Reduction of obesity-associated white adipose tissue inflammation by rosiglitazone is associated with reduced non-alcoholic fatty liver disease in LDLr-deficient mice

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Obesity is associated with chronic low-grade inflammation that drives the development of metabolic diseases, including non-alcoholic fatty liver disease (NAFLD). We recently showed that white adipose tissue (WAT) constitutes an important source of inflammatory factors. Hence, interventions that attenuate WAT inflammation may reduce NAFLD development. Male LDLr−/− mice were fed a high-fat diet (HFD) for 9 weeks followed by 7 weeks of HFD with or without rosiglitazone. Effects on WAT inflammation and NAFLD development were analyzed using biochemical and (immuno)histochemical techniques, combined with gene expression analyses. Nine weeks of HFD feeding induced obesity and WAT inflammation, which progressed gradually until the end of the study. Rosiglitazone fully blocked progression of WAT inflammation and activated PPARγ significantly in WAT. Rosiglitazone intervention did not activate PPARγ in liver, but improved liver histology and counteracted the expression of genes associated with severe NAFLD in humans. Rosiglitazone reduced expression of pro-inflammatory factors in WAT (TNF-α, leptin) and increased expression of adiponectin, which was reflected in plasma. Furthermore, rosiglitazone lowered circulating levels of pro-inflammatory saturated fatty acids. Together, these observations provide a rationale for the observed indirect hepatoprotective effects and suggest that WAT represents a promising therapeutic target for the treatment of obesity-associated NAFLD.

The prevalence of obesity has increased dramatically over the last 30 years and metabolic disorders associated with obesity have become a major health and economic problem worldwide1. Obesity is associated with a state of low-grade chronic inflammation, frequently referred to as systemic inflammation or metabolic inflammation 2, which is thought to drive the development of several metabolic diseases including non-alcoholic fatty liver disease (NAFLD)3,4. We recently showed that adipose tissue is a critical source of inflammation in obesity and causally involved in NAFLD progression5. However, it is unclear whether suppression of adipose tissue inflammation would attenuate NAFLD progression.

White adipose tissue (WAT) is the primary site of energy storage. This storage function involves expansion of WAT through adipocyte hyperplasia (increase in cell number) and adipocyte hypertrophy (increase in cell size)6. Adipocyte hypertrophy is closely associated with WAT inflammation: in an in vitro experiment with isolated primary human adipocytes7, only very hypertrophic cells were found to secrete MCP-1, a key mediator of immune cell recruitment into WAT. Consistent with this observation, adipocyte hypertrophy is associated with infiltration of macrophages and formation of crown-like structures (CLS)8, a histological hallmark of inflamed WAT. Notably,
a strong increase in CLS is observed at the time point at which a WAT depot has reached its maximal mass as shown very recently in a model of diet-induced obesity. It is thought that the inflamed WAT is less insulin sensitive, which enhances lipolysis of stored fat, thereby contributing to ectopic fat deposition and the development of liver steatosis. In line with this, Kolak and colleagues have shown that obese patients with inflamed WAT have more liver fat than equally obese subjects without WAT inflammation. In addition to the increased fat flux, inflamed WAT may produce inflammatory factors that can contribute to systemic inflammation and promote the progression from liver steatosis to non-alcoholic steatohepatitis (NASH). However, experimental support for a causal role of WAT in the development of NASH has long been lacking. Recently we have shown that surgical removal of inflamed abdominal (epididymal) WAT in mice reduced lobular inflammation and attenuated NASH development, suggesting that WAT constitutes an possible target for the treatment of NASH.

WAT inflammation may be reduced via the nuclear hormone receptor peroxisome proliferator-activated receptor-γ (PPAR-γ) which is predominantly expressed in adipose tissue, controlling inflammatory and metabolic processes. Previous studies in humans and animals provide indication that pharmacological activators of PPAR-γ such as rosiglitazone may reduce the inflammatory state of WAT in obesity. We herein investigated whether rosiglitazone intervention can reduce manifest WAT inflammation and would attenuate subsequent NAFLD development. To do so, we first determined the time point at which WAT inflammation develops during high-fat diet treatment in LDLr−/− mice. Subsequently, we studied the therapeutic effect of rosiglitazone on WAT inflammation and associated NAFLD development.

Results

WAT inflammation starts in epididymal WAT during high-fat diet-induced obesity. After 16 weeks, CLS formation was most pronounced in epididymal WAT (eWAT) (Fig. 1A), while CLS were hardly observed in mesenteric WAT (mWAT) and inguinal WAT (iWAT). Quantitative analysis showed a marked increase in CLS number in eWAT (p < 0.05; Fig. 1B). CLS number correlated with eWAT mass (r = 0.80, p < 0.001, not shown) and with average adipocyte size, a measure of adipocyte hypertrophy (r = 0.61, p < 0.01; Fig. 1C). The average adipocyte size in eWAT was greater than in mWAT and iWAT (not shown). Hence, eWAT is most susceptible to develop CLS, with substantial inflammation established after 9 weeks of high-fat feeding.

Rosiglitazone attenuates WAT inflammation independent of obesity and targets WAT. Mice were treated with high-fat diet for 9 weeks to induce obesity (Table 1). At this time point, intervention with rosiglitazone was started. The caloric intake was comparable between the HFD control group and the HFD + Rosi group (14.6 ± 0.7 and 13.4 ± 0.6 kcal/day, respectively). Continuous high-fat feeding increased fasting plasma glucose, while rosiglitazone had a significant lowering effect (Table 1). Rosiglitazone also significantly lowered fasting plasma insulin and HOMA-IR relative to HFD mice (Table 1). Weight gain and total fat mass were comparable between HFD and HFD + Rosi (Table 1), indicating that the observed metabolic effects were independent of obesity.
The expression of anti-inflammatory M1 macrophage markers CD11c and CCR2 (Fig. 2D). In addition, rosiglitazone increased the expression of genes involved in inflammatory and oxidative stress pathways as shown by microarray analysis (Supplement 2). This analysis demonstrated a highly significant affected the expression of 1049 genes (FDR < 0.05), of which 71 are established PPARγ-regulated genes (including fatty acid transporter protein 1, fatty acid binding proteins, perilipin, uncoupling protein-1, acyl-CoA synthetase) (for detailed list, see Supplement 2). By contrast, microarray analysis of corresponding livers showed no activation of PPARγ from this microarray analysis (Supplement 3). Altogether, these data demonstrated that rosiglitazone significantly activated PPARγ; in WAT and attenuated high-fat diet-induced WAT inflammation.

Rationale for the hepatoprotective effects of rosiglitazone. In eWAT, rosiglitazone blocked the HFD-induced gene expression of leptin and TNFα (Fig. 4A,B). These effects were paralleled in plasma; HFD + Rosi reduced concentrations of leptin and TNFα (Supplement 5). By contrast, rosiglitazone fully restored the HFD-induced decrease in adiponectin gene expression in eWAT (Fig. 4C) which was also reflected in plasma (Supplement 5).

| Parameter | Chow | REF | HFD | HFD + Rosi |
|-----------|------|-----|-----|-----------|
| BW gain (g) | 3.2 ± 0.5 | 9.1 ± 1.8a | 17.5 ± 0.9b | 17.1 ± 1.2b |
| Total adiposity (g) | 1.0 ± 0.1 | 2.6 ± 0.6a | 4.4 ± 0.3b | 4.1 ± 0.2b |
| Glucose (mM) | 11.1 ± 0.3 | 12.5 ± 0.7a | 15.0 ± 0.5b | 10.6 ± 0.2c |
| Insulin (ng/ml) | 0.7 ± 0.2 | 2.9 ± 0.8a | 4.65 ± 0.9b | 1.4 ± 0.2c |
| HOMA-IR | 8.0 ± 2.4 | 43.1 ± 12.8a | 78.6 ± 16.1b | 16.3 ± 2.5c |

Table 1. Metabolic parameters of experimental groups. Abbreviations: Chow, mice fed a chow diet for 16 weeks; REF, reference, mice receiving a HFD for 9 weeks to define condition prior to intervention; HFD, control mice after 16 weeks of HFD; HFD + Rosi, rosiglitazone-treated mice (intervention from 9–16 weeks). a, Significantly different from chow; b, Significantly different from chow and REF; c, Significantly different from HFD (all, p < 0.05).
In addition, rosiglitazone prevented the high-fat diet-induced increase in total saturated fatty acids in plasma (Fig. 4D). In line with this, total NEFA were significantly increased (by 26%, \( p < 0.05 \)) in the HFD group, whereas no significant increase was observed in the HFD + Rosi group (11%, n.s.). More specifically, plasma concentrations of palmitic acid (C16:0) and stearic acid (C18:0) were not increased in HFD + Rosi (Fig. 4D).

Since WAT inflammation correlated with WAT mass and adipocyte hypertrophy we analyzed effects of rosiglitazone on eWAT, iWAT and mWAT in more detail (Fig. 4E). During intervention with rosiglitazone, eWAT mass did not further increase while iWAT mass almost doubled, indicating a shift of fat mass from eWAT towards iWAT. Despite the increase in iWAT mass, this depot did not become inflamed (Fig. 4F). Quantification of adipocyte size showed that the expansion in iWAT was mainly attributable to an increase in adipocyte number rather than adipocyte size (Fig. 4G). This suggests that increased capability of iWAT to store fat may prevent the
Figure 3. Effects of rosiglitazone intervention on NAFLD development. (A) Representative photomicrographs of HE-stained liver sections of REF, HFD and HFD + Rosi. (B) Biochemical analysis of hepatic triglyceride content. Histological quantification of (C) microvesicular steatosis and (D) macrovesicular steatosis show that steatosis was ameliorated by rosiglitazone compared with HFD (n = 7–10/group). (E) TNFα gene expression in liver was diminished in rosiglitazone-treated mice (n = 7–8/group). (F) Gene expression of fibrotic genes determined by RT-PCR. Rosiglitazone reduced HFD-induced expression of Col1a1, Col1a2 and TIMP-1. (G) Onset of fibrosis in Sirius Red-stained liver cross-sections in HFD mice, but not in HFD + Rosi. Pictures are shown in magnification x100. Data are mean ± SEM, *p < 0.05. Mean expression of RT-PCR data was set 1 for chow-fed mice.
### Table 2. Microarray analysis of hepatic gene expression profile based on genes identified in human NAFLD.

The table lists the genes that were recently reported to be associated with NAFLD severity in humans. HFD feeding of LDLr−/− mice resulted in a significant effect on 16 genes compared to chow (arrows indicate significant up- (↑) or downregulation (↓)). Rosiglitazone counteracted the effect of a HFD as shown by the comparison of HFD + Rosi vs. HFD.

| Probe ID | Gene symbol | Gene name | HFD vs. Chow | p-value | HFD + Rosi vs. HFD | p-value |
|----------|-------------|-----------|--------------|---------|-------------------|---------|
| ILMN_2635229 | Thbs2 | thrombospondin 2 | 1.740 | 9.47E-06 | 0.650 | 4.62E-04 |
| ILMN_2764588 | Igbp7 | insulin-like growth factor binding protein 7 | 1.465 | 1.79E-05 | 0.834 | 3.52E-02 |
| ILMN_1217309 | Tax1bp3 | Tax1 (human T-cell leukemia virus type I) binding protein 3 | 1.360 | 7.45E-04 | 0.826 | 3.28E-02 |
| ILMN_2866901 | Efpemp2 | epidermal growth factor-containing fibulin-like extracellular matrix protein 2 | 1.556 | 7.62E-04 | 0.751 | 2.70E-02 |
| ILMN_2636424 | Itgbl1 | integrin, beta-like 1 | 1.528 | 1.11E-03 | 0.905 | 4.33E-01 |
| ILMN_274556 | Dkk3 | dickkopf homolog 3 (Xenopus laevis) | 1.402 | 1.16E-03 | 0.744 | 4.27E-03 |
| ILMN_1258629 | Col3a1 | collagen, type III, alpha 1 | 1.528 | 1.11E-03 | 0.905 | 4.33E-01 |
| ILMN_2866901 | Efpemp2 | epidermal growth factor-containing fibulin-like extracellular matrix protein 2 | 1.556 | 7.62E-04 | 0.751 | 2.70E-02 |
| ILMN_2636424 | Itgbl1 | integrin, beta-like 1 | 1.528 | 1.11E-03 | 0.905 | 4.33E-01 |
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| ILMN_1258629 | Col3a1 | collagen, type III, alpha 1 | 1.528 | 1.11E-03 | 0.905 | 4.33E-01 |

Reference: 19.
Development of hypertrophy and associated inflammation in eWAT, and may thereby contribute to beneficial effects of rosiglitazone on NAFLD development.

**Discussion**

Recent findings indicate that inflamed (abdominal) WAT plays a causal role in the development of NASH in the context of obesity. WAT may thus constitute a new target for intervention. Compounds that specifically target and quench WAT inflammation have not been developed yet. We therefore used rosiglitazone, an activator of PPAR-γ.
inflammatory cascades leading to activation of TNFα hepatic stellate cells42, providing mechanistic support for a crucial role of inflammatory lipid mediators in NASH.

that conditioned medium from palmitic acid-treated hepatocytes induces the expression of pro-fibrotic genes in unhealthy obese patients who had more ectopic liver fat at a comparable body mass index28. This suggests that the human setting. inflammation concurrent with histologic NASH) may facilitate preclinical research that aims at translation to the experimental conditions established herein (HFD-induced obesity, hyperinsulinemia, WAT deletion in hepatocytes or macrophages
tal effects: Recent knock-out studies have shown that targeted PPARγ deletion in hepatocytes or macrophages protected mice against high-fat induced steatosis21, while deletion of PPARγ in adipose tissues increased liver steatosis upon high-fat feeding22. Furthermore, rosiglitazone treatment remained effective in mice lacking PPARγ specifically in the liver23, supporting the view that adipose tissue is an important site of thiazolidinedione action. Consistent with our findings, beneficial effects of rosiglitazone in NAFLD were also observed in aged (12 months old) LDLr−/− mice that develop a more severe disease phenotype than young mice (3 months old) as used herein24. However, this study did not examine the effects of rosiglitazone in a therapeutic (intervention) setting and its effects in adipose tissue were not analyzed. In the study by Gupte and colleagues24, the diet was supplemented with cholest erol which may explain some of the differences observed on liver gene expression and inflammation. Dietary cholesterol has been shown to be a strong inducer of inflammatory gene expression in the liver25,26. For instance, treatment with a HFD supplemented with small amounts (0.2% w/w) of cholesterol triggered Kupffer cell activation and inflammatory gene expression after already 2 weeks in LDLr−/− mice, whereas the same diet without cholesterol hardly had an effect on liver inflammation26. High-fat diets without cholesterol supplementation induce liver inflammation typically at a slower pace and, importantly, this liver inflammation is at least partly mediated by the inflamed white adipose tissue (WAT)27. However, it is unclear to what extent WAT may contribute to liver inflammation when cholesterol is added to a high-fat diet.

We found that eWAT is more prone to develop chronic inflammation than mWAT or iWAT. This observation may be related to the fact that adipocytes in eWAT are more prone to hypertrophy than those in other adipose depots28. In the present study, CLS numbers in eWAT correlated with adipocyte size supporting the importance of adipocyte hypertrophy in the development of WAT inflammation4-8. Consistent with this, metabolically healthy obese subjects were found to have significantly smaller adipocytes compared with metabolically unhealthy obese patients who had more ectopic liver fat at a comparable body mass index28. This suggests that the ability to expand WAT through mechanisms of adipocyte hyperplasia may prevent: a) WAT inflammation and b) ectopic fat accumulation, thereby contributing to a healthy metabolic state.

We observed that rosiglitazone stimulated hyperplasia specifically in subcutaneous WAT thereby preventing adipocyte hypertrophy, which is also observed in patients treated with thiazolidinediones29,30. Consequently, this depot did not become inflamed even though its mass was much greater than in control animals, as is seen in humans treated with rosiglitazone31. The observed stimulation of hyperplasia specifically in iWAT by rosiglitazone may be explained by depot-specific regulation of perilipin, which is essential for enlargement of lipid droplets. Kim and co-workers showed that perilipin protein expression increased after rosiglitazone treatment in subcutaneous adipose tissue, but did not change in visceral adipose tissue32.

Clinical trials have shown that treatment with thiazolidinediones can improve liver histology in patients with NASH33,34. However, the underlying mechanisms mediating the beneficial effects of thiazolidinediones in NASH development are unclear. Data from the present study support the view that rosiglitazone may attenuate the development of NASH via an effect on WAT. Several studies showed that infiltration of macrophages into WAT is strongly associated with NAFLD development10,35,36. More specifically, an increase in CD11c + CD206 + and CCR2 + macrophages in WAT is associated with enhanced production of pro-inflammatory adipokines and cytokines in WAT, and NASH severity36. Herein we show that rosiglitazone intervention reduced the expression of pro-inflammatory M1 markers, CD11c and CCR2 and increased the expression of anti-inflammatory M2 marker, Arginase-1. An increase in Arginase-1 expression has also been observed in HFD-fed Sv129 mice after treatment with rosiglitazone37, but rosiglitazone did not alter the expression of CD11c which may be related to the relatively short intervention period. Long-term rosiglitazone treatment in ob/ob mice resulted in lower CD11c expression level in WAT37, consistent with our findings. Analysis of CLS in the present study shows that long-term rosiglitazone intervention attenuates WAT inflammation by reducing CLS numbers (Fig. 2B), rather than altering the activation state of immune cells within a CLS (as determined CD11c and CCR2 immunoreactivity).

Our study indicates that the hepatoprotective effects on NASH by rosiglitazone may at least partly be mediated by adipokines, since plasma leptin and TNFα levels were reduced and plasma adiponectin levels were increased. It is known that leptin can exert pro-inflammatory effects and can activate hepatic stellate cells thereby promoting fibrosis38. TNFα plays a crucial role in human and animal NAFLD and neutralization of TNFα activity attenuated the disease39. For instance, adiponectin is a potent TNFα-neutralizing cytokine that counteracts inflammation that is relevant for NASH progression38,39. It has been demonstrated that also saturated fatty acids can activate inflammatory cascades leading to activation of TNFα40. We found that the saturated fatty acids; palmitic acid and stearic acid were markedly increased by high-fat feeding and reduced with rosiglitazone. Notably, these fatty acids are also increased in patients with diagnosed NASH41. Furthermore, surgical excision of inflamed WAT in mice lowered palmitic acid in plasma and reduced progression towards NASH37. In vitro experiments have shown that conditioned medium from palmitic acid-treated hepatocytes induces the expression of pro-fibrotic genes in hepatic stellate cells42, providing mechanistic support for a crucial role of inflammatory lipid mediators in NASH.

We also observed that rosiglitazone attenuated the HFD-induced hepatic expression of the genes encoding for Col1a1, Col1a2 and TIMP-1. This hepatoprotective effect of rosiglitazone was further substantiated by an effect on genes that are associated with severity of human NAFLD as shown by Moylan et al.19. These findings support the view that the experimental conditions established herein (HFD-induced obesity, hyperinsulinemia, WAT inflammation concurrent with histologic NASH) may facilitate preclinical research that aims at translation to the human setting.
In all, intervention with rosiglitazone reduces WAT inflammation, lowers circulating inflammatory mediators and attenuates NAFLD progression. These effects were independent of total adiposity and body weight, indicating that adipose tissue quality (i.e. inflammatory state) rather than absolute mass is critical for NAFLD development. Our results suggest that intervention in WAT may present a new therapeutic option for the treatment of NAFLD.

**Methods**

**Animal experiments.** All animal experiments were approved by the institutional Animal Care and Use Committee of the Netherlands Organization of Applied Scientific Research (Zeist, The Netherlands; approval number DEC2935) and were conducted in accordance with the Dutch Law on Animal Experiments, following international guidelines on animal experimentation. Mice (aged 12–14 weeks at the start of the experiment) had ad libitum access to food and water.

*Time-course study.* Male LDLr−/− mice were fed a high-fat diet (HFD: 45 kcal% lard fat, D12451, Research Diets, New Brunswick, NJ, USA) and were sacrificed after 0, 9 and 16 weeks to collect epididymal WAT (eWAT), mesenteric WAT (mWAT) and inguinal WAT (iWAT). Tissues were prepared essentially as reported.

*Intervention study.* Tissues and plasma were obtained from a large cohort study in which rosiglitazone and other interventions (e.g. fenofibrate) were analyzed. Briefly, one group (n = 9) was sacrificed after 9 weeks of HFD to define the condition prior to intervention (reference, REF). The remaining mice continued on HFD (HFD, n = 13) or HFD supplemented with 0.01% w/w rosiglitazone (HFD + Rosi, n = 9, Avandia, GSK, Zeist, The Netherlands). A separate control group was kept on chow as a baseline control for microarray and RT-PCR gene expression analysis. In week 16, all animals were sacrificed and WAT depots and liver were collected. Mice (n = 2) that did not become obese after 9 weeks of high-fat feeding (i.e. body weight gain 50% less than group mean), were excluded from the analyses.

**Histological, biochemical, metabolomics and gene expression analyses.** Briefly, WAT characteristics and NAFLD development were quantified histologically as described. Immunohistochemistry was performed on frozen, acetone-fixed WAT sections using primary antibodies specific for CCR2 (PA5-23044, Thermo Fisher Scientific, Rockford, IL, USA) and CD11c (BD553800, BD Biosciences, San Diego, CA, USA). After incubation, biotinylated antibodies were detected by incubation with streptavidin–HRP using Nova Red as a substrate (both, Vector Laboratories, Burlingame, CA, USA). All sections were counterstained with hematoxylin. Immunopositive cells were quantified in four different cross-sections per mouse using ImageJ. Intrahepatic triglyceride concentrations were analyzed by high performance thin-layer chromatography (HPTLC). Plasma parameters were determined with commercially available assays as previously specified. Plasma fatty acids were determined by gas chromatography/mass spectrometry (GC/MS). The plasma concentration of total free non-esterified fatty acids (NEFAs) was determined with NEFA-HR kit (Instruchemie, Delfzijl, The Netherlands). Illumina microarray gene expression and subsequent pathway analysis of eWAT and liver was performed following established protocols. To analyze potential off-target effects of rosiglitazone in the liver, an upstream transcriptional activator analysis was performed. Microarray data were validated and confirmed by RT-PCR and changes in expression were calculated using the comparative Ct (ΔΔCt) method, expressed as fold-change relative to chow.

**Statistical analysis.** All data are presented as mean ± SEM. Data were analyzed using one-way ANOVA and least significant difference (LSD) post-hoc test. Non-normally distributed data were analyzed by Kruskal-Wallis followed by Mann-Whitney U post-hoc test. Correlations were determined by Spearman's rank correlation. Statistically significant differences in plasma fatty acids over time within HFD and HFD + Rosi were analyzed using Student's paired t-test. Statistical tests were performed using Graphpad Prism software (version 6, Graphpad Software Inc., La Jolla, USA). *P* < 0.05 was considered statistically significant.

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**Author Contributions**

P.Y.W., T.K. and R.K. designed the study. P.M., M.C.M., W.L. and L.V. performed the experiments. P.M., P.Y.W., J.H.v.B., T.K. and R.K. interpreted the data. P.M. and R.K. wrote the manuscript. All authors were involved in critical revision of the drafted manuscript and approved the final version.

**Additional Information**

**Supplementary information** accompanies this paper at http://www.nature.com/srep

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