Transcriptome analysis to investigate the link between obesity and its metabolic complications
Wolfs, Marcel Guillaume Maria

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Chapter 4

Adipose tissue expression of Leptin, Rarres 2 (a.k.a. Chemerin), and Angiopoietin 2 is associated with human Non-Alcoholic SteatoHepatitis (NASH)

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In preparation
Abstract

Obesity is characterized by the expression and release of signalling molecules (adipokines) from adipose tissue. For some adipokines the plasma levels correlate with aspects of Non-Alcoholic Fatty Liver Disease (NAFLD), implying that release of these adipokines from adipose tissue in obesity directly contributes to NAFLD. Therefore, we investigated mRNA expression levels of adipokines in adipose tissue and liver of severely obese individuals with varying degrees of NAFLD.

The mRNA expression levels of 48 adipokines were measured in subcutaneous adipose tissue (SAT), visceral adipose tissue (VAT), and liver of 93 severely obese individuals. Some 14 adipokines were predominantly expressed in adipose tissue, while 11 adipokines were predominantly expressed in liver and the remaining 23 adipokines had a more variable expression pattern. Expression in VAT, but not in SAT or liver, of RARRES2 (R=-0.39; P=3.7 × 10^{-4}), ANGPT2 (R=0.37; P=8.9 × 10^{-4}), and LEP (R=0.39; P=4.3 × 10^{-4}) was significantly correlated to NAFLD, in particular lobular inflammation, independent of BMI. Expression of all other adipokines in VAT or SAT was not significantly correlated to any aspect of NAFLD. Hepatic expression of IL1RN, SERPINE1 (a.k.a. PAI-1), CXCL10, and IGF1 was significantly correlated to aspects of NAFLD, independent of BMI.

These results reveal a role for expression of LEP, RARRES2, and ANGPT2 from visceral adipose tissue in NAFLD, in particular with respect to lobular inflammation. Although proposed to be adipokines, the role of IL1RN, SERPINE1, CXCL10, and IGF1 in NAFLD seems to be related to processes within the liver. In addition, this study has identified ANGPT2, LEP, and RARRES2 mRNA expression in visceral adipose tissue as potential novel markers for NAFLD in severely obese individuals.
Introduction

Obesity is associated with Non-Alcoholic Fatty Liver Disease (NAFLD). The benign form of NAFLD is characterized by hepatic fat accumulation or hepatic steatosis (1). However, some 25–40% of NAFLD patients show progression from simple steatosis to Non-Alcoholic SteatoHepatitis (NASH) (2). NASH is characterized by asymptomatic hepatic inflammation and cytologic ballooning (3). Due to the growing obesity epidemic, NASH is becoming a serious health problem, because at least 50% of NASH patients will develop fibrosis (2) and around 25% of these individuals will progress to severe liver pathology such as liver cirrhosis and hepatocellular carcinoma (4).

Studies in transgenic mouse models have indicated that processes within the adipose tissue initiated in obesity are the most likely suspects to be causally involved in NAFLD. Noteworthy, inactivation of the regulatory gene Mapk8 (a.k.a JNK1) in adipose tissue of mice resulted in altered liver function (5), and similarly adipocyte-specific ablation of the Fas gene resulted in protection against diet-induced NAFLD (6).

Altered expression and release of signalling molecules within adipose tissue (adipokines) is a hallmark of obesity. These adipokines can enter the bloodstream and reach the liver (7), and their changed expression in obesity might thus provide a link between obesity and NAFLD. In particular adipokines derived from visceral adipose tissue (VAT) are considered to have such a role since VAT is directly connected with the liver through the portal vein, and particularly visceral adiposity is related to obesity complications including NAFLD (8-10). Indeed, plasma levels of some adipokines were reported to be related to aspects of NAFLD (11-15).

However, many adipokines are ubiquitously expressed, which means that a link between adipokine plasma levels and NAFLD does not necessarily provide evidence that release of these adipokines from adipose tissue links obesity to NAFLD. Furthermore the individual role and importance of several adipokines is unclear.

Therefore, in the current study, we studied 93 severely obese individuals with varying degrees of NAFLD. In these individuals, mRNA levels of 48 genes advocated to function as an adipokine (16-19) were measured in subcutaneous adipose tissue, visceral adipose tissue, and liver. We subsequently studied the association between mRNA expression of these genes and important aspects of NAFLD, i.e. steatosis, fibrosis, lobular inflammation, portal inflammation, cytologic ballooning, glycogenated nuclei, and large lipogranulomas. Our aims were to identify adipokines...
which mRNA expression levels in adipose tissue are related to these important aspects of NAFLD.
Materials and methods

Study population

The severely obese individuals with a BMI ranging from 30 to 74 were recruited as described earlier (20) from 2006 to 2009. They underwent elective bariatric surgery at the Department of General Surgery, Maastricht University Medical Centre (Maastricht, the Netherlands). Subjects using anti-inflammatory drugs or having acute or chronic inflammatory diseases, degenerative diseases, and subjects reporting alcoholic intake >10 g/day, were excluded. In this way, we included 93 obese subjects for further histologic assessment of liver pathology. Blood parameters and clinical traits of the study subjects are shown in supplemental table 1. This study was approved by the Medical Ethics Board of Maastricht University Medical Centre. Informed written consent was obtained from each individual.

Histologic assessment of liver pathology

Liver biopsies were formalin fixed and paraffin embedded. Biopsies were analysed by an experienced pathologist who was unaware of the clinical and biochemical data of the individuals. According to the histological scoring system described by Kleiner et al. (3), each individual was scored for seven different histologic parameters of liver pathology, including steatosis, fibrosis, inflammation (lobular inflammation, large lipogranulomas, portal inflammation), liver cell injury (ballooning) and others (glycogenated nuclei). There were notable differences in the degree of liver pathology (Table 1). Correlations between the liver phenotypes and sex, age, and BMI are shown in supplemental figure 1.

Tissue sampling, histology preparation, and mRNA isolation

Tissue sampling and mRNA isolation were performed as described earlier (20). Wedge biopsies from subcutaneous adipose tissue, visceral adipose tissue, and liver were taken during bariatric surgery. mRNA was isolated using the Qiagen Lipid Tissue Mini Kit (Qiagen, Hilden, Germany, 74804) and mRNA quality and concentration were assessed with an Agilent Bioanalyzer (Agilent Technologies, Waldbronn, Germany, 5067-1521). mRNA integrity numbers (RIN) of these samples ranged between 4.5 and 9.3 (average 7.5), 5.8 and 8.7 (average 7.5), and 6.2 and 9.4 (average 7.6) for liver, SAT, and VAT respectively.
mRNA pre-hybridization processing and hybridization

mRNA pre-hybridization processing and hybridization were performed as described earlier (20). Starting with 200 ng of mRNA, the Ambion Illumina TotalPrep Amplification Kit was used for anti-sense RNA synthesis, amplification, and purification, according to the manufacturer’s protocol (Applied Biosystems/Ambion, Austin, TX, USA). 750 ng of complementary RNA was hybridized to Illumina HumanHT12 BeadChips (Illumina, San Diego, CA, USA) and scanned on the Illumina BeadArray Reader. These micro arrays contain 48,813 different probes targeting 37,812 different genes; some genes are targeted by more than one probe.

Data normalization and quality control

Quantile-quantile normalization was applied to all genome-wide data from liver, SAT, and VAT using LIMMA package (version 3.4.5) in R (version 2.11.1) (R foundation for statistical computing, Vienna, Austria). Only samples were included that passed quality control filtering, which was based on the median probe intensity, general behaviour of known housekeeping genes, and principal component analysis over the samples. Available genome-wide genotype data were used to rule out sample mix-ups (21), and qRT-PCR was performed to estimate the technical quality of the micro array (20). We obtained reliable RNA measures for 74 liver samples, 83 SAT samples and 77 VAT samples. All expression data has been made freely available by submission to GEO under GSE22070 (SAT data), and GSE22071 (VAT data). Liver expression data will be made available soon.

mRNA analysis of adipokines

We defined adipokines as genes that [1] are secreted by adipose tissue, [2] have the potential to enter the bloodstream and reach the liver, and [3] are related to features of the metabolic syndrome, for instance through a relation between their plasma levels and obesity, NAFLD, or type 2 diabetes. By mining literature we identified 48 genes that met these criteria (16-19). These 48 genes were targeted by 69 probes on the micro array.

In order to obtain insight into tissue-specific expression of adipokines, we conducted hierarchical cluster analysis and correlation analysis for the adipokine expression in liver, SAT and VAT tissues. For a better illustration, we took the average intensity of multiple probes per gene, if it applies. All analyses were done using
R (version 2.14.1) (R foundation for statistical computing, Vienna, Austria). The heat-map and hierarchical cluster of adipose expression in three tissues was plotted using R package gplots (version 2.10.1). The distance was calculated using Euclidean distance and the samples and genes were clustered using the “complete” method. The inter-tissue correlation of adipokines was computed using Spearman correlation. We performed permutation analysis to determine the empirical threshold for correlation at FDR 0.05 level. For an expression matrix (n number of genes × m number of samples) from a tissue type, we randomly reordered the m columns for the expression matrix of liver, SAT and VAT, respectively. We then recalculated the Spearman correlation for the permuted expression values. This permutation was repeated 1000 times and we determined the empirical P-value threshold 0.01 at FDR 0.05 level.

To find correlations between histological features of the liver and mRNA expression of genes encoding adipokines in three tissue types, we computed Spearman correlations between liver histology scores and the expression level of each adipokine probe from each tissue type. The significance of correlation was determined as $P < 5.9 \times 10^{-4}$ that corresponds to FDR 0.05 level, using 1000× permutation method. For each permutation, we kept the expression matrix intact but reordered the columns of the phenotypic matrix ($\kappa$ number of liver histological parameters × $\mu$ number of samples).

Considering the strong inter-tissue correlation of some adipokines, for the adipokines with expression in a certain tissue type significantly correlated with liver histology, we further computed partial correlations conditional on the correlation structures of their expression in the other two tissues. We first coded the Spearman correlation coefficient between a given liver histology $P$ and the expression of a gene in three tissues types (T1, T2 and T3) as $r_{PT_1}$, $r_{PT_2}$, and $r_{PT_3}$ respectively and the inter-tissue correlation of gene expression as $r_{T_1T_2}$, $r_{T_1T_3}$, and $r_{T_2T_3}$ respectively. The first order partial correlation between $P$ and $T_1$ expression, conditional on $T_2$ is then given by: $r_{PT_1|T_2} = \frac{r_{PT_1} - r_{PT_2}r_{T_1T_2}}{\sqrt{(1-r_{PT_2}^2)(1-r_{T_1T_2}^2)}}$. The second-order partial correlation between $P$ and $T_1$ expression, conditional on both $T_2$ and $T_3$ is then formulated as: $r_{PT_1|T_2T_3} = \frac{r_{PT_1} - r_{PT_2}r_{T_1T_2} - r_{PT_3}r_{T_1T_3} + r_{PT_2}r_{T_1T_2}r_{T_1T_3}}{\sqrt{(1-r_{PT_2}^2)(1-r_{PT_3}^2)(1-r_{T_1T_2}^2)(1-r_{T_1T_3}^2)}}$. The P-values were then calculated for the partial correlation coefficients. The significance of partial correlation was determined as $P < 0.01$ at FDR 0.05 level, using 1000× permutations that permutated both the expression matrix and phenotypic matrix as described above.

BMI is one of the risk factors for NAFLD and we observed that three liver histologic parameters were significantly correlated with BMI (supplemental figure...
1). To assess whether the correlation of adipokine and liver histology is dependent on BMI, we performed conditional regression analysis to regress out the effect of BMI. For each significant correlation between a liver histology and the expression level of an adipokine in a tissue type, we first regressed out the effect of BMI on the expression intensity by linear regression model. We then subjected the residuals to the Spearman correlation analysis with the liver histology. The significance for the correlation between residuals and liver histology was controlled at $P<0.01$ at FDR 0.05 level, using 1000× permutation analysis.

We used Cytoscape software to visualize the significant correlations.
Results

Histologic assessment of NAFLD and NASH

For each of the 93 severely obese individuals we scored seven features of NAFLD, including hepatic steatosis, fibrosis, inflammation (lobular inflammation, large lipogranulomas, portal inflammation), hepatocyte death (ballooning), and glycogenated nuclei, according to the method proposed by Kleiner et al. (Methods and Materials, (3)). Among them, 25 severely obese individuals showed no signs of NAFLD and 8 individuals showed simple steatosis with no features of NASH. The remaining 60 individuals had steatosis accompanied by varying degrees of NASH (Table 1). To investigate the role of possible confounding factors, we first tested the correlation between features of NAFLD and age, sex, and BMI. We observed that BMI was correlated with the degree of steatosis ($P=2.4 \times 10^{-4}$), fibrosis ($P=2.8\times10^{-4}$), and hepatocyte ballooning ($P=1.0 \times 10^{-3}$), but no correlations were observed for sex and age. We further observed many correlations between different features of NAFLD, with the strongest correlation observed between steatosis and lobular inflammation ($P=1.76\times10^{-15}$), which remained significant after correction for BMI ($P=2.2\times10^{-16}$) (supplemental figure 1). Thus, patients with severe steatosis have more severe NASH and this relation is independent of BMI.

Table 1: Summary of liver pathology of the study population.

<table>
<thead>
<tr>
<th></th>
<th>Total number of individuals scored</th>
<th>No. of individuals with score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Steatosis (0–3)</td>
<td>93</td>
<td>25</td>
</tr>
<tr>
<td>Fibrosis (0–3)</td>
<td>91</td>
<td>62</td>
</tr>
<tr>
<td>Lobular inflammation</td>
<td>88</td>
<td>36</td>
</tr>
<tr>
<td>Large lipogranulomas</td>
<td>92</td>
<td>70</td>
</tr>
<tr>
<td>Portal inflammation</td>
<td>93</td>
<td>72</td>
</tr>
<tr>
<td>Ballooning (0–2)</td>
<td>92</td>
<td>49</td>
</tr>
<tr>
<td>Glycogenated nuclei</td>
<td>90</td>
<td>74</td>
</tr>
</tbody>
</table>

*Data are the number of individuals scored for different grades of hepatic steatosis and 6 features of NASH as described by Kleiner et al. (3). The table also shows for each trait the total number of individuals that were successfully scored.*
The expression of adipokines in liver, SAT and VAT contributes to NAFLD

To characterize the role of adipose tissue in NAFLD, we determined genome-wide gene expression profiles of liver, visceral adipose tissue (VAT), and subcutaneous adipose tissue (SAT), that were simultaneously obtained during bariatric surgery (20, 22). After quality control and normalization (Methods and Materials), a total of 74 liver samples, 83 SAT samples, and 77 VAT samples were retained for further analysis. Genome-wide gene expression profiles differed between tissues (supplemental figure 2), but in none of the tissues clustering of individuals based on genome-wide gene expression profiles showed obvious relationships with liver disease scores (supplemental figure 3).

To understand the role of adipokines in NAFLD development, we confined our analyses to a set of 48 adipokines that were selected from the literature (see methods section). Expression levels of most of these adipokines differed between tissues, and we could classify tissue samples based on gene expression (Figure 1). Furthermore, 26 adipokines were not co-expressed between any pair of tissues, and the expression of the other 22 adipokines showed correlation between different tissues, but mainly between the two adipose tissues, SAT and VAT (Figure 1).

In order to determine which adipokines in which tissue type had expression levels correlated to features of NAFLD, we calculated Spearman correlation coefficients between each feature of NAFLD and the expression of adipokines in each tissue. At $P < 5.9 \times 10^{-4}$ (FDR <0.05) level, expression of seven adipokines correlated with five different features of NAFLD (Table 2, Figure 2). Two remaining features of NAFLD that did not correlate with adipokine expression levels were large lipogranulomas and glycogenated nuclei.

The correlation between expression of adipokines and features of NAFLD was tissue-specific, except for the correlation between expression of RARRES2 (a.k.a. Chemerin) and lobular inflammation (Table 2, Figure 2). These findings were in line with the observed tissue-specific expression of adipokines. To further validate the tissue-specificity of correlations between adipokine expression and features of NAFLD, we conducted partial correlation analyses (Table 2, Figure 2), i.e. we determined the correlation between expression of the adipokines and features of NAFLD in one tissue, conditional on the expression of the same adipokine in the other two tissues for all possible tissue combinations (Methods and Materials). The partial correlation between expression of RARRES2 in VAT and lobular inflammation remained
Crosstalk between adipose tissue and liver

statistically significant. On the contrary, the partial correlation between expression of RARRES2 in SAT and lobular inflammation was very weak. For the other adipokines, results of the partial correlation analyses did not differ from the initial correlation analyses. Thus for all the adipokines except RARRES2, we found their expression only in one specific tissue correlating with features of NAFLD (Table 2, Figure 2).

Next, we investigated whether BMI acted as a confounding factor underlying the correlations between expression of adipokines and features of NAFLD. To this end, we calculated Spearman correlation coefficients between each feature of NAFLD and the expression of adipokines in each tissue corrected for BMI. The BMI correction strongly diminished the effects of LEP expression in VAT on ballooning and steatosis and the effect of ILIRN expression in SAT on hepatocyte ballooning (Table 2, Figure 2). The BMI correction had no significant effect on the other correlations between adipokine expression and NAFLD.

Thus, our analyses revealed a relation between tissue-specific expression of seven adipokines and features of NAFLD, that were largely independent of BMI. These findings provide new insights into the mechanisms underlying the development of NAFLD.
Figure 1: The expression of adipokines in liver, SAT and VAT.

A). Heat-map diagram of differential gene expression in three tissue types. Each column represents a tissue sample and each row represents a single gene. The heat-map was plotted using the function heatmap.2 from R package gplots (version 2.10.1), with distance function euclidean and the complete cluster method. Expression levels are coloured green for low intensities and red for high intensities. At the top of the heat-map is the hierarchical clustering order of the tissue samples. The tissue samples are coloured red for liver samples, green for visceral adipose tissue and blue for subcutaneous adipose tissue. The left side of the heat-map shows the hierarchical clustering order of genes, whereas the right side shows the gene name. The genes clusters are coloured red for higher expression in liver than in adipose tissue and green for lower expression in liver than in adipose tissues. The genes names are coloured differently based on their co-expression between tissues as in B).

B). Venn diagram showing co-expression of genes between tissues. The expression of 22 genes were significantly correlated between at least one pair of tissues at FDR <0.05 level. The genes are coloured differently dependent on the tissue-type of their correlation.
Five liver characteristics were correlated with the expression of at least one adipokine. The three characteristics that correlated with BMI are shown in blue. Two histologies showed no correlation with BMI (indicated in red). Each gene node indicates the expression of a gene in a certain tissue type. If its expression positively correlated with liver histology, the correlation is shown as a red line with arrow head; if its expression negatively correlated with liver histology, the correlation is shown as a green line with bar head. Solid lines indicate tissue-specific correlations, which remained significant after correcting for the expression of that gene in other tissue types; dashed line indicate correlations that may not be tissue-specific, i.e. the correlation was not significant after correcting for the gene expression in other tissue types. “BMI” indicates a BMI-dependent correlation, i.e. after correcting for BMI, the correlation is not significant any more. The correlation of adipokine expression between tissue types is shown as a blue line without head.

Figure 2: The correlation network for adipokines.
Table 2: The Adipokines correlated with NAFLD.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Trait</th>
<th>Tissue</th>
<th>Correlation (P-value)</th>
<th>Tissues corrected</th>
<th>BMI corrected</th>
<th>Role and relation to NAFLD in literature</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANGPT2</td>
<td>Lobular inflammation</td>
<td>VAT</td>
<td>0.42 (1.0 x 10^-5)</td>
<td>0.37 (9.3 x 10^-4)</td>
<td>0.37 (7.9 x 10^-4)</td>
<td>Involved in vascular remodelling (antagonist of Tie2 receptor). ANGPT2 plasma levels are increased in individuals with obesity and type 2 diabetes.*</td>
<td>(23-26)</td>
</tr>
<tr>
<td>LEP</td>
<td>Lobular inflammation</td>
<td>VAT</td>
<td>0.40 (2.3 x 10^-4)</td>
<td>0.39 (4.5 x 10^-4)</td>
<td>0.30 (7.6 x 10^-4)</td>
<td>Appetite control through hypothalamus; also peripheral immune functions.</td>
<td>(13, 27-34)</td>
</tr>
<tr>
<td></td>
<td>Ballooning</td>
<td>VAT</td>
<td>0.37 (4.9 x 10^-4)</td>
<td>0.38 (6.5 x 10^-4)</td>
<td>0.16 (0.15)</td>
<td>Leptin plasma levels are increased in individuals with high BMI and hepatic steatosis, but have been reported not to be associated with NASH.**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Steatosis</td>
<td>VAT</td>
<td>0.41 (1.3 x 10^-4)</td>
<td>0.42 (1.4 x 10^-4)</td>
<td>0.26 (0.018)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RARRES2</td>
<td>Lobular inflammation</td>
<td>VAT</td>
<td>-0.51 (1.5 x 10^-6)</td>
<td>-0.40 (3.1 x 10^-4)</td>
<td>-0.43 (8.7 x 10^-5)</td>
<td>Involved in macrophage recruitment and in adipocyte differentiation.</td>
<td>(35-40)</td>
</tr>
<tr>
<td>(a.k.a.</td>
<td>Lobular inflammation</td>
<td>SAT</td>
<td>-0.37 (5.6 x 10^-4)</td>
<td>-0.13 (0.24)</td>
<td>-0.32 (2.8 x 10^-3)</td>
<td>RARRES2 plasma levels are increased in individuals with features of metabolic syndrome including NAFLD and NASH.</td>
<td></td>
</tr>
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</table>

Continued on Next Page...
Table 2 – Continued

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Correlation (P-value)</th>
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<th>BMI corrected</th>
<th>Role and relation to NAFLD in literature</th>
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<tr>
<td>Portal</td>
<td>VAT</td>
<td>-0.37</td>
<td>0.25</td>
<td>0.28</td>
<td>VAT</td>
<td>RARRES2 is mostly expressed in liver. Conclusive evidence supporting a role as an adipokine is lacking.***</td>
<td></td>
</tr>
<tr>
<td></td>
<td>inflammation</td>
<td>(5.9×10⁻⁴)</td>
<td>(0.028)</td>
<td>(0.01)</td>
<td></td>
<td></td>
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<tr>
<td>Steatosis</td>
<td>VAT</td>
<td>-0.39</td>
<td>0.24</td>
<td>0.28</td>
<td>VAT</td>
<td>RARRES2 mRNA expression in VAT is modestly correlated with RARRES2 plasma levels. (36)</td>
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<tr>
<td></td>
<td></td>
<td>(2.9×10⁻⁴)</td>
<td>(0.036)</td>
<td>(0.012)</td>
<td></td>
<td></td>
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<tr>
<td><em>IL1RN</em></td>
<td>Lobular</td>
<td>0.45</td>
<td>0.38</td>
<td>0.45</td>
<td></td>
<td>Modulates the immune and inflammatory related activities of interleukin 1A and 1B.****</td>
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<tr>
<td></td>
<td>inflammation</td>
<td>(4.1×10⁻⁵)</td>
<td>(8.4×10⁻⁴)</td>
<td>(4.8×10⁻⁵)</td>
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<td></td>
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<tr>
<td>Ballooning</td>
<td>SAT</td>
<td>0.38</td>
<td>0.34</td>
<td>0.01</td>
<td></td>
<td>IL1RN plasma levels as well as <em>IL1RN</em> liver mRNA expression are correlated to features of metabolic syndrome including NASH. (41, 42)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2.6×10⁻⁴)</td>
<td>(1.7×10⁻³)</td>
<td>(0.93)</td>
<td></td>
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<tr>
<td><em>CXCL10</em></td>
<td>Steatosis</td>
<td>0.38</td>
<td>0.39</td>
<td>0.35</td>
<td></td>
<td>Stimulation of monocytes, natural killer and T-cell migration, and modulation of adhesion molecule expression.</td>
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<td></td>
<td></td>
<td>(4.9×10⁻⁴)</td>
<td>(5.9×10⁻⁴)</td>
<td>(1.1×10⁻³)</td>
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<td>Tissues corrected</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>BMI corrected</td>
<td></td>
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<tr>
<td>Fibrosis</td>
<td>Liver</td>
<td>0.39</td>
<td>0.41</td>
<td>CXCL10 plasma levels are associated with the presence of necro-inflammatory foci in the liver in mice fed an MCD diet, and were related to the progression of Alcoholic SteatoHepatitis.</td>
<td>(43, 44)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(2.9×10⁻⁴)</td>
<td></td>
<td></td>
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<tr>
<td>IGFI</td>
<td>Steatosis</td>
<td>-0.44</td>
<td>-0.45</td>
<td>Similar to insulin in function and structure. IGFI plasma levels are decreased in individuals with hepatic steatosis.</td>
<td>(45)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(3.6×10⁻⁵)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SERPINE1</td>
<td>Lobular inflammation</td>
<td>0.55</td>
<td>0.48</td>
<td>Inhibitor of fibrinolysis. SERPINE1 plasma levels are higher, and enzyme activity is increased in individuals with NAFLD and NASH, as compared to healthy controls.</td>
<td>(46-49)</td>
</tr>
<tr>
<td>(a.k.a PAI-1)</td>
<td></td>
<td></td>
<td>0.54</td>
<td></td>
<td>(50, 51)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(2.5×10⁻⁷)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(1.5×10⁻⁵)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(4.9×10⁻⁷)</td>
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</tr>
</tbody>
</table>

Continued on Next Page...
Table 2 – Continued

<table>
<thead>
<tr>
<th>Gene</th>
<th>Trait</th>
<th>Tissue Correlation (P-value)</th>
<th>Tissues corrected</th>
<th>BMI corrected</th>
<th>Role and relation to NAFLD in literature</th>
<th>References</th>
</tr>
</thead>
</table>

The table shows all adipokines that are correlated with liver histology (FDR < 0.05). For these adipokines also partial correlation coefficients adjusted for tissue type and BMI are shown; the significance threshold for these partial correlation coefficients is 0.28 (P = 0.01). * We are the first to provide evidence that ANGPT2 expression in VAT is related to NASH, in particular to lobular inflammation. ** In contrast to other studies investigating Leptin plasma levels, we provide evidence that LEP expression in VAT is correlated to lobular inflammation, and that this correlation remains significant after correction for BMI. *** We are the first to provide evidence that RARRES2 expression in VAT is related to NASH, in particular to lobular inflammation. **** IL1RN could act as a protective mechanism (assuming IL1A and IL1B induce damage). Upregulation of IL1RN could reflect the presence and action of a feedback system as a response to damage.
Discussion

In this study, we have shown that expression levels of \textit{ANGPT2}, \textit{RARRES2}, and \textit{LEP} in VAT, but not in SAT or liver, are correlated to NAFLD, in particular to lobular inflammation. These correlations strongly support the hypothesis that altered expression and release of \textit{ANGPT2}, \textit{RARRES2}, and \textit{LEP} in VAT, but not in SAT or liver, is a hallmark of NAFLD. On the other hand, we have also found that several proposed adipokines were predominantly expressed in the liver and that expression of \textit{IL1RN}, \textit{CXCL10}, \textit{IGF1}, and \textit{SERPINE1} (a.k.a. PAI-1) in the liver, but not in VAT or SAT, were correlated to features of NAFLD. Likely, these genes are related to NAFLD through a mechanism within the liver, instead of acting as a factor primarily released for (visceral) adipose tissue. Thus for some proposed adipokines we confirmed their role as an adipokine, whereas for other proposed adipokines we found evidence that they are related to NAFLD through mechanisms within the liver.

Compared to previous studies an important strength of our approach is that we investigated a direct link between adipose tissue physiology and NAFLD. Most previous studies investigated relations between NAFLD and systemic adipokine plasma levels (references are listed in Table 2). However, systemic adipokine plasma levels likely do not reflect the levels of adipokines that reach the liver through the portal vein, since adipokine levels in the circulation and the portal vein are different (7, 52). Thus our approach to investigate relations between mRNA levels in adipose tissue and NAFLD provides a better insight into the role of the adipose tissue in NAFLD. A further improvement of our approach could be to measure adipokine (protein) plasma levels in the portal vein. However, we did not acquire blood from the portal vein to perform such measurements.

Another strength of our approach was that we compared severely obese individuals with various degrees of NAFLD without including lean people. Thereby, we likely reduced the number of confounders that show different expression in adipose tissue in obese conditions, but that are not related to NAFLD. Furthermore we used both SAT and VAT, and we found that only VAT expression of adipokines was related to aspects of NAFLD, which is in line with the established relation between NAFLD and particularly visceral adiposity (8-10).

Our data provide no conclusive evidence that the adipokines correlated to lobular inflammation have a direct effect on the liver. In obesity, adipose tissue releases not only adipokines, but also excessive amounts of fatty acids, which greatly contribute to the development of hepatic steatosis (53). \textit{ANGPT2}, \textit{RARRES2}, and \textit{LEP} have
been reported to have an autocrine or paracrine function in adipose tissue (54-64), which might affect adipose tissue physiology in such a way that the flux of fatty acids from the adipose tissue to the liver increases, thereby increasing the chance to develop NASH. On the other hand, there is also evidence that ANGPT2, RARRES2, and LEP can have a direct role on liver pathophysiology by inducing inflammation (65-70). Thus, additional experiments are needed to confirm the exact role of ANGPT2, RARRES2, and LEP expression in VAT, in relation to the induction of lobular inflammation.

A striking difference between our results and results from previous studies is seen for RARRES2. Although we report a negative correlation between expression of RARRES2 in VAT and lobular inflammation, several earlier studiers reported a positive correlation between RARRES2 plasma levels and features of the metabolic syndrome, e.g. obesity and type 2 diabetes (references are listed in Table 2). Several explanations for this discrepancy can be given. First, our study included only severely obese individuals, whereas other studies often included lean individuals. Since RARRES2 is strongly correlated to BMI, inclusion of lean individuals could introduce bias. Second, as seen in our own data, hepatic expression of RARRES2 largely exceeds expression of RARRES2 in adipose tissue. Therefore, plasma levels of RARRES2 in the circulation might not reflect adipose tissue RARRES2 release, which is underlined by a modest correlation between RARRES2 mRNA expression in VAT and RARRES2 plasma levels (36). Third, RARRES2 protein is subjected to alternative forms of post-translational modification, which might lead to release of specific RARRES2 isoforms from adipose tissue (71, 72). Thus, the relation between RARRES2 and NAFLD seems to be very complex. Studies investigating effects of tissue-specific knock-outs in animal models should be undertaken to unravel the functions of this apparently important gene.

In contrast with the assumption that all 48 genes investigated in this study function as an adipokine, we found evidence that IL1RN, CXCL10, IGF1, and SERPINE1 are related to NAFLD through mechanisms within the liver. However, although these genes thus do not likely function as an adipokine, their hepatic expression levels reflect aspects of NAFLD. Therefore, plasma levels of these secreted molecules, if tightly linked to hepatic expression, could represent potential novel biomarkers for NASH. Indeed, plasma levels of these genes have been reported to be a biomarker for NASH (41-49). Thus our data support the view that plasma levels of IL1RN, CXCL10, IGF1, and SERPINE1 are biomarkers for NASH. In addition expression levels in VAT of ANGPT2, RARRES2, and LEP in VAT could represent
novel biomarkers for NASH. However, further research to validate these molecules as biomarkers for NASH is required.

In conclusion, our data revealed a role for expression of ANGPT2, RARRES2, and LEP from VAT in NAFLD, in particular with respect to lobular inflammation. Although proposed to be adipokines, the roles of IL1RN, CXCL10, IGF1, and SERPIN1 in NAFLD seems to be related to processes within the liver.
Crosstalk between adipose tissue and liver

Acknowledgements

This work was financially supported by grant 2006.00.007 of the Dutch Diabetes Foundation, IOP genomics grant IGE05012A, and a Transnational University Limburg grant. We are indebted to Yanti Slaats (NUTRIM School for Nutrition, Toxicology and Metabolism, University Medical Centre Maastricht, the Netherlands) for collecting tissue samples, to Ann Driessen (Department of Pathology, University Medical Centre Maastricht, the Netherlands) for assessing histological features of the liver, and to Pieter van der Vlies, Marcel Bruinenberg, and Bahram Sanjabi (Department of Genetics, University Medical Centre Groningen, The Netherlands) for technical expertise.
### Supplemental table 1: Clinical and plasma parameters of the study population.

<table>
<thead>
<tr>
<th></th>
<th>Mean (SD) / No. of individuals</th>
<th>Minimal / maximal values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td>26/67</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>44.2 (9.7)</td>
<td>18-67</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>46.1 (9.5)</td>
<td>30.7-73.6</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>6.45 (1.98)</td>
<td>4.3-14.5</td>
</tr>
<tr>
<td>HbA₁c (%)</td>
<td>6.54 (1.35)</td>
<td>5.1-12.1</td>
</tr>
<tr>
<td>Insulin (mU/l)</td>
<td>19 (10.6)</td>
<td>3.8-53</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.08 (1.12)</td>
<td>3-9.8</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>0.98 (0.37)</td>
<td>0.5-2.8</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.21 (1)</td>
<td>1.1-7.4</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>2.22 (1.98)</td>
<td>0.63-16.4</td>
</tr>
<tr>
<td>NEFA (nmol/l)</td>
<td>0.63 (0.3)</td>
<td>0.12-1.66</td>
</tr>
<tr>
<td>ALAT (U/l)</td>
<td>26.5 (16)</td>
<td>6-124</td>
</tr>
<tr>
<td>ASAT (U/l)</td>
<td>24.7 (12.4)</td>
<td>7-72</td>
</tr>
<tr>
<td>C-reactive protein (mg/l)</td>
<td>10.2 (8.1)</td>
<td>1-37</td>
</tr>
</tbody>
</table>

Data are means ± standard deviation (SD) and the minimal and maximal values for each trait. NEFA, non-esterified fatty acid; ALAT, alanine aminotransaminase; ASAT, aspartate aminotransaminase.
Crosstalk between adipose tissue and liver

Supplemental figure 1: The correlation of hepatic pathologies of NAFLD.
A). The correlation between hepatic pathologies of NAFLD and sex, age and BMI.
B). The inter-correlation among the hepatic pathologies of NAFLD. The correlation was computed with Spearman correlation. Each ellipse represents a level curve of the density of a bivariate normal with the matching correlation; thus a smaller ellipse represents a stronger correlation. Correlations significant at P<0.01 are indicated in blue.
Supplemental figure 2: PCA plots of gene expression in different tissue types.

We applied a principal component analysis (PCA) based on expression correlation matrix among three different tissue samples from all subjects. Each dot presents a tissue sample from a certain subject. The colours represent the tissue types: red for liver, blue for SAT and green for VAT. The PCA analysis clearly demonstrates a global difference in gene expression among tissue types.
Supplemental figure 3: Hierarchical clustering of the individuals based on global gene expression in liver, SAT and VAT.

A). The hierarchical cluster of individuals based on gene expression in liver. B). The hierarchical cluster based on gene expression in SAT. C). The cluster based on gene expression in VAT. The heatmap below the cluster represent the sex, age, BMI and seven hepatic pathologies of NAFLD. The color scheme range from white (low score) to red (high score).
References


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