Adipose tissue dysfunction signals progression of hepatic steatosis towards nonalcoholic steatohepatitis in C57Bl/6 mice

Short title: Adipose tissue dysfunction and NASH

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**Objective:** Nonalcoholic fatty liver disease (NAFLD) is linked to obesity and diabetes, suggesting an important role of adipose tissue in the pathogenesis of NAFLD. Here we aim to investigate the interaction between adipose tissue and liver in NAFLD, and identify potential early plasma markers that predict NASH.

**Research Design and Methods:** C57Bl/6 mice were chronically fed a high fat diet to induce NAFLD and compared with mice fed low fat diet. Extensive histological and phenotypical analyses coupled with a time-course study of plasma proteins using multiplex assay was performed.

**Results:** Mice exhibited pronounced heterogeneity in liver histological scoring, leading to classification into 4 subgroups: LF-low (LFL) responders displaying normal liver morphology, LF-high (LFH) responders showing benign hepatic steatosis, HF-low (HFL) responders displaying pre-NASH with macrovesicular lipid droplets, and HF-high (HFH) responders exhibiting overt NASH characterized by ballooning of hepatocytes, presence of Mallory bodies, and activated inflammatory cells. Compared to HFL responders, HFH mice gained weight more rapidly and exhibited adipose tissue dysfunction characterized by decreased final fat mass, enhanced macrophage infiltration and inflammation, and adipose tissue remodelling. Plasma haptoglobin, IL-1β, TIMP-1, adiponectin and leptin were significantly changed in HFH mice. Multivariate analysis indicated that in addition to leptin, plasma CRP, haptoglobin, eotaxin and MIP-1α early in the intervention were positively associated with liver triglycerides. Intermediate prognostic markers of liver triglycerides included IL-18, IL-1β, MIP-1γ and MIP-2, whereas insulin, TIMP-1, GCP-2 and MPO emerged as late markers.

**Conclusions:** Our data support the existence of a tight relationship between adipose tissue dysfunction and NASH pathogenesis and point to several novel potential predictive biomarkers for NASH.

Obesity is associated with a number of metabolic perturbations that increase risk for type 2 diabetes, coronary heart disease, and liver dysfunction. These metabolic perturbations, collectively referred to as the metabolic syndrome, include hypertension, dyslipidemia, and insulin resistance. Additionally, metabolic syndrome is often characterized by non-alcoholic fatty liver disease (NAFLD) (1).

It is evident that obesity represents a state of chronic low-grade inflammation that likely originates in the adipose tissue. Upon fat expansion, macrophages and other leukocytes infiltrate the adipose tissue and account for secretion of various cytokines and adipokines (2; 3). As many of these cytokines reduce insulin sensitivity, the elevated inflammatory status may provide a mechanistic explanation for the well-established link between obesity and insulin resistance (4). Alternatively, the complications of obesity may be traced to aberrant storage of lipids in non-adipose tissues, which can profoundly disturb organ function (5).

Excess storage of fat in liver is the hallmark of NAFLD, which refers to a wide histological spectrum of liver diseases ranging from hepatic steatosis to pathological steatohepatitis (NASH) and fibrotic complications (6). Steatosis alone is
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considered to be relatively innocuous but prognosis is much more grim for NASH, which might progress to cirrhosis and liver cancer (7). Several theories have been proposed to explain why steatosis occasionally progresses to NASH. One popular model is the two-hit hypothesis, in which the first hit is the accumulation of fat in the hepatocytes that renders the liver more susceptible to second hits comprised of inflammatory insults or oxidative stress (7). Alternatively, progression of steatosis to NASH may be stimulated by cellular lipotoxicity mediated by lipotoxic fatty acids, cholesterol and/or ceramides (8).

Since NAFLD is strongly linked to obesity, an important role of adipose tissue in the pathogenesis of NAFLD is suspected. Indeed, growing evidence indicates that proteins secreted from adipose tissue may be implicated in NAFLD (9). To gain insight into the nature of the interaction between adipose tissue and liver in the context of obesity-related NAFLD, and to identify potential early plasma markers that predict steatosis and/or NASH, we subjected C57Bl/6 mice to a chronic high fat diet to induce NAFLD and coupled extensive histological and phenotypical analyses with a time-course study of plasma proteins using multiplex assay. The results indicate a tight relationship between adipose tissue dysfunction and NASH pathogenesis and point to several novel potential predictive biomarkers for NASH.

MATERIAL AND METHODS

Animal Experiment. Twenty male C57Bl/6JolaHsd (short: C57Bl/6) mice at 8 weeks of age were purchased from Harlan (Horst, the Netherlands) and housed individually. Detailed info about the mouse strain is available at: http://www.harlan.com/research_models_and_services/research_models_by_product_type/inbred_mice/c57bl6j_inbred_mice.hl After 3 weeks on low fat diet (LFD), mice were divided into two weight-matched groups. One group continued on the LFD while the other group switched to a high fat diet (HFD), containing 10 or 45% energy percent triglycerides, respectively (D12450B and D12451, Research Diets, New Brunswick, USA) for 21 weeks. Lard in these diets was replaced by palm oil. Palm oil is devoid of cholesterol, which may have pro-inflammatory properties. Food intake was measured by weighing the pellets once per week. Mice were housed individually allowing for assessment of food consumption of individual mice. Food intake was averaged over the 20 week intervention and multiplied by the caloric value of the feed to determine energy intake in Kcal/mouse/day. Numbers were subsequently averaged per group. At week 0,2,4,8,12,16 and 20, tail vein plasma samples were collected after a 6h fast. Two mice within the HFD group died before the end of the experiment for reasons unrelated to the dietary intervention. After 21 weeks, ad-libitum fed mice were anaesthetized using isofluorane. Blood was collected by orbital puncture, followed by sacrifice via cervical dislocation. Liver and epididymal white adipose tissues were removed, weighed, and immediately frozen in liquid nitrogen. For histology, liver was frozen with OCT compound and adipose tissue samples were fixed in 10% formalin and processed for paraffin-embedding. Animal experiments were approved by the local animal ethics committee at Wageningen University.

Hepatic Triglyceride Content Determination. Liver triglycerides were determined enzymatically as previously described (10).

RNA extraction, Real-Time PCR and Affymetrix Microarrays. Techniques were employed as previously described (11). Microarray data were analyzed as previously described (12). Genes with a p-value<0.05 were considered significantly regulated. Array data have been submitted to the Gene
Expression Omnibus (Accession number pending). Gene Set Enrichment Analysis (GSEA) was used to identify significantly regulated pathways (13). Gene sets with a False Discovery Rate<0.25 were considered significant.

**Plasma Measurements.** Plasma concentrations of multiple chemokines were measured with multiplex technologies (Rodent Map 2.0, Rules Based Medicine, Austin, TX, USA). Plasma free fatty acids and ALT were measured with commercially available kits from Instruchemie (Delfzijl, The Netherlands). Plasma leptin and insulin levels were measured using kits from Linco (St. Louis, MO, USA)

**Liver Immunohistochemistry.** For oil red O staining, 5µm frozen liver sections were air dried for 30 minutes, followed by fixation in formal calcium (4% formaldehyde, 1% CaCl2). Oil red O staining was performed using standard protocols. Haematoxylin and eosin staining of frozen liver sections was carried out as described [http://www.ihcworld.com/histology.htm](http://www.ihcworld.com/histology.htm). Collagen was stained using fast green FCF/sirius red F3B.

For Immunohistochemistry, 5µm frozen liver sections were cut, dried overnight, fixed in acetone for 10 minutes and washed in PBS prior to use. For visualisation of hepatic stellate cell activation, rabbit anti-GFAP polyclonal antibody was used (Dako, Glostrup, Denmark). For detection of macrophages/monocytes, a rat polyclonal anti-Cd68 antibody (Serotec, Oxford, UK) was used. Sections were incubated 30 minutes at room temperature with GFAP (1:1000 dilution) or Cd68 (1:100 dilution) primary antibodies, followed by incubation for 30 minutes at room temperature with anti-rabbit peroxidase-conjugated En Vision (Dako, GFAP) or 1:20 diluted anti-rat IgG peroxidase-conjugated secondary antibodies (Serotec, C6d68). Visualization of the complex was done using 3-amino-9-ethylcarbazole (AEC) chromogen for 15 minutes, followed by staining with Haematoxylin. Negative controls were performed by omitting the primary antibody.

**Immunohistochemistry of adipose tissue.** 5µm-thick paraffin-embedded sections were cut, dried 30 minutes at 37°C and washed in PBS prior to use. Staining of adipose tissue macrophages was carried out using an antibody against Cd68 as described above. Hematoxylin and eosin staining of sections was done using standard protocols. Collagen was stained using fast green FCF/sirius red F3B.

**Multivariate RBM data analysis.** The data obtained from the commercial RBM multiplex analysis (70 proteins measured in 126 plasma samples) contained a number of technically unreliable entries for specific samples and proteins that were: a) lower than the least detectable dose (LDD): replaced by 0.5xLDD, b) not detectable (i.e. not measured on the standard curve): replaced by 0.1xLDD or c) not measurable (i.e. not sufficient sample material available): replaced by 0.1xLDD. Proteins were removed if they did not contain more than 50% reliable entries for 2 or more groups of the totally available 14 groups (7 time points x 2 diets). This approach assures that potentially relevant group differences are retained in the data. Of the 70 proteins screened, 19 were excluded, 18 contained more than 50% reliable entries for at least 2 groups and 33 proteins contained 100% reliable entries for all groups. One mouse was removed from the dataset for MVA on the basis that some plasma proteins display atypical outlying values.

Multiway Partial Least Squares (PLS), or NPLS, is a multivariate statistical technique that is an extension of standard PLS analysis that is able to analyze data across different time points (14-16). Both methods are able to analyze large numbers of variables in small sample sizes by reducing the dimensionality of the data. In contrast to standard two-
dimensional data (matrix), multiway data can be represented as a collection of matrices (a cube) where each matrix contains data from one specific time-point. For NPLS the data were centered across the samples (i.e. zero mean) to remove offsets followed by autoscaling for the variables (i.e. zero mean and a standard deviation of 1 to remove arbitrary differences in measurement scales (16). Validation of the NPLS models using a double cross-validation strategy and the selection of most important proteins from the models have been performed as previously described (17). Because, (double) cross-validation relies on a random subdivision of data, the complete validation procedure was repeated five times on basis of different random subdivisions of the data during cross-validation. A model was considered to be reliable if at least 4 (out of 5) models could be calculated with an $R^2$ larger than 0.7.

**Statistical Analyses.** Statistical significant differences were calculated using a Student’s T-test. The cut-off for statistical significance was set at a $P<0.05$. NPLS was performed using the N-way toolbox (http://www.models.life.ku.dk/nwaytoolbox) in combination with Matlab 7.1.0, release 14 (The Mathworks, Natick,MA,USA) and home-made software.

**RESULTS**

A subpopulation of C57Bl/6 mice fed HFD develops NASH. To study the effect of chronic high fat feeding on liver metabolic functions, C57Bl/6 mice were fed a low fat diet (LFD) or high fat diet (HFD) for 21 weeks. Mice fed the HFD gained more weight compared to mice fed LFD (Fig.1A), which was already evident after 2 weeks. Enhanced weight gain in mice fed HFD may be related to increased energy intake (Fig.1B). After 21 weeks, weight of the epididymal fat pad, which was assumed to reflect overall adiposity of the animals, was markedly higher in mice fed HFD (Fig.1C).

To characterize the effect of HFD on hepatic steatosis, liver sections were stained with H&E (Fig.1D) and Oil Red O (Fig.1E). Remarkably, a marked heterogeneity in fat accumulation and histology was observed within each group. Scoring of the sections by a pathologist (T.R.) indicated different stages of NAFLD and led to classification of mice into four subgroups, which surprisingly but not deliberately ended up being of approximately equal size. These subgroups were LF low (LFL) responders (n=4) which display normal liver morphology, LF high (LFH) responders (n=6) which develop benign hepatic steatosis, HF low (HFL) responders (n=4) which show a pre-NASH phenotype of macrovesicular lipid droplets, and finally HF high (HFH) responders (n=4) which develop overt NASH characterized by ballooning of hepatocytes, presence of Mallory bodies, and activated inflammatory cells (Fig.1D, inset).

Quantitation of hepatic triglycerides confirmed the heterogeneity between the subgroups, with HFH mice accumulating the highest amount of triglycerides (Fig.1F). Consequently, liver/bodyweight ratio was increased specifically in HFH mice, indicating hepatomegaly (Fig.1G). Finally, plasma alanine-aminotransferase activity was highest in the HFH subgroup, reflecting increased liver damage (Fig.1H).

NASH-related Metabolic Pathways are exclusively impaired in HFH Responders. To correlate changes in liver functions with gene expression, expression profiling was performed on individual mouse livers. Microarray data were processed according to subgroups, with LFL mice serving as reference group for calculation of fold-change and p-values. The most dramatic effects were observed in HFH responders as shown by changes in expression of >3000 genes (Fig.2A). To identify genes regulated exclusively in HFH responders, we selected genes that were statistically significantly
regulated in HFH vs. all subgroups but unchanged in other comparisons. This “HFH responder gene expression signature” comprised 388 upregulated and 319 downregulated genes. One dominant pathway within the HFH expression signature was lipid metabolism, illustrated by the marked induction of Cidec and Mogat1 (Fig.2B). Other lipid metabolism genes such as CD36 and PPARγ increased gradually from LFL to HFH, correlating with hepatic triglycerides (Fig.2B, Fig.1F). An other pathway well-represented within the HFH expression signature was inflammation, as shown by marked and specific induction of acute phase genes orosomucoid, serum amyloid-A and lipocalin-2 in the HFH subgroup (Fig.2B), and confirmed by qPCR (Fig.2C). Finally, many genes in the HFH expression signature were related to fibrosis, including Ctgf, collagens, metalloproteases and Timp1 (Fig.2B,C). Expression analysis of individual mice within the HFH group showed uniform induction of genes involved in the above-mentioned pathways (Fig.2B, right panel). GSEA indicated that while pathways related to lipid metabolism were upregulated in all subgroups compared to LFL mice, with most prominent effects observed in HFH mice, numerous pathways of inflammation, cell cycle and oxidative stress were specifically induced in HFH mice (Supplemental table 1 in the online appendix available at http://diabetes.diabetesjournals.org). The complete microarray dataset is available at http://humannutrition2.wur.nl/duval2010.

The elevated inflammatory status in HFH livers was corroborated by immunostaining for macrophage marker Cd68 (Fig.3A). Early fibrosis was detected in one HFH mouse (Fig.3B). Finally, hepatic stellate activation was demonstrated in HFH mice by GFAP immunostaining (Fig.3C). Overall, these analyses support induction of inflammation and fibrosis in HFH responders, indicating NASH.

**HFH Responder Mice exhibit Adipose Tissue Dysfunction.** Mice classified as high responders also gained the most bodyweight (Fig.4A), likely related to increased food intake (Fig.4B). Indeed, a positive correlation was found between final bodyweight and hepatic triglycerides (Fig.4C). Remarkably, despite increased weight gain, weight of the epididymal fat pad at sacrifice was markedly lower in HFH compared to HFL mice (Fig.4D). As expected, leptin expression in adipose tissue mirrored adiposity (Fig.4E), which was also true for the plasma free fatty acids (Fig.4F). Evaluation of the morphology of the epididymal fat pad in HFH mice after H&E staining revealed atrophied adipocytes surrounded by inflammatory cells, which was hardly observed in HFL responders (Fig.4G). Cd68 immunostaining indicated increased presence of macrophages in HFH mice (Fig.4H), which was supported by expression of F4/80 and Cd68 (Fig.5). In contrast, expression of the anti-inflammatory adipokine adiponectin was markedly reduced in HFH mice, as was resistin (Fig.5). Interestingly, expression of adipogenic (PPARγ,FABP4) and lipogenic (DGAT2,SREBP-1,Fatty acid synthase) marker genes was significantly downregulated in HFH mice compared to HFL mice, suggesting adipose tissue dysfunction. Finally, collagen staining revealed fibrotic adipose tissue in HFH mice (Fig.4I), which was supported by increased expression of TIMP-1 (Fig.5). These data suggest that HFH responders, classification of which is entirely determined by liver histology, exhibit adipose tissue dysfunction characterized by decreased fat mass, enhanced macrophage infiltration, inflammation, and adipose tissue remodelling.

**Plasma Biomarkers are significantly associated with Liver Triglycerides.** To find early biomarkers that may predict NASH in C57Bl/6 mice and that may serve as potential mediators between adipose tissue dysfunction and NASH, plasma was collected at different
time-points of diet intervention and assayed for 70 plasma proteins using multiplex analysis. Levels of most plasma proteins were not consistently different between the subgroups. One exception was the acute phase protein haptoglobin, which was elevated in HFH mice after 12 weeks of diet intervention (Fig.6A). Similarly, plasma levels of the fibrosis marker TIMP-1 started to deviate at week 12 and further increased until the end. Remarkably, IL-1β and leptin levels were already elevated in HFH after 2 weeks and this pattern was maintained throughout the intervention. Finally, plasma insulin levels indicated that HFH mice became hyperinsulinemic from week 12, suggesting development of insulin resistance.

To systematically screen for plasma biomarkers that predict liver triglycerides, multivariate analysis was performed using Multiway Partial Least Squares. The results of multivariate analysis between plasma proteins at different time points and liver triglycerides are depicted in Fig.6B and Supplemental table 2. In addition to leptin, plasma levels of CRP, eotaxin, haptoglobin, and MIP-1α early in the intervention were positively associated with liver triglycerides at 20 weeks. Intermediate prognostic markers of liver triglycerides included IL-18, IL-1β, MIP-1γ and MIP-2, whereas insulin, TIMP-1, GCP-2 and MPO emerged as late markers. Throughout the diet intervention, highest regression coefficients were obtained for CRP, haptoglobin, leptin and IL-1β (Supplemental table 2). Adiponectin was not significantly associated with liver triglycerides. The complete multivariate dataset is available at http://humannutrition2.wur.nl/duval2010.

Besides potentially serving as predictive biomarkers of liver triglycerides, these proteins may provide a functional link between adipose tissue dysfunction and NAFLD.

**DISCUSSION**

C57Bl/6 mice fed HFD represent a popular animal model for human obesity and insulin resistance. Despite development of hepatic steatosis and other features of patients with NAFLD (18), except for a recent report the model has not been extensively used to study NAFLD (19). As expected, high fat feeding increased adipose tissue mass and hepatic fat storage. Consistent with previous data showing considerable variability in the obese and diabetic phenotype (20; 21), we observed marked heterogeneity in bodyweight gain. Additionally, the magnitude of fat storage and NAFLD scoring differed markedly between the mice, giving rise to four well-distinguishable groups. Detailed histological and gene expression analysis indicated that HFH mice exhibit NASH. Accordingly, detailed study of these HFH responders may give novel insight into the development and progression of NAFLD.

Although humans have no epididymal fat pads, we studied epididymal adipose tissue because it represents the most commonly used fat depot in mouse studies, is easily accessible, can be accurately weighed, and is relatively homogenous. Unfortunately, we did not have access to DEXA or MRI to be able to measure fat percentage, lean body mass and fat distribution. Remarkably, HFH mice, despite showing highest weight gain, had significantly less epididymal fat after 21 weeks compared to HFL mice, which likely reflects differences in overall adiposity. An important question is why there is an apparent limit to the expansion of adipose tissue in HFH mice, and especially what is the link with NASH. Obesity-related adipocyte hypertrophy is known to be associated with adipose inflammation characterized by infiltration of macrophages and other leukocytes, appearance of so-called crown-like structures, and increased expression of several inflammatory markers (2; 3; 22; 23). How adipose tissue inflammation develops during obesity is not clear but a role of
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hypertrophy, hypoxia and adipocyte cell death has been suggested (23; 24). Compared to HFL mice, adipose tissue of HFH mice showed more pronounced inflammation as shown by increased macrophage staining and expression of inflammatory marker genes, and increased collagen staining, suggesting fibrosis. Furthermore, decreased adipocyte size and increased cell death were observed in HFH mice. Since HFH mice are classified entirely based on liver histology, these data indicate a strong link between inflammatory and morphological changes in adipose tissue and progression of steatosis to NASH. Consequently, adipose tissue “failure” or dysfunction may signal progression of hepatic steatosis towards NASH. These data support the previous suggestion that loss of adipose tissue mass due to remodeling contributes to hepatic steatosis induced by HF-feeding (23), and thereby strengthen an emerging view that obesity starts to cause metabolic problems when adipose tissue cannot fully meet demands for additional storage, leading to fat accumulation in other organs such as muscle, liver, and β-cells causing lipotoxicity (25-27). The limited expandability of adipose tissue may be related to adipose tissue fibrosis and associated disproportionate accumulation of extracellular matrix components (22).

Multivariate longitudinal analysis of plasma proteins yielded a number of candidates that may serve as prognostic markers for NAFLD and NASH. In addition, these proteins may provide insight into the functional link between adipose tissue dysfunction and NAFLD. Besides leptin, the best predictive markers were the acute phase proteins CRP and haptoglobin, and MIP-1α (Ccl3). Plasma CRP was previously proposed as a diagnostic marker for NASH (28). In one study, hs-CRP was significantly elevated in patients with NASH compared to patient with only steatosis (29). Furthermore, hs-CRP correlated with the severity of fibrosis in NASH. In another study, plasma CRP was not helpful in diagnosis of NASH in severely obese patients, possibly because adipose tissue contributes to plasma CRP in obesity.

Hardly any data exist on the relation between plasma haptoglobin and NASH. Haptoglobin was included in a composite biomarker combining 13 parameters to predict NASH (30). Interestingly, in a recent study plasma haptoglobin showed a negative correlation with fibrosis stage (31). There are no reports on the association between plasma MIP-1α and hepatic steatosis or NASH. However, it was reported that MIP-1α mRNA in human liver is positively associated with liver fat (32).

Another good predictive marker for liver triglycerides was IL-1β. Plasma IL-1β levels were significantly elevated in HFH mice already after 2 weeks of HFD, probably because of elevated production in adipose tissue. Recently, we showed that IL-1β may promote steatosis in mice by inhibiting PPARα activity (33). Whether adipose tissue-derived IL-1β links adipose tissue dysfunction and NASH requires further study.

Recent studies indicate that IL-18 promotes hepatic steatosis in mice (34; 35). Interestingly, patients with NAFLD were found to have significantly elevated plasma IL-18 levels (36). According to our multivariate analysis, plasma IL-18 from week 8 onwards was significantly associated with liver triglycerides. Overall, further research into the potential use of plasma CRP, haptoglobin, MIP-1α, eotaxin, and IL-18 as prognostic biomarkers for NAFLD in humans is warranted.

One of the late biomarkers to emerge from our study was TIMP-1. Specifically, plasma TIMP-1 levels started to deviate in the HFH mice after 12 weeks of HFD. Since TIMP-1 expression was increased in HFH mice in both liver and adipose tissue, it is unclear which tissue primarily contributes to increased plasma levels. TIMP-1 is used extensively as a marker of fibrosis related to
viral hepatitis (38). However, its use in the context of NAFLD is very limited. According to our data, plasma TIMP-1 levels may have potential as biomarker for NASH. Consistent with this notion, plasma TIMP-1 was recently shown to be a valuable component of a composite predictive marker of NASH in human (39).

An adipokine that has been extensively linked to NAFLD is adiponectin (37; 38). Besides its anti-steatotic role (39; 40), adiponectin has potent anti-inflammatory effects in liver (37). In humans, plasma adiponectin, either alone or as ratio to plasma leptin (41), has shown promise as diagnostic marker for NASH, although it should be validated in larger cohorts of patients (9). In the present study, plasma adiponectin was not significantly associated with liver triglycerides.

Overall, the best predictive marker for liver triglycerides, which was also clearly elevated in HFH mice, was leptin. Portal infusion with leptin was shown to increase hepatic triglycerides in rats (42). Furthermore, leptin appears to be one of the key regulators of inflammation and progression to fibrosis in NASH (43-46). Although some studies have found elevated plasma leptin levels in patients with NASH (47; 48), other studies have not, thus somewhat questioning the potential of plasma leptin as a noninvasive marker for diagnosis of NASH in humans (49).

Hyperleptinemia in HFH mice is expected to decrease food intake. However, energy intake was higher in HFH compared to other subgroups, suggesting existence of leptin resistance, at least centrally in the hypothalamus. In contrast, leptin resistance is expected to be absent from liver, in which chronically elevated leptin levels may promote NASH by stimulating hepatic triglyceride storage, inflammation and fibrosis (38). Leptin resistance may thus be the basis for hyperleptinemia and hyperphagia in HFH mice, leading to accelerated weight and fat gain and consequent adipose tissue dysfunction.

The significant heterogeneity in the response to HFD in C57Bl/6 mice has been previously reported (20; 21). The underlying reason for the large heterogeneity is uncertain but may be related to copy number variations in the mouse genome (50), and perhaps specifically by differences in the copy number of the Ide gene encoding the insulin-degrading enzyme (51). Alternatively, the variation in phenotype after HFD may be mediated by epigenetic mechanisms (21), giving rise to variable adipose expression of specific genes. However, the importance of epigenetic mechanisms in controlling expression of one of these genes was later discounted (52). Overall, the relative importance of genetic, epigenetic and environmental factors in the response to high fat feeding in C57Bl/6 mice is still unclear.

In conclusion, we show that a subset of C57Bl/6 mice fed HFD composed of palm oil develop NASH. Our data support the existence of a tight relationship between adipose tissue dysfunction and NASH pathogenesis and point to several novel potential predictive biomarkers for NASH.

Author contributions. CD, SK and MM designed the research, CD, SK, BA, and RS collected data. CD, UT, MB, TR and SK analyzed the data. CD and SK wrote manuscript. All authors reviewed/edited manuscript.

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Figure Legends

Figure 1: A subpopulation of mice fed HFD develops NASH. A) Changes in bodyweight in C57Bl/6 mice fed LFD (open squares, n=10) or HFD (closed squares, n=8). B) Mean energy intake of mice fed LFD or HFD during 21 weeks of dietary intervention. C) Weight of epididymal fat pad after 21 weeks of dietary intervention. Error bars reflect standard deviation. * = significantly different from mice fed LFD according to Student’s t-test (P<0.05). Haematoxylin and eosin staining (D) and oil red O staining (E) of representative liver sections of the 4 subgroups (see text). F) Liver triglyceride concentration. G) Liver weight (expressed as percentage of total bodyweight). H) Activity of alanine aminotransferase (glutamate pyruvate transaminase) in plasma. Error bars reflect standard deviation. Bars with different letters are statistically different (P<0.05 according to Student’s t-test). Number of mice per group: n=4 (LFL, HFL, HFH), n=6 (LFH).

Figure 2: Upregulation of inflammatory and fibrotic gene expression in HFH responder mice. A) Number of genes up- or down-regulated in the various subgroups in comparison to the LFL mice, as determined by Affymetrix GeneChip analysis. Genes with a p-value below 0.05 were considered significantly regulated. B) Heat map showing changes in expression of selected genes involved in lipid metabolism, inflammation and fibrosis in liver. Mean expression in LFL mice was set at 1. Gene expression changes in individual mice within the HFH group are shown on the right. C) Changes in gene expression of selected genes as determined by real-time quantitative PCR. Mean expression in LFL mice was set at 100%. Error bars reflect standard deviation. Bars with different letters are statistically different (P<0.05 according to Student’s t-test). Number of mice per group: n=4 (LFL, HFL, HFH), n=6 (LFH).

Figure 3: (Immuno)histochemical staining confirms enhanced inflammation and early fibrosis in HFH mice. A) Immunohistochemical staining of macrophage activation in representative liver section of HFL and HFH mice using antibody against the specific macrophage marker Cd68. B) Collagen staining using fast green FCF/sirius red F3B. C) Staining of stellate cell activation using antibody against GFAP.

Figure 4: Adipose dysfunction in HFH mice. A) Bodyweight changes in the 4 subgroups during the 21 week dietary intervention. White squares: LFL, Light grey squares: LHF, dark grey squares: HFH, black squares: HFH. B) Mean daily energy intake. C) Positive correlation between final bodyweight and liver triglyceride concentration (P<0.05). D) Weight of epididymal fat depot. E) Adipose tissue leptin mRNA expression as determined by qPCR. Mean expression in LFL mice was set at 100%. F) Plasma free fatty acid levels. Error bars reflect standard deviation. * = significantly different from HFL mice according to Student’s t-test (P<0.05). Number of mice per group: n=4 (LFL, HFL, HFH), n=6 (LFH). G) Haematoxylin and eosin staining of representative adipose tissue sections. H) Immunohistochemical staining of macrophages using antibody against Cd68. I) Collagen staining using fast green FCF/sirius red F3B.

Figure 5: Change in adipose gene expression indicate adipose tissue dysfunction. Adipose tissue mRNA expression of a selected group of genes was determined by quantitative real-time PCR after 21 weeks of dietary intervention. Mean expression in LFL mice was set at 100%. Error bars reflect standard deviation. * = significantly different from HFL mice according to Student’s t-test (P<0.05). Number of mice per group: n=4 (LFL, HFL, HFH), n=6 (LFH).

Figure 6: Plasma proteins as early predictive biomarker for NASH in C57Bl/6 mice. A) Plasma concentration of haptoglobin, TIMP-1, IL-1β, leptin and insulin were determined by multiplex assay at specific time points during the 21 weeks of dietary intervention after a 6h fast. White
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squares: LFL, Light grey squares: LFH, dark grey squares: HFL, black squares: HFH. Error bars reflect standard deviation. * = significantly different from HFL mice according to Student’s t-test (P<0.05). Number of mice per group: n=4 (LFL, HFL, HFH), n=6 (LFH). B) Graphs illustrating the result of multivariate analysis showing the association of protein plasma concentrations at various time points with final liver triglyceride content. Significant proteins display an inverse RSD value higher than 2 (bold line indicates the inverse RSD threshold value of 2).

Figure 1
Adipose tissue dysfunction and NASH

Figure 2
Figure 3

A  HFL  HFH

B

C
Figure 6