WT mice were fed a HFD containing 45 energy% fat for 11 weeks, and body weight of the mice was followed during the HFD intervention. FcR γ-chain−/− mice gained less body weight than WT mice (Fig. 1A). In line with this observation, weights of isolated fat pads were decreased in FcR-γ-chain−/− mice (gonadal fat -65%; P<0.001, subcutaneous fat -64%; P<0.001, visceral fat -60%; P<0.001) (Fig. 1B).

FcR γ-chain−/− adipocytes are smaller and have an improved differentiation capacity ex vivo
A decrease in fat pad mass can be caused by smaller adipocytes and/or by a decreased number of adipocytes. To investigate adipocyte morphology, adipocyte size distribution, volume-weighted mean adipocyte diameter and total adipocyte number were determined in isolated adipocytes from gonadal, subcutaneous and visceral fat pads from FcR γ-chain−/− and WT mice fed a HFD for 11 weeks. In FcR γ-chain−/− mice the distribution was shifted to the left for all three fat pads (Fig. 2A-C), indicating less hypertrophic adipocytes. Indeed, in FcR γ-chain−/− mice volume-weighted mean cell diameter was decreased in gonadal (gWAT) (-26%; P<0.001), subcutaneous (sWAT) (-29%; P<0.001) and visceral (vWAT)
white adipose tissue (Fig. 2D) compared to WT mice. In addition to smaller adipocytes, also a decreased number of adipocytes was found in gW AT (-27%; P<0.001) and sW AT (-17%; P<0.05) of FcR γ-chain−/− mice (Fig. 2E). To investigate differentiation capacity, isolated preadipocytes from gonadal and subcutaneous fat pads of FcR γ-chain−/− and WT mice were differentiated in adipogenic medium and TG accumulation was measured. An increased differentiation capacity was observed for gonadal preadipocytes of FcR γ-chain−/− mice (+147%; P<0.05) (Fig. 2F).

FcR γ-chain−/− mice have decreased inflammation in white adipose tissue
To investigate whether functional FcR are important for white adipose tissue inflammation, mRNA expression of several inflammatory genes was measured in gonadal white adipose tissue (gWAT) of FcR γ-chain−/− and WT mice. A decreased expression of F4/80 (-46%; P<0.05), CD68 (-70%; P<0.001), monocyte chemotactic protein-1 (MCP-1) (-83%; P<0.001), interleukin 6 (IL-6) (-51%; P<0.01) and interleukin 10 (IL-10) (-58%; P<0.01) was found in FcR γ-chain−/− mice, which indicates decreased inflammation (Fig. 3A). Immunohistochemistry using an antibody that recognizes the macrophage antigen F4/80, showed crown like structures (CLS) in gonadal adipose tissue of WT mice, but not in gonadal adipose tissue of FcR-γ-chain−/− mice (Fig. 3B).

FcR γ-chain−/− mice have decreased HFD-induced insulin resistance
To investigate whether functional FcR play a role in diet-induced insulin resistance, plasma glucose and insulin levels of overnight fasted FcR γ-chain−/− and WT mice that had been on a HFD for 11 weeks were measured. Both glucose (-28%; P<0.001) and insulin (-47%; P<0.001) levels were significantly decreased in FcR-γ-chain−/− mice compared to WT mice.
Figure 2. FcR γ-chain<sup>−/−</sup> adipocytes are smaller and have an improved adipogenic capacity. After 11 weeks of HFD feeding, adipocytes were isolated from gonadal (gWAT), subcutaneous (sWAT) and visceral (vWAT) white adipose tissues of wild-type (WT) and FcR γ-chain<sup>−/−</sup> mice. Adipocyte size distribution was determined (A-C). Values are means ± SD (n= 9-10 per group). Volume-weighted mean diameter (D), total number of adipocytes (E) and adipogenic capacity (F) was determined. Values are means ± SD (n=9-10); *P<0.05, ***P<0.001.
Fig. 4A-B, suggesting that FcR-γ-chain-/- mice have decreased HFD-induced insulin resistance. To further investigate the insulin sensitivity, a hyperinsulinemic euglycemic clamp was performed. This analysis revealed a significant increase in glucose disposal rate in FcR γ-chain-/- mice during the hyperinsulinemic period (+20 %; P<0.05) (Fig. 4C), indicating that HFD-induced peripheral insulin resistance is decreased in these mice compared to WT controls. Insulin mediated suppression of HGP was similar in both groups (Fig. 4D), indicating similar hepatic insulin sensitivity after HFD.

**FcR γ-chain-/- mice have equal food intake and energy expenditure**

To investigate how FcR γ-chain-/- mice are protected against diet-induced obesity indirect calorimetry measurements were performed in the mice during week 5 and 10 of the HFD intervention. Fig. 5 shows results of week 5, which were similar to the results of week 10. The batch of mice that was used for indirect calorimetry measurements did not differ significantly in body weight at week 5, only body weight gain differed significantly (body weight was 30.6
Figure 4. FcR \( \gamma \)-chain\(^{-/}\) mice are protected against HFD-induced insulin resistance. A second set of wild-type (WT) and FcR \( \gamma \)-chain\(^{-/}\) mice was fed a HFD for 11 weeks and after an overnight fast plasma glucose (A) and insulin levels (B) were determined. Values are means ± SD (n=8); *** \( P < 0.001 \). A hyperinsulinemic euglycemic clamp was performed in wild-type (WT) and FcR \( \gamma \)-chain\(^{-/}\) mice. Rate of disappearance during basal and hyperinsulinemic period (C) and hepatic glucose production during hyperinsulinemic period (D) were determined. Values are means ± SD (n=8); * \( P < 0.05 \).

± 2.2 vs. 31.3 ± 1.1, body weight gain was 3.0 ± 0.8 vs. 5.0 ± 0.8; \( P < 0.001 \). Differences in calorimetry during week 5 could thus not be due to differences in body weight. Nocturnal and diurnal data were analyzed separately to distinguish between periods of low (diurnal) and high (nocturnal) physical activity. During the diurnal period no differences were observed in total energy expenditure (EE), respiratory exchange ratio (RER), absolute fatty acid (FA\(_{\text{ox}}\)) and absolute carbohydrate (CH\(_{\text{ox}}\)) oxidation rates both at week 5 and 10. Food intake tended to be lower in the FcR \( \gamma \)-chain\(^{-/}\) mice (\( p = 0.08 \)) during week 10, but was not different during week 5 (Fig. 5A-E). Ambulatory physical activity during the diurnal period was not different between groups (data not shown) both at week 5 and 10. During the nocturnal period, ambulatory physical activity was higher in FcR \( \gamma \)-chain\(^{-/}\) mice, both at week 5 and 10 (week 5: +33%, week 10: +47%; \( P < 0.05 \)). This higher physical activity did not result in an increased EE in FcR \( \gamma \)-chain\(^{-/}\) mice. Measured food intake and EE were not different, however the average
RER was increased in FcR γ-chain−/− mice, both at week 5 and 10 (week 5: +3%, week 10: +2%; P<0.05) which was reflected in a somewhat lower absolute FAox rate and a somewhat higher CHox rate (Fig. 5A-E). This higher CHox rate in FcR γ-chain−/− mice compared to WT controls corresponds to the increased insulin sensitivity that was observed.

In addition, the energy balance was determined for FcR γ-chain−/− and WT mice by calculating [food intake (kcal/24h)] – [energy expenditure (kcal/24h)]. No significant

Figure 5. FcR γ-chain−/− mice have equal food intake and energy expenditure. The second set of wild-type (WT) and FcR γ-chain−/− mice was subjected to indirect calorimetry measurements during week 5 of HFD intervention. Food intake (FI) (A), energy expenditure (B), respiratory exchange rates (C), fat oxidation (D) and carbohydrate oxidation levels (E) were measured. Values are means ± SD (n=8); *P<0.05.
differences in energy balance were observed, both during week 5 and 10 (week 5: +8.9 ± 1.8 vs. +10.0 ± 5.5, week 10: +3.4 ± 1.8 vs. +4.4 ± 3.8).

**FcR γ-chain−/− mice have decreased plasma lipid levels and decreased liver lipid levels**

To further unravel the mechanisms underlying the decreased adiposity of the FcR γ-chain−/− mice, plasma lipid parameters were determined during week 11 of HFD feeding. Plasma TG, TC and PL levels were significantly decreased in FcR γ-chain−/− mice (-26%; P<0.05, -39% and -24 %; P<0.001) (Table 2). In addition liver lipids were measured, demonstrating decreased liver TG content in FcR γ-chain−/− mice (-60%; P<0.001) (Fig. 6A). mRNA levels of acyl-Coenzyme A oxidase 1 (Acox1) and carnitine palmitoyltransferase 1a (Cpt1a), 2 genes involved in β-oxidation, were decreased in FcR γ-chain−/− mice (-66%; P<0.001 and -59%; P<0.01) (Fig. 6B). Furthermore, keton bodies in plasma were measured during week 9 and 11 of the HFD intervention in 2 batches of mice. Equal or decreased levels of keton bodies were observed, respectively (1.06 ± 0.25 versus 1.02 ± 0.28 and 0.90 ± 0.27 versus 1.23 ± 0.14; P<0.01). Together these results indicate decreased β-oxidation in FcR γ-chain−/− mice.

**FcR γ-chain−/− mice have decreased postprandial response**

Decreased adiposity in FcR γ-chain−/− mice might also be caused by absorption problems. To investigate this hypothesis, FcR γ-chain−/− and WT mice were given an oral bolus of 200 μl olive oil after 7 weeks of HFD and plasma TG levels were monitored in time. FcR γ-chain−/− mice had a decreased postprandial response compared to WT mice (Fig. 7A).

![Figure 6](image-url). FcR γ-chain−/− mice have decreased plasma lipid levels and decreased liver lipid levels. After 11 weeks of HFD, liver was dissected of wild-type (WT) and FcR γ-chain−/− mice and liver lipids were determined (A). Values are means ± SD (n=5); ***P<0.001. RNA was isolated from WT and FcR γ-chain−/− livers and mRNA levels of acyl-Coenzyme A oxidase 1 (Acox1) and carnitine palmitoyltransferase 1a (Cpt1a) were measured (B). Values are means ± SD (n=9-10); **P<0.01, ***P<0.001.
Table 2. FcR γ-chain−/− mice have decreased plasma lipid levels on high fat diet.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>FcR γ-chain−/−</th>
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<tr>
<td>TG (mM)</td>
<td>1.10 ± 0.30</td>
<td>0.81 ± 0.21*</td>
</tr>
<tr>
<td>TC (mM)</td>
<td>4.23 ± 0.30</td>
<td>2.56 ± 0.40***</td>
</tr>
<tr>
<td>PL (mM)</td>
<td>2.99 ± 0.36</td>
<td>2.26 ± 0.38***</td>
</tr>
<tr>
<td>FA (mM)</td>
<td>1.08 ± 0.22</td>
<td>1.21 ± 0.24</td>
</tr>
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</table>

Plasma was obtained from overnight fasted mice during week 11 of high fat diet intervention (n=10). Plasma triglycerides (TG), total cholesterol (TC), phospholipids (PL) and free fatty acids (FA) were measured. Values are means ± SD. *P<0.05, ***P<0.001.

Figure 7. FcR γ-chain−/− mice have decreased postprandial response. After 7 weeks of HFD, the postprandial response triglyceride (TG) was determined in a third set of wild-type (WT) and FcR γ-chain−/− mice (A). Values are means ± SD (n=9); *P<0.05, **P<0.01, ***P<0.001. During week 6 of HFD intervention feces was collected of these mice and TG content was determined in fecal samples of WT and FcR γ-chain−/− mice (B). Values are means ± SD (n=7-9).

Figure 7B. Differences in fecal TG content were observed (Fig. 7B). These data indicate that intestinal lipid absorption kinetics are affected in FcR γ-chain−/− mice.

5.4 DISCUSSION

The study presented here is to our knowledge the first to indicate that FcR associated antibody effector pathways are involved in the development of diet-induced insulin resistance. We show that after a HFD, FcR γ-chain−/− mice become less obese and develop less adipose tissue inflammation and peripheral insulin resistance compared to WT controls. As the FcR γ-chain is crucial for full activation of IgG and IgE antibody effector pathways, our results demonstrate that FcR associated antibody effector pathways might play a key role in
these processes. Our results are in line with a role of Ig in the development of HFD-induced insulin resistance, which has recently been shown by Winer et al. 10.

The FcR γ-chain−/− mice showed a reduced body weight gain and decreased adipocyte size as compared to HFD-fed WT mice. Since body weight and adipocyte size per se are determinants of insulin sensitivity, these effects can explain at least part of the observed decreased HFD-induced insulin resistance. However, the calorimetry measurements demonstrated a higher CH_ox rate in FcR γ-chain−/− mice during week 5 when the FcR γ-chain−/− and WT mice did not differ in body weight. As a high CH_ox rate corresponds to increased insulin sensitivity, these data may suggest that the Fc γ-chain per se plays a role in diet-induced insulin resistance and is not only a consequence of the effects on body weight. In the study of Winer et al., B-lymphocyte deficient mice showed improved glucose tolerance and insulin sensitivity compared to HFD-fed WT mice, although they did not differ from WT mice in weight gain nor adipocyte size upon HFD feeding. Their study suggests that B-lymphocyte derived Ig are responsible for a body weight-independent effect on HFD-induced insulin resistance. In another recent study using B-lymphocyte deficient mice on BalbC background (BcKO mice), body fat stores were assessed after a HFD and the BcKO mice did show reduced inguinal and epididymal fat stores compared to their heterozygous littermate controls 16.

The fact that FcR γ-chain−/− mice are relatively protected against HFD-induced obesity cannot be explained by differences in food intake nor energy expenditure. However, intestinal lipid absorption may be affected in FcR γ-chain−/− mice as indicated by a decreased postprandial response to an olive oil load. In line with a reduction of the intestinal lipid flux, circulating plasma TG and liver TG were both decreased and genes involved in β-oxidation were also decreased. Despite the reduced postprandial TG response, fecal fat secretion was not different from WT mice. Combined, these observations may indicate that the lipids that were not absorbed postprandially as plasma TG in the FcR γ-chain−/− mice, were still being metabolized in the intestine, most likely by the intestinal microbiota. Since intestinal microbiota are predominantly anaerobic, energy expended via this pathway will not be measured by gas-based calorimetry equipment. Although the TG/FA breakdown products from the intestinal microbiota (such as short chain FA) will be absorbed and used as fuel, the net energy output will be much lower as the microbial conversion reactions cost a lot of energy. This loss of energy may also contribute to the reduced body weight gain in HFD-fed FcR γ-chain−/− mice.

Recently, it was found that in the absence of IgA, the major antibody class in the gut, and in the presence of microbiota, the intestinal epithelium down regulates its metabolic functions and up regulates its immune functions, which ultimately results in lipid malabsorption. These effects were also observed in B-lymphocyte deficient mice. Moreover, duodenal samples of immunodeficient humans display a down regulation of metabolic genes and an up regulation of genes involved in immunity in intestinal epithelium 16. The TG absorption problems found in the FcR γ-chain−/− mice, which also represent an immune-compromised model, are completely in line with these observations. However, we do not know whether IgA is involved in the observed phenotype of the FcR γ-chain−/− mice, since in mice the FcR for IgA – i.e FcαR- has never been identified, in contrast to in humans. Humans express a γ-chain associated high
affinity receptor for IgA, FcαRI (CD89). However, this FcαRI, expressed on myeloid cells such as macrophages, neutrophils and eosinophils, has never been found in mice.

Besides its association with different FcR, the FcR γ-chain is also associated with several receptors involved in innate immunity including NKR-P1C, PIR-A and IL3R. NKR-P1C is expressed on natural killer cells and mediates cytotoxicity against tumor cells. The paired immunoglobulin like receptor-A (PIR-A), which is present on monocytes and macrophages, dendritic cells, mast cells and certain B lymphocytes subsets expressing natural antibodies, is able to activate these cells upon interaction with major histocompatibility complex (MHC) class I molecules. Additionally, the γ-chain is a constitutive component of IL-3R and is necessary for IL-3-induced production of IL-4 by basophils. Together these findings indicate that besides a signaling role in the antibody mediated effector mechanisms, the FcR γ-chain plays also an important role in innate immunity, which could contribute to the metabolic phenotype of the FcR γ-chain−/− mice that we observed.

However, we postulate that the FcR γ-chain−/− mice is a suitable model to study antibody effector mechanisms despite its association to other receptor types. In case FcR are involved in HFD-induced obesity and insulin resistance, the FcR γ-chain−/− mice should show a phenotype. Our experiments indicate that FcR γ-chain−/− mice indeed have a phenotype, which is a first indication for a major role for FcR mediated Ig effector pathways in HFD-induced obesity and insulin resistance and therefore they provide multiple avenues for future studies.

In conclusion, our data provide direct evidence for a role of the FcR γ-chain in HFD-induced obesity and insulin resistance. Although, we cannot exclude that non-FcR mediated mechanisms are responsible for this effect, our data suggests a major role for FcR mediated Ig effector pathways in HFD obesity and insulin resistance. Our data also clearly indicate that FcR γ-chain mediated pathways are important in multiple tissues involved in HFD-induced pathology, including adipose tissue and the intestine.

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