Universiteit Antwerpen

THE ROLE OF HEPATOCELLULAR AUTOPHAGY IN NON-ALCOHOLIC FATTY LIVER DISEASE

DE ROL VAN HEPATOCELLULAIRE AUTOFAGIE IN NIET-ALCOHOLISCHE LEVERVERVETTING

Promotoren: prof dr. Sven M. Francque
prof. dr. Peter P. Michielsen

Begeleiders: prof. dr. Benedicte Y. De Winter
Ing. Joris G. De Man

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Wilhelmus J. Kwanten

CHAPTER 7

AUTOPHAGY, UPR AND NAFLD RELATED GENE EXPRESSION ANALYSIS IN AN OBESE PATIENT COHORT

Analysed within a larger multicentre study of gene expression in obese patients with NAFLD.

(confidential data, under embargo)

ABSTRACT

Background & aims

Whereas controversy arises on the role of hepatic autophagy on hepatic steatosis in animal studies, the knowledge of autophagy in patients with non-alcoholic fatty liver disease (NAFLD) remains limited. Moreover, autophagy is a highly dynamic process and paired follow-up data may significantly contribute to gaining insight in the alterations in autophagy linked to phenotypically changes. Therefore, autophagy was studied together with the unfolded protein response (UPR), an other cellular homeostatic mechanism, in a large cohort of obese patients.

Methods

Patients of the obesity clinic of a tertiary hospital underwent a metabolic and hepatic work-up, during which a liver biopsy was performed in case of suspected NAFLD. The genes related to autophagy and UPR, determined by gene-array, were investigated paired-wise in relationship with NAFLD-severity at baseline and after 1-year follow-up.

Results

The alterations in the genes related to autophagy and UPR were minor. However, distinct patterns of gene expressions, mostly related to metabolism, could be observed corresponding to the degree of steatosis and the presence of NASH. The expression of CREBP3L3 and IGFBP1 were most distinct. Patients with NASH at baseline showed 28 genes differentially expressed compared to baseline when NASH was resolved at follow-up, of which PAI-1 was the most strongly reduced.

Conclusions

The NAFLD-severity related with a specific signature of differentially expressed genes mostly related to metabolic pathways. Successful treatment of NASH affects many genes after 1-year follow-up. Differences in autophagy or UPR, two important cellular homeostatic processes, however, were only minor.

(This chapter contains confidential data, under embargo)

INTRODUCTION

In previous chapters we described the importance of autophagy on cellular homeostasis and its potential role within the pathophysiology of NAFLD. Its interaction with another important cellular housekeeping process, the UPR, was discussed as well. Both processes are implicated in NAFLD amongst other liver diseases [21,150]this signaling pathway can either result in the recovery of homeostasis or can activate a cascade of events that ultimately result in cell death. The UPR/endoplasmic reticulum (ER. Even though the effects of autophagy on the liver and the metabolism in the whole-body are acknowledged as substantial, its exact role on the lipid metabolism in the liver is debated [31,106]. Besides, given the fact that knowledge of autophagy is almost exclusively based on *in vitro* and animal experiments and that results cannot be simply translated between species and from animal models to patients [198], there is a clear need for data in patients with NAFLD. Currently, there is a paucity of studies that looked to autophagy in patients with NAFLD, mainly due to methodological restrictions, such as difficulties to detect autophagy or autophagic flux "*in vivo*" and the ethical considerations related to repetitive liver biopsies in patients [198]. Nevertheless, both pathology-based [39,73] and rt-qPCR-based studies [74,75]we aimed to assess the relationship between endoplasmic reticulum (ER suggest that autophagy is compromised in patients with NASH.

In the current chapter we present the results of a study on the expression pattern of genes related to NAFLD, autophagy and the UPR in liver biopsies of a large cohort of well-defined patients with NAFLD. We looked specifically to potential differences related to the stages of NAFLD (i.e. NAFL, NASH ± significant fibrosis). Moreover, to our knowledge this is the first study looking to alterations in follow-up biopsies in patients that underwent bariatric surgery or followed lifestyle intervention. Hence, alterations in the expressed genes or gene profiles could be described in function of the histological alterations within the NAFLD-spectrum.

MATERIAL AND METHODS

Patients and liver biopsy

The liver biopsies used in this study were part of a large obese study cohort, as

described previously [222]. Briefly, patients with overweight or obesity were prospectively recruited (study approved by the Ethical Committee of the Antwerp University Hospital file 6/25/125) and underwent a metabolic and liver-specific work-up. Patients were excluded in case of significant alcohol use or other (concurrent) liver disease. When, based on clinico-anamnestic data, biochemistry and/or ultrasound findings, NAFLD was suspected a liver biopsy was proposed since a biopsy is still the golden standard for the accurate diagnosis and staging of NAFLD. For those patients undergoing bariatric surgery a liver biopsy was proposed in all cases [223,224]. After additional written informed consent, the liver biopsy was performed percutaneously (16 G Menghini) or peri-operatively (14 G Tru-Cut) in case of bariatric surgery.

After 1 year of treatment (which included a multidisciplinary weight management program with emphasis on lifestyle adaptations and without prescription of any specific anti-obesity drug, or, in patients who met the reimbursement criteria, bariatric surgery) patients had a full reassessment, including a repeat biopsy in those who consented.

All biopsies were routinely stained and scored by two experienced pathologists blinded to any clinical data. The different histological features of NAFLD were assessed using the NASH Clinical Research Network (NASH CRN) Scoring System. NASH was defined, according to the current guidelines, as having a combination of steatosis, inflammation and ballooning all present at some degree [5,225]. If steatosis was present but the criteria for NASH were not met, the patient was classified as NAFL. Patients were subsequently divided in 4 groups: obese without NAFLD and without fibrosis, NAFL without fibrosis, NASH with no to low degree of fibrosis (F≤2) and NASH with advanced fibrosis (≥F3) (Table 7.1). Patients were also compared according to no (grade 0) versus high degree (grade 2-3) of steatosis, and according to the absence versus the presence of NASH (Table 7.1). Those who could not be classified within the given criteria of a certain variable were discarded for that analysis. When a follow-up biopsy was available and patients had NASH at baseline, the resolution of NASH was defined as the disappearance of ballooning (score 0), together with the disappearance or persistence of only mild lobular inflammation (score 0 or 1). Meanwhile the fibrosis score had to be equal or less compared to baseline [226] (Table 7.1).

Variable	Groups	Criteria
NAFLD stage	No NAFLD NAFL NASH NASH + advanced fibrosis	S=0, B=0, I=0, F=0 S≥1, B OR I =0, F ≤2 S≥1 AND B≥1 AND I≥1, F ≤2 S≥1 AND B≥1 AND I≥1, F ≥3
Steatosis	Steatosis No steatosis	S≥2, F≤2 S=0, F≤2
NASH	NASH No NASH	S≥1 AND B≥1 AND I≥1, F=any S=0 OR B=0 OR I=0, F≤2
Response	Response Non-response	Previously NASH + S=any AND B=0 AND I≤1, F=not increased compared to baseline Not fulfilling the criteria of Response

Table 7.1 Patient classification *Patients were analysed according to different variables, as described in material and methods. This table summarises the different criteria used for patient classification. Individual features of the histological classification was done according the NASH CRN scoring system, the definition of NAFL, NASH was according to the present guidelines [5,225] and response to therapy if (NASH at baseline) was according to the recently proposed definition [226]. NAFLD, non-alcoholic fatty liver disease; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; S, steatosis; B, ballooning; I, inflammation; F, fibrosis.*

RNA extraction and RNA arrays

The RNA extraction and RNA array of the liver biopsies were performed as previously described [222]. In brief, RNA was extracted by guanidinium thiocyanate/phenol/chloroform extractions. RNA was purified and hybridised to Affymetrix HuGeneST2.0 arrays after cDNA labelling. Normalisation was performed using the RMA algorithm. Genes studied with a relation to autophagy and the UPR were based on commercial available pathway-focussed PCR arrays, as well as a set of (predominantly metabolic) genes related to NAFLD (full list in table S1). Those genes that were significantly upregulated or repressed by more than 1.2-fold were subsequently classified via functional enrichment analysis using the Metascape gene annotation and analysis resource (http://www.metascape.org *[accession date 25th of July 2016]*) [227]. Categorisation was according to the Kyoto Encyclopedia of Genes and Genomes (KEGG); a minimum of 3 genes needed to correspond to a given pathway.

Statistical analyses and data presentation

All data were analysed by SPSS (version 22, IBM SPSS Statistics software, Armonk (NY), USA). Independent Student's-t-test or one-way ANOVA was used to compare baseline values, with post-hoc Dunnett's comparison (compared to obese non-steatotic patients) when appropriate. In case of non-parametric data, limited to patient's characteristics, Kruskal-Wallis test was used. The follow-up data were compared with a paired Student's-t-test, or in case of non-parametric data with a Wilcoxon signed rank test. Categorical data (i.e. histological features) were compared with a Fisher exact test, or a Wilcoxon signed rank test comparing follow-up with baseline scores. Two-tailed probabilities were calculated; p-values <0.05 were considered statistically significant.

RESULTS

Main characteristics at baseline

In total 231 gene arrays met the quality criteria (e.g. proper hybridisation) and were considered for analysis. Of these 152 were baseline samples while 79 were samples from the follow-up biopsies. Histological classification within the given criteria for the NAFLD stages (Table 7.1) was not possible in 37 samples (e.g. isolated inflammation without ballooning or solely low degree of fibrosis), making 194/231 datasets eligible for further analysis.

The baseline patient-cohort existed out of 138 obese patients, of which the characteristics are presented in Table 7.2. The majority of patients was diagnosed with NASH, of which only a minority was faced with advanced fibrosis (8.6% of NASH, 6.5% of total). Patients were significantly different in relation to the NAFLD-severity for their BMI, waist, waist-hip ratio, total and visceral fat mass and for blood levels of transaminases (AST, ALT), HDL-cholesterol, fasting glucose, fasting C-peptide and the calculated HOMA-IR.

Parameter	No NAFLD n= 16	NAFL n= 18	NASH F1-F2 n= 95	NASH F3-F4 n= 9	p
Sex (Male/ Female)	2/14	2/16	42/53	4/5	
Age (y)	39 [30–45]	43 [37–51]	47 [35–54]	37 [28–58]	
BMI (kg/m^2)	36.80 [33.38–40.93]	39.85 [38.40–47.25]	39.10 [35.60–42.10]	43.70 [40.20–44.90]	<0.01
Waist circumference (cm)	112.50 [102.13–118.75]	118.50 [111.13–124.13]	120.75 [114.00–129.00]	128.00 [124.75–133.75]	<0.001
Waist-hip ratio	0.92 [0.85–1.00]	0.94 [0.89–1.02]	1.00 [0.92–1.06]	1.02 [0.98–1.12]	<0.001
Fat percentage (%)	51.80 [45.85–55.48]	51.65 [48.65–55.50]	48.10 [41.50–52.90]	49.10 [46.55–53.20]	NS
Total AT (cm^2)	796.5 [553.0-873.0]	888.5 [748.8-1013.0]	822.5 [733.3-954.3]	926.0 [880.0-971.5]	<0.01
Visceral AT (cm^2)	130.0 [96.8-165.5]	199.5 [146.3-260.3]	207.0 [156.0-263.5]	229.0 [192.5-340.5]	<0.001
Subcut. AT (cm^2)	643.5 [445.0-709.5]	681.0 [581.5-799.5]	626.0 [509.8-701.3]	655.0 [622.0-795.5]	NS
CRP (mg/dL)	0.83 [0.20–1.25]	0.59 [0.33–0.95]	0.42 [0.28–0.88]	0.38 [0.27–0.90]	NS
AST (U/L)	20.00 [17.25–24.75]	22.00 [17.25–25.00]	26.00 [20.00–36.25]	27.00 [17.00–76.50]	0.015
ALT (U/L)	26.00 [21.50–35.50]	25.50 [22.50–35.50]	41.00 [31.00–57.00]	50.00 [32.00–89.50]	<0.001
ALP (U/L)	71.00 [54.00–94.75]	78.00 [69.25–100.50]	77.00 [64.00–89.50]	84.00 [64.50–115.50]	NS
GGT (U/L)	29.50 [23.75–56.25]	31.50 [26.75–39.75]	37.50 [28.75–52.25]	36.00 [31.50–61.50]	NS
Bilirubin total (mg/dL)	0.40 [0.31–0.52]	0.50 [0.36–0.62]	0.50 [0.41–0.70]	0.44 [0.33–0.80]	NS
Total cholesterol (mg/dL)	197.00 [161.50–225.75]	207.00 [166.50–228.50]	203.00 [177.00–220.00]	220.00 [161.00–265.00]	NS
HDL cholesterol (mg/dL)	51.00 [40.25–65.50]	53.50 [41.00–59.25]	42.00 [38.00–51.00]	39.00 [33.50–56.50]	0.020
LDL cholesterol (mg/dL)	110.80 [77.88–132.75]	111.10 [95.35–147.50]	119.00 [103.00–146.00]	124.60 [98.00–175.50]	NS
Triglycerides (mg/dL)	138.00 [95.50–219.25]	133.00 [107.75–160.75]	148.00 [119.00–186.00]	156.00 [102.50–260.00]	NS

Fasting glucose (mg/dL)	80.50 [75.25–85.50]	78.50 [74.75–86.00]	84.00 [79.00–92.00]	88.00 [85.50–108.50]	<0.01
Fasting insulin (µU/mL)	12.20 [8.70–16.60]	15.55 [11.50–18.88]	16.80 [11.35–23.23]	18.80 [15.80–27.60]	NS
Fasting C-peptide (nmol/L)	0.95 [0.72–1.13]	1.03 [0.93–1.21]	1.12 [0.95–1.43]	1.33 [1.14–1.62]	0.031
HOMA-IR	2.53 [1.61–3.60]	3.21 [2.26–4.01]	3.59 [2.32–5.21]	4.35 [3.28–5.83]	0.046

Table 7.2 Baseline patient characteristics *Variables are presented as median and the inter-quartile range [25%-75%]. Groups were compared with one-way ANOVA or Kruskal Wallis when appropriate. HOMA-IR was calculated as [fasting-insulin (mU/L) x fasting glucose (mmol/L)]/22.5 ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AT, adipose tissue; BMI, body-mass index, CRP, C-reactive protein; GGT, γ-glutamyltransferase; HDL, high-density lipoprotein; HOMA-IR, homeostasis model of assessment insulin resistance; LDL, low-density lipoprotein; NS, not significant; subcut., subcutaneous*

NAFLD-severity results in differently expressed gene patterns

Of the studied genes, in total 17 genes were significantly altered compared to obese controls without NAFLD. Patients with NAFL, i.e. only steatosis, had 2 genes differently expressed (Table 7.3). Both the CREB3L3 gene, a gene with properties comparable to ATF6 [148]a homeostatic signalling network that orchestrates the recovery of ER function, and failure to adapt to ER stress results in apoptosis. Progress in the field has provided insight into the regulatory mechanisms and signalling crosstalk of the three branches of the UPR, which are initiated by the stress sensors protein kinase RNA-like ER kinase (PERK, and IGFBP1, of which the corresponding protein is a carrier for IGF1 and which tunes the IGF activities [228], were significantly downregulated. Interestingly, these genes were altered as well in patients with NASH. The expression of CREB3L3 decreased with an increasing NAFLD-severity. The IGFBP1 expression was 1.85 times lower in NASH F3-F4 as well, but did not reach significance within this group (p=0.45).

When patients with NASH F1-F2 were compared to obese controls, 12 genes were differently expressed (Table 7.3). Except for FASN, a gene involved in de novo lipogenesis [206], all genes had lower expression levels compared to obese controls. Functional enrichment analyses of these genes revealed that the AMPK-signalling pathway was most linked to these altered expressed genes (Table 7.4). The AMPK system senses the energy status of the cell and surprises energy-consuming pathways, and activates meanwhile ATP-producing pathways, such as glycolysis and fatty acid oxidation [229]. The other pathways that were enriched, could mainly be linked to glucose handling.

Of the 12 genes that were significantly different in NASH F1-F2, 3 genes remained significantly different in patients with NASH F3-F4 (Table 7.3). The relative alterations of these 3 genes were all larger than the relative changes in the NASH F1-F2 group. Furthermore, 5 other genes were significantly different within these patients compared to the obese controls. The pathways most linked to the observed alterations in advanced NAFLD were all linked to fatty acid and glucose related pathways (Table 7.4)

Gene	NAFL (n=18)	NASH F1/F2 (n=95)	NASH F3/F4 (n=9)
ACACA	NC	NC	↗ 1.24 *
ACSL5	NC	↘ 1.30 ***	↘ 1.48 ***
CREB3L3	↘ 1.33 *	↘ 1.44 ***	↘ 1.70 ***
ERN1	NC	↘ 1.44 **	NC
FASN	NC	↗ 1.31 *	↗ 2.03 ***
FOXO1	NC	↘ 1.31 *	NC
G6PC	NC	↘ 1.34 *	NC
GK	NC	NC	↘ 1.41 **
HPRT1	NC	NC	↘ 1.22 *
IGFBP1	↘ 1.84 *	↘ 2.40 ***	NC
INSIG2	NC	↘ 1.37 *	NC
LDLR	NC	↘ 1.43 *	NC
LEPR	NC	↘ 1.23 *	NC
LEPROT	NC	↘ 1.23 *	NC
PKLR	NC	NC	↗ 1.25 *
PPARA	NC	NC	↘ 1.26 *
PPARGC1A	NC	↘ 1.29 *	NC

Table 7.3 Significant differences in gene expression compared to obese non-steatotic controls *Fold changes of all genes that were significantly upregulated or repressed (>1.2 fold) compared to obese non-steatotic controls are listed. Upregulation is marked with upwards pointing arrow and in blue, downregulation with a downward arrow and in orange. One-way ANOVA, post-hoc Dunnett correction.*
NC, no significant change.

KEGG Pathway	p-value	Count	Gene list
NASH F1-F2 compared to No NAFLD			
AMPK signalling pathway	1.01E-06	5	FASN, FOXO1, G6PC, LEPR, CREB3L3
Adipocytokine signalling pathway	1.06E-03	3	G6PC, LEPR, ACSL5
Glucagon signalling pathway	2.02E-03	3	FOXO1, G6PC, CREB3L3
Insulin resistance	2.02E-03	3	FOXO1, G6PC, CREB3L3
Insulin signalling pathway	3.34E-03	3	FASN, FOXO1, G6PC
NASH F3-F4 compared to No NAFLD			
Fatty acid biosynthesis	3.84E-06	3	ACACA, FASN, ACSL5
Fatty acid metabolism	1.15E-04	3	ACACA, FASN, ACSL5
PPAR signalling pathway	2.32E-04	3	GK, PPARA, ACSL5
Glucagon signalling pathway	5.50E-04	3	ACACA, PPARA, CREB3L3
AMPK signalling pathway	7.96E-04	3	ACACA, FASN, CREB3L3
Insulin signalling pathway	9.58E-04	3	ACACA, FASN, PKLR
Steatosis compared to No steatosis			
AMPK signalling pathway	1.26E-09	7	CPT1A, FASN, FOXO1, G6PC, LEPR, PPARGC1A, CREB3L3
Adipocytokine signalling pathway	3.79E-07	5	CPT1A, G6PC, LEPR, PPARGC1A, ACSL5
Glucagon signalling pathway	1.63E-06	5	CPT1A, FOXO1, G6PC, PPARGC1A, CREB3L3
Insulin resistance	1.80E-06	5	CPT1A, FOXO1, G6PC, PPARGC1A, CREB3L3
Insulin signalling pathway	2.97E-04	4	FASN, FOXO1, G6PC, PPARGC1A
Fatty acid metabolism	4.60E-04	3	CPT1A, FASN, ACSL5
Longevity regulating pathway	2.99E-03	3	FOXO1, PPARGC1A, CREB3L3
NASH compared to No NASH			
AMPK signalling pathway	3.59E-08	6	CPT1A, FASN, FOXO1, G6PC, PPARGC1A, CREB3L3
Glucagon signalling pathway	7.85E-07	5	CPT1A, FOXO1, G6PC, PPARGC1A, CREB3L3
Insulin resistance	7.85E-07	5	CPT1A, FOXO1, G6PC, PPARGC1A, CREB3L3
Adipocytokine signalling pathway	1.00E-05	4	CPT1A, G6PC, PPARGC1A, ACSL5
Insulin signalling pathway	1.27E-04	4	FASN, FOXO1, G6PC, PPARGC1A
Fatty acid metabolism	2.47E-04	3	CPT1A, FASN, ACSL5
Longevity regulating pathway	1.62E-03	3	FOXO1, PPARGC1A, CREB3L3

Table 7.4 Pathways of significantly differentially expressed genes *Functional enrichment analysis of genes differentially expressed according to the given comparison. The respective KEGG-pathway and corresponding genes are displayed. p-values show Benjamini-Hochberg adjusted p-values of the enrichment analysis.*

Steatosis or NASH mostly affect metabolic pathways

Because the major debate of autophagy within the pathophysiology of NAFLD is concentrated around the issue whether it contributes to lipid accumulation or not [31], a subanalysis was performed between patients with no steatosis (n=16) and those with a high degree of steatosis (score ≥2, n=66), leaving patients with low grade of steatosis out of the analysis. Patients with advanced fibrosis were excluded to prevent potential confounding (Table 7.1), as the grade of steatosis is usually low in NAFLD with advanced fibrosis [7]

Patients with steatosis had 3 genes with a relatively higher expression compared to patients without steatosis (Table 7.5), whilst 14 genes were relatively lower expressed. The pathways affected by steatosis are shown in Table 7.4. The AMPK signalling pathways was most affected, followed by the adipocytokine signalling pathway and insulin related pathways.

Since steatosis *per se* is considered relatively innocent, whilst NASH is associated with increased morbidities and mortality, a comparison between patients with and without NASH was performed as well (see Table 7.1 for criteria). Patients with NASH (n=104) had in total 14 genes differently expressed compared to those without NASH (n=34) (Table 7.6). Again, FASN was upregulated and as in the previous functional analyses the AMPK signalling pathway was primary involved in this comparison (Table 7.4). The other pathways involved were similar to those found in comparison based on steatosis, though changed position in significance.

Gene	Fold Change
ACSL5	↘ 1.34 **
ACTB	↗ 1.23 **
CPT1A	↘ 1.22 *
CREB3L3	↘ 1.43 *
CXCR4	↘ 1.32 *
ERN1	↘ 1.52 **
FASN	↗ 1.29 *
FOXO1	↘ 1.38 **
G6PC	↘ 1.32 *
GANC	↘ 1.22 *
IGFBP1	↘ 2.35 ***
INHBE	↗ 1.20 *
INSIG2	↘ 1.42 *
LDLR	↘ 1.45 *
LEPR	↘ 1.25 **
LEPROT	↘ 1.25 **
PPARGC1A	↘ 1.31 **

Table 7.5 (left) Significant differences in gene expression comparing steatotic with non-steatotic patients *All genes that were significant upregulated or repressed (>1.2 fold) compared to non-steatotic patients are listed. Upregulation is marked with upwards pointing arrow and in blue, downregulation with a downward arrow and in orange. Independent Student's-t-test.*

Gene	Fold Change
ACSL5	↘ 1.23 ***
CAPN3	↘ 1.22 *
CPT1A	↘ 1.22 *
CREB3L3	↘ 1.27 *
ERN1	↘ 1.36 **
FASN	↗ 1.27 *
FOXO1	↘ 1.29 *
G6PC	↘ 1.25 **
GANC	↘ 1.23 *
IGFBP1	↘ 1.77 **
INSIG2	↘ 1.25 *
LDLR	↘ 1.28 *
PDK4	↘ 1.23 **
PPARGC1A	↘ 1.20 *

Table 7.6 (right) Significant differences in gene expression comparing NASH with non-NASH patients *All genes that were significantly upregulated or repressed (>1.2 fold) compared to non-NASH patients are listed. Upregulation is marked in blue, downregulation in orange. Independent Student's-t-test.*

The resolution of NASH strongly affects several pathways

Thirty-six out of 56 follow-up samples could be linked to a baseline sample, of which 28 patients had NASH at baseline. These 28 patients were further analysed depending on the evolution of NASH in time (whether NASH resolved or not). One patient of these 28 patients had advanced fibrosis (F3) at baseline, though evolved to NAFL at the follow-up. Patient characteristics are presented in table 7.7 and 7.8. Even though both non-responders and responders managed to improve their BMI and had an overall improvement of metabolic parameters, the effects in the non-responders were substantially less than those in the responders, underlining the impact of sufficient weight loss on NAFLD and metabolic parameters. It should be noted also that the proportion of patients that underwent bariatric surgery amongst the responders was higher and that the baseline histology amongst responders was proportionally less severe, especially for the factor ballooning.

	Non-responder (n=17)			**Responder (n=11)**		
Parameter	**Baseline**	**Follow-up**	**p**	**Baseline**	**Follow-up**	**p**
Bariatric surgery (n/N)	1/17			8/11		
Sex (Male/ Female)	6/11			5/6		
Age (y)	42 [27–58]			49.00 [43.00–60.00]		NS
BMI (kg/m^2)	35.30 [33.55–43.30]	34.70 [31.40–39.10]	<0.001	37.80 [32.80–41.00]	27.75 [25.75–30.58]	<0.001
Waist circumference (cm)	117.00 [110.50–123.50]	108.00 [104.50–115.50]	<0.01	119.50 [114.00–123.50]	100.00 [90.00–105.00]	<0.001
Waist-hip ratio	0.95 [0.91–1.05]	0.95 [0.89–1.02]		1.02 [1.00–1.06]	0.94 [0.88–1.01]	<0.001
Fat percentage (%)	49.20 [42.30–53.15]	43.30 [38.15–49.85]	<0.001	47.20 [38.20–52.10]	28.85 [19.78–43.83]	<0.001
Total AT (cm^2)	740.0 [653.5–918.5]	669.0 [602.5–780.0]	<0.01	824.0 [744.0–903.0]	463.0 [310.0–568.0]	<0.001
Visceral AT (cm^2)	189.0 [163.5–235.0]	161.0 [118.5–213.0]	<0.001	263.0 [208.0–312.0]	97.0 [73.0–150.0]	<0.001
Subcut. AT (cm^2)	535.0 [448.0–671.5]	545.0 [410.0–590.5]	0.011	572.0 [511.0–635.0]	318.0 [249.0–422.0]	<0.001
CRP (mg/dL)	0.52 [0.20–1.00]	0.26 [0.11–0.43]	<0.01	0.43 [0.15–1.13]	0.10 [0.03–0.39]	0.013
AST (U/L)	36.50 [23.75–49.00]	28.00 [22.00–32.00]	<0.01	28.00 [27.00–43.00]	24.00 [14.00–28.00]	NS
ALT (U/L)	43.00 [26.00–70.50]	30.00 [20.50–47.00]	0.014	41.00 [34.00–71.00]	31.00 [18.00–39.00]	0.045
ALP (U/L)	70.00 [52.00–88.00]	77.00 [44.00–88.00]	NS	85.00 [73.00–102.00]	84.00 [61.00–109.00]	NS
GGT (U/L)	28.50 [22.00–47.50]	27.00 [20.00–32.00]	0.029	42.00 [29.00–47.00]	30.00 [21.00–42.00]	0.026
Bilirubin total (mg/dL)	0.60 [0.50–0.65]	0.49 [0.39–0.59]	NS	0.50 [0.32–0.70]	0.60 [0.40–0.98]	<0.01
Total cholesterol (mg/dL)	206.00 [185.50–249.00]	207.00 [162.75–239.00]	NS	204.00 [177.00–220.00]	187.00 [164.25–211.50]	NS
HDL cholesterol (mg/dL)	43.00 [36.50–53.50]	43.50 [34.25–65.00]	NS	46.00 [38.00–65.00]	63.50 [46.75–69.75]	<0.01
LDL cholesterol (mg/dL)	134.00 [99.50–161.50]	125.60 [87.00–154.60]	NS	120.20 [103.00–138.60]	102.90 [80.70–131.00]	NS
Triglycerides (mg/dL)	167.00 [119.50–204.00]	132.00 [98.00–170.50]	NS	156.00 [130.00–244.00]	93.00 [78.75–138.75]	<0.01
Fasting glucose (mg/dL)	81.00 [75.00–86.00]	85.00 [80.50–93.00]	0.039	82.00 [78.00–90.00]	81.50 [73.50–90.00]	NS
Fasting insulin (µU/mL)	14.20 [8.65–20.15]	15.70 [12.80–21.25]	NS	15.45 [9.80–16.70]	5.55 [3.40–8.98]	<0.01
Fasting C-peptide (nmol/L)	1.02 [0.79–1.29]	0.96 [0.85–1.17]	NS	1.12 [0.86–1.18]	0.57 [0.51–0.79]	<0.001
HOMA-IR	2.84 [1.67–4.27]	3.15 [2.56–4.73]	NS	2.99 [1.95–3.92]	1.26 [0.62–1.88]	<0.01

Table 7.7 Patient characteristics at baseline and follow-up in patients with baseline NASH

Variables are presented as median and the interquartile range [25%-75%]. Groups were compared with a paired Student's-t-test, or with a Wilcoxon signed rank test. HOMA-IR was calculated as [fasting-insulin (mU/L) x fasting glucose (mmol/L)]/22.5

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AT, adipose tissue; BMI, body-mass index, CRP, C-reactive protein; GGT, γ-glutamyltransferase; HDL, high-density lipoprotein; HOMA-IR, homeostasis model of assessment insulin resistance; LDL, low-density lipoprotein; NS, not significant; subcut., subcutaneous

	Non-responder (n=17)			**Responder (n=11)**				
Parameter	**Baseline**	**Follow-up**	**p**	**Baseline**	**Follow-up**	**p**	**p2**	**p3**
Steatosis			*NS*				0.046	<0.001
0	0	0		0	9			
1	6	6		5	2			
2	3	10		2	0			
3	8	1		4	0			
Ballooning			*NS*				<0.001	<0.001
0	0	0		0	11			
1	6	11		8	0			
2	11	6		3	0			
			NS				<0.001	<0.001
0	0	0		0	11			
1	11	9		5	0			
2	3	8		4	0			
3	3	0		2	0			
Fibrosis			*NS*				*NS*	*NS*
0	10	12		4	10			
1	4	2		3	1			
2	3	3		3	0			
3	0	0		1	0			
4	0	0		0	0			

Table 7.8 Histological characteristics at baseline and follow-up in patients with baseline NASH *The numbers of patients are given for each histological parameter. p denotes the significance of the difference between follow-up and baseline scores (Wilcoxon signed rank test). p2 and p3 denote the significance of the difference between responders and non-responders for their baseline and follow-up scores respectively (Fisher exact).*
NS, not significant

Gene	Non-responder (n = 17/28)	Responder (n = 11/28)
ACLY	NC	↘ 1.27 **
ATF6	NC	↘ 1.22 **
ATG4A	NC	↘ 1.22
CD36	NC	↘ 1.32 ***
CEBPB	NC	↘ 1.25 *
CPT1A	NC	↘ 1.50 ***
CTSS	NC	↘ 1.49 **
CXCR4	NC	↘ 1.81 **
ERN1	NC	↘ 1.48 **
FASN	↗ 1.85 **	NC
FGF21	NC	↘ 1.64 **
FOXO1	NC	↘ 1.53 **
GABARAPL1	NC	↘ 1.66 **
GCK	↘ 1.25 *	NC
HPRT1	NC	↗ 1.21
HSPA4L	NC	↘ 1.26 *
IGF1	NC	↗ 1.66 **
IGFBP1	↗ 1.54 *	↘ 3.59 *
LDLR	NC	↘ 2.32 **
PDK4	NC	↘ 1.24
PPA1	NC	↘ 1.24 ***
PPARA	NC	↗ 1.40 **
PPARGC1A	NC	↘ 1.61 **
RB1	NC	↘ 1.23 **
SCD	↗ 1.37 *	NC
SERPINE1	NC	↘ 6.52 ***
SOCS3	NC	↘ 1.24 **
STAT3	NC	↘ 1.26 ***
TGFB1	NC	↘ 1.22 **
USP14	NC	↘ 1.21 **
WIPI1	NC	↘ 1.29 **

Table 7.9 Gene expression between baseline and follow-up of patients with NASH at baseline, according to response to treatment *All genes that were significant upregulated or repressed (>1.2 fold) compared to baseline are listed for both responders and non-responders to treatment. Upregulation is marked in blue, downregulation in orange. Dependent Student's-t-test. NC, no significant change.*

KEGG Pathway	p-value	Count	Gene list
Insulin resistance	6.77E-08	7	CD36, CPT1A, FOXO1, PPARA, STAT3, SOCS3, PPARGC1A
AMPK signalling pathway	7.96E-08	7	CD36, CPT1A, FASN, FOXO1, IGF1, SCD, PPARGC1A
Adipocytokine signalling pathway	8.83E-08	6	CD36, CPT1A, PPARA, STAT3, SOCS3, PPARGC1A
Glucagon signalling pathway	3.19E-05	5	CPT1A, FOXO1, GCK, PPARA, PPARGC1A
Pathways in cancer	8.35E-05	7	FOXO1, IGF1, RB1, STAT3, TGFB1, CXCR4, FGF21
FoxO signalling pathway	8.35E-05	5	FOXO1, IGF1, STAT3, TGFB1, GABARAPL1
Insulin signalling pathway	8.90E-05	5	FASN, FOXO1, GCK, SOCS3, PPARGC1A
PPAR signalling pathway	1.43E-04	4	CD36, CPT1A, PPARA, SCD
AGE-RAGE signalling pathway in diabetic complications	5.80E-04	4	FOXO1, SERPINE1, STAT3, TGFB1
Hepatitis C	1.54E-03	4	LDLR, PPARA, STAT3, SOCS3
Fatty acid metabolism	1.62E-03	3	CPT1A, FASN, SCD
Non-alcoholic fatty liver disease (NAFLD)	2.11E-03	4	ERN1, PPARA, TGFB1, SOCS3
Pancreatic cancer	3.57E-03	3	RB1, STAT3, TGFB1
Prolactin signalling pathway	4.01E-03	3	GCK, STAT3, SOCS3
Melanoma	4.01E-03	3	IGF1, RB1, FGF21
Prostate cancer	7.04E-03	3	FOXO1, IGF1, RB1
Longevity regulating pathway	7.78E-03	3	FOXO1, IGF1, PPARGC1A
HIF-1 signalling pathway	9.61E-03	3	IGF1, SERPINE1, STAT3
Toxoplasmosis	1.35E-02	3	LDLR, STAT3, TGFB1
Hepatitis B	2.38E-02	3	RB1, STAT3, TGFB1
Protein processing in endoplasmic reticulum	3.39E-02	3	ERN1, HSPA4L, ATF6
Transcriptional misregulation in cancer	3.71E-02	3	CEBPB, FOXO1, IGF1
Tuberculosis	3.71E-02	3	CEBPB, CTSS, TGFB1

Table 7.10 Pathways of significantly differentially expressed genes *Functional enrichment analysis of genes differentially expressed in patients with resolution of NASH compared to those with persisting NASH. The respective KEGG-pathway and corresponding genes are displayed. p-values show Benjamini-Hochberg adjusted p-values of the enrichment analysis.*

Amongst the non-responders 4 genes were differently expressed between the baseline and the follow-up, of which 3 demonstrated a higher expression level (Table 7.9). Pathway analysis could not link these 4 genes to a specific pathway, but both FASN and SCD contribute to fatty-acid biosynthesis [206]. The gene GCK is involved in the primary step of glycolysis [206], and was expressed at lower levels compared to baseline.

The expression of genes was largely affected when patients were able to clear their NASH, affecting 28 genes (Table 7.9). The corresponding affected pathways are presented in Table 7.10, showing that the genes linked to insulin resistance were most involved besides a mix of different pathways that are mainly related to fatty acid handling, glucose handling and tumourigenesis. Analysis on the genes that were more than 1.5 fold up- or downregulated did not essentially change the involved pathways (data not shown). Intriguingly, IGFBP1 showed opposite effects in non-responders and responders, with strong downregulation when NASH resolved. The expression of the LDL-receptor was also strongly downregulated compared to baseline in responders, in line with the improved lipid profile. However, the most attenuated gene was SERPINE1 (also known as PAI-1), an inhibitor of fibrinolysis [13]but also has systemic consequences. More specifically, evidence points out that NAFLD has to be considered as a significant independent risk factor for subclinical and clinical cardiovascular disease (CVD, that was 6.5 times lower at follow-up compared to baseline.

DISCUSSION

The main goal of this study was to investigate whether we could get more insight in the role of several pathways, in particular those related to autophagy and the UPR, in the pathophysiology of NAFLD by studying differentially expressed genes in relationship with the NAFLD-severity via a gene-array analysis in a large patient cohort. The most differences in gene expression compared to obese patients that did not have NAFLD could be detected in patients with NASH F1-F2 (Table 7.4).

Of interest are the alterations of CREB3L3 and insulin growth factor binding protein 1 (IGFBP1) in NAFLD. The CREB3L3 gene, also known as CREBH, is a transcription factor that has ATF6-like properties [148]a homeostatic signalling network that orchestrates the recovery of ER function, and failure to adapt to ER stress results in apoptosis. Progress in the field has provided insight into the regulatory mechanisms and signalling crosstalk of the three branches of the UPR, which are initiated by the stress sensors protein kinase RNA-like ER kinase (PERK. CREBP3L3 promotes gluconeogenetic genes and promotes plasma clearance of TG [206]. The loss of CREBP3L3 is known to cause steatosis and hypertriglyceridemia [230], which is in concordance with the observed correlation between NAFLD-severity and relative decrease in CREBP3L3 (Table 7.3). Resolution of NASH at follow-up, however, did not reveal significant increase of this gene. IGFBPs are carrier proteins for the hormone IGF, prolonging their half-life, and are able to determine the tissue-specific responsiveness to the IGF signals in a context-dependent way [228]. IGFBP1 is predominantly expressed in the liver [228,231], its serum levels are inversely correlated with the degree of steatosis [231] and the circulating levels decrease in response to insulin and even more in case of hepatic insulin resistance [232]. In line with this inverse relationship, IGFBP1 levels decreased in NAFL and NASH F1-F2 (Table 7.3). Patients with NASH were indeed shown to have lower gene expression levels of IGFBP1 [233,234], also in line with our data (Table 7.6). Steatosis alone suppresses IGFBP1-expression even more than NASH [234], though this phenomenon was not detected in our data (Table 7.3). The opposite findings in non-responders (upregulated) and responders (strongly downregulated) are contrasting the aforementioned relationship between steatosis and IGFBP1, though we lack a clear explanation for this observation. Most likely other regulatory mechanisms are involved, since IGBP's act context-dependent.

In patients that were successfully treated in the obesity clinic and showed resolution of NASH, also the alterations of the LDL receptor (LDLR) and serpin family E member 1 (SERPIN1) caught the eye. The downregulation of LDLR, the receptor for the LDL lipoprotein, seems contra-intuitive concerning the lower expression levels in NASH F1-F2 compared to controls or in presence of steatosis as well (Table 7.3 and 7.5). However, the lower levels can be explained by increasing intracellular lipid levels that negatively regulate the LDLR [236]through nonalcoholic steatohepatitis (NASH, whereas an explanation for the lower levels after resolution of NASH can be found in increased insulin sensitivity and improved LDL-receptor binding capacities after significant weight loss (>10%) [237]. SERPIN1, better known as plasminogen activator inhibitor 1 (PAI-1), is an important inhibitor of the fibrinolysis and hence contributes to the advanced thromboembolic risk in patients with NAFLD [13]but also has systemic consequences. More specifically, evidence points out that NAFLD has to be considered as a significant independent risk factor for subclinical and clinical cardiovascular disease (CVD. The plasma levels and expression of PAI-1 are positively related to NASH [238,239]. The 6.5-fold reduction in the expression of this gene upon a treatment response is promising as it might support the hypothesis that the increased cardiovascular morbidity in NASH can be reversible when returning to normal liver or steatosis alone [13]

In contrast to previous reports on enrichment of autophagy and URP related genes [75] and the aforementioned pathology-based studies, we were not able to detect many alterations in genes related to autophagy, with the exception of ATG4A and GABARAPL1 in case of NASH-resolution (Table 7.8). ATG4 and GABARAPL1 proteins are both involved in the formation of autophagosomes [27]. The absence of involvement of other autophagy-related genes impedes further conclusions on the involvement of autophagy within the studied patient population. Moreover, the measurement of autophagy via PCR alone can be representative, though ideally has to be complemented with other techniques (e.g. Western blot, immunohistochemistry) as well [198]. In the current study on human samples, this possibility was inevitably hampered by the limited size of the spare liver biopsy samples that were merely fully used for RNA-extraction. Of the UPR related genes, CREB3L3 (see above) and ERN1, encoding for the ER stress sensor IRE1, were affected in the same manner. Interestingly, endoplasmic reticulum to nucleus signalling 1 (ERN1) did not increase after NASH resolution, but was downregulated. The exact mechanisms behind this phenomenon are unclear, but might be explained by the fact that the ER stress pathways can be both the consequence and the cause of steatosis [240].

Since autophagy and UPR are crucial in cellular homeostasis, baseline alterations of these pathways most likely interfere with health and lead to severe disturbances early in life. Hence, (near-)normal functioning of these key-regulators in the majority of an adult population can be expected compared to other less fundamental pathways. This does, however, not preclude a potential effect of modulation of autophagy and/or the UPR on NAFLD and its related metabolic conditions.

The pathway analysis of the genes that were significantly differently expressed in the different analyses were mainly pointing in the direction of a downregulation and were related to insulin-glucose related pathways and the AMPK signalling pathway (Tables 7.4 and 7.9). AMPK plays a key role in (cellular) metabolism by regulating glucose homeostasis, lipid metabolism, protein synthesis and oxidative metabolism. Given this central role, stimulation of AMPK is an important target for treatment in NAFLD [229]. Other gene-arrays also found differences within pathways related to cell adhesion/migration, organisation of the extracellular matrix and liver regeneration/proliferation [233,234,241]. Genes involved in these pathways were, however, mostly not included in our analysis. A larger analysis that includes these pathways as well would be of interest. Furthermore, as mentioned before, observations in pre-clinical animal models do not by definition translate into humans and hence into patients. Very recently it was shown that the gene expression showed a major heterogeneity between different animal models, and the comparability of genes or expressed pathways with human data was low [242].

This study is faced with some limitations. The majority of the detected differences are small and about 1.3-1.4 times changed compared to the designated comparator. Some propose more stringent criteria (e.g ≥1.5 or ≥2.0) and different cutoffs may have a major impact on the interpretation of the results [243]so is the preprocessing of microarray to transcriptomics. Microarray data suffers from several normalization and significance problems. Arbitrary fold change (FC. However, smaller alterations within 1 pathway might have a greater (clinical) impact than alterations of a single gene [233]. Secondly, the majority of the included patients were diagnosed with

NASH, while the other patient groups were smaller. This relative overrepresentation might have hampered the detection of additional differences between groups that became outweighed. However, these data represent real-life data of a tertiary obesity clinic, potentially resulting in the detection of NAFLD in an early phase. On the other hand, patients originating from obesity clinic have less likely advanced fibrosis compared with hepatology clinics where admission is more likely related to advanced liver disease. Finally, other co-morbidities, the previous medical history and the medication use were not included in this analysis, while these might all influence the biological processes. Nevertheless, this study has also some strengths. First, it exists out of a large well-described patient cohort, with both bariatric and non-bariatric interventions and including an obese non-steatotic population as an internal control population. The latter makes confounding of genes involved in obesity, one of the main risk factors of NAFLD, less likely. Follow-up biopsies are mostly performed in the setting of clinical trials, while real-life studies with a follow-up biopsy outside the setting of bariatric surgery are rare. Moreover, to the best of our knowledge, this is the first study to date that not only cross-sectionally assesses baseline liver biopsies, but that also has longitudinal gene expression data in a subset of patients and that investigates differently expressed genes in paired liver biopsies, demonstrating significant changes in relation to the resolution of NASH.

Taken together, this study looking at differently expressed genes in relation to NAFLD-severity showed that, while NAFL-patients did differ in just 2 genes, NASH-patients with a low degree fibrosis or NASH-patients with more advanced fibrosis differed considerable from obese patients without NAFLD. The differences were mostly related to metabolic pathways, including AMPK. Furthermore, we were able to look at differently expressed genes after 1-year follow-up and demonstrated that successful treatment of NASH affects many genes, of which the decrease of PAI-1 was most notable. Differences in autophagy or UPR, two important cellular homeostatic processes, however, were only minor.

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SUPPLEMENTARY TABLES

Gene Symbol (HGNC)	GeneID (Entrez)	Full Name
ABCA1	19	ATP-binding cassette, sub-family A (ABC1), member 1
ABCB6	10058	ATP-binding cassette, sub-family B (MDR/TAP), member 6
ACACA	31	acetyl-Coenzyme A carboxylase alpha
ACADL	33	acyl-Coenzyme A dehydrogenase, long chain
ACLY	47	ATP citrate lyase
ACOX1	51	acyl-Coenzyme A oxidase 1, palmitoyl
ACSL5	51703	acyl-CoA synthetase long-chain family member 5
ACSM3	6296	acyl-CoA synthetase medium-chain family member 3
ACTB	60	actin, beta
ADIPOR1	51094	adiponectin receptor 1
ADIPOR2	79602	adiponectin receptor 2
ADM2	79924	adrenomedullin 2
AKT1	207	v-akt murine thymoma viral oncogene homolog 1
AMBRA1	55626	autophagy/beclin-1 regulator 1
AMFR	267	autocrine motility factor receptor
APOA1	335	apolipoprotein A-I
APOB	338	apolipoprotein B (including Ag(x) antigen)
APOC3	345	apolipoprotein C-III
APOE	348	hypothetical LOC100129500; apolipoprotein E
APP	351	amyloid beta (A4) precursor protein
ASNS	440	asparagine synthetase
ATF4	468	activating transcription factor 4 (tax-responsive enhancer element B67); activating transcription factor 4C
ATF6	22926	activating transcription factor 6
ATF6B	1388	activating transcription factor 6 beta
ATG10	83734	ATG10 autophagy related 10 homolog (S. cerevisiae)
ATG12	9140	ATG12 autophagy related 12 homolog (S. cerevisiae)
ATG13	9776	KIAA0652
ATG14	22863	ATG14 autophagy related 14 homolog (S. cerevisiae)
ATG16L1	55054	ATG16 autophagy related 16-like 1 (S. cerevisiae)

ATG16L2	89849	ATG16 autophagy related 16-like 2 (S. cerevisiae)
ATG3	64422	ATG3 autophagy related 3 homolog (S. cerevisiae)
ATG4A	115201	ATG4 autophagy related 4 homolog A (S. cerevisiae)
ATG4B	23192	ATG4 autophagy related 4 homolog B (S. cerevisiae)
ATG4C	84938	ATG4 autophagy related 4 homolog C (S. cerevisiae)
ATG4D	84971	ATG4 autophagy related 4 homolog D (S. cerevisiae)
ATG5	9474	ATG5 autophagy related 5 homolog (S. cerevisiae)
ATG7	10533	ATG7 autophagy related 7 homolog (S. cerevisiae)
ATG9B	285973	ATG9 autophagy related 9 homolog B (S. cerevisiae)
ATP5C1	509	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1
ATXN3	4287	ataxin 3
B2M	567	beta-2-microglobulin
BAD	572	BCL2-associated agonist of cell death
BAK1	578	BCL2-antagonist/killer 1; BCL2-like 7 pseudogene 1
BAX	581	BCL2-associated X protein
BCL2	596	B-cell CLL/lymphoma 2
BCL2L1	598	BCL2-like 1
BECN1	8678	beclin 1, autophagy related
BEX2	84707	brain expressed X-linked 2
BID	637	BH3 interacting domain death agonist
BNIP3	664	BCL2/adenovirus E1B 19kDa interacting protein 3
CALR	811	calreticulin
CANX	821	calnexin
CAPN3	825	calpain 3, (p94)
CASP3	836	caspase 3, apoptosis-related cysteine peptidase
CASP8	841	caspase 8, apoptosis-related cysteine peptidase
CCT4	10575	chaperonin containing TCP1, subunit 4 (delta)
CD36	948	CD36 molecule (thrombospondin receptor)
CDKN1B	1027	cyclin-dependent kinase inhibitor 1B (p27, Kip1)
CDKN2A	1029	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)
CEBPB	1051	CCAAT/enhancer binding protein (C/EBP), beta
CLN3	1201	ceroid-lipofuscinosis, neuronal 3
CNBP	7555	CCHC-type zinc finger, nucleic acid binding protein
CPT1A	1374	carnitine palmitoyltransferase 1A (liver)
CPT2	1376	carnitine palmitoyltransferase 2
CREB3	10488	cAMP responsive element binding protein 3
CREB3L3	84699	cAMP responsive element binding protein 3-like 3
CTSB	1508	cathepsin B
CTSD	1509	cathepsin D
CTSS	1520	cathepsin S
CXCR4	7852	chemokine (C-X-C motif) receptor 4
CYP2E1	1571	cytochrome P450, family 2, subfamily E, polypeptide 1
CYP7A1	1581	cytochrome P450, family 7, subfamily A, polypeptide 1
DAPK1	1612	death-associated protein kinase 1
DDIT3	1649	DNA-damage-inducible transcript 3
DERL1	79139	Der1-like domain family, member 1
DGAT2	84649	diacylglycerol O-acyltransferase homolog 2 (mouse)
DNAJB9	4189	DnaJ (Hsp40) homolog, subfamily B, member 9
DNAJC10	54431	DnaJ (Hsp40) homolog, subfamily C, member 10
DNAJC3	5611	DnaJ (Hsp40) homolog, subfamily C, member 3
DRAM1	55332	DNA-damage regulated autophagy modulator 1
DRAM2	128338	DNA-damage regulated autophagy modulator 2
EDEM1	9695	ER degradation enhancer, mannosidase alpha-like 1
EIF2A	83939	eukaryotic translation initiation factor 2A, 65kDa
EIF2AK3	9451	eukaryotic translation initiation factor 2-alpha kinase 3
EIF4G1	1981	eukaryotic translation initiation factor 4 gamma, 1
ERN1	2081	endoplasmic reticulum to nucleus signaling 1
ERN2	10595	endoplasmic reticulum to nucleus signaling 2
ERP44	23071	endoplasmic reticulum protein 44
ESR1	2099	estrogen receptor 1
FABP1	2168	fatty acid binding protein 1, liver
FABP3	2170	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)
FABP5	2171	fatty acid binding protein 5
FADD	8772	Fas (TNFRSF6)-associated via death domain
FAS	355	Fas (TNF receptor superfamily, member 6)
FASN	2194	fatty acid synthase
FBXO6	26270	F-box protein 6
FGF21	26291	fibroblast growth factor 21

FOXA2	3170	forkhead box A2
FOXO1	2308	forkhead box O1
G6PC	2538	glucose-6-phosphatase, catalytic subunit
G6PD	2539	glucose-6-phosphate dehydrogenase
GAA	2548	glucosidase, alpha; acid
GABARAP	11337	GABA(A) receptor-associated protein
GABARAPL1	23710	GABA(A) receptor-associated protein like 1
GABARAPL2	11345	GABA(A) receptor-associated protein-like 2
GANAB	23193	glucosidase, alpha; neutral AB
GANC	2595	glucosidase, alpha; neutral C
GAPDH	2597	glyceraldehyde-3-phosphate dehydrogenase-like 6
GCK	2645	glucokinase (hexokinase 4)
GCLC	2729	glutamate-cysteine ligase, catalytic subunit
GINS2	51659	GINS complex subunit 2 (Psf2 homolog)
GK	2710	glycerol kinase
GSK3B	2932	glycogen synthase kinase 3 beta
HDAC1	3065	histone deacetylase 1
HDAC6	10013	histone deacetylase 6
HERPUD1	9709	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1
HGS	9146	hepatocyte growth factor-regulated tyrosine kinase substrate
HMGCR	3156	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
HNF4A	3172	hepatocyte nuclear factor 4, alpha
HPRT1	3251	hypoxanthine phosphoribosyltransferase 1
HSP90AA1	3320	heat shock protein 90kDa alpha (cytosolic), class A member 1
HSPA2	3306	heat shock 70kDa protein 2
HSPA4	3308	heat shock 70kDa protein 4
HSPA4L	22824	heat shock 70kDa protein 4-like
HSPA5	3309	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)
HSPA8	3312	heat shock 70kDa protein 8
HSPH1	10808	heat shock 105kDa/110kDa protein 1
HTRA2	27429	HtrA serine peptidase 2
HTRA4	203100	HtrA serine peptidase 4
HTT	3064	huntingtin
IFNG	3458	interferon, gamma
IGF1	3479	insulin-like growth factor 1 (somatomedin C)
IGFBP1	3484	insulin-like growth factor binding protein 1
IL10	3586	interleukin 10
IL1B	3553	interleukin 1, beta
IL6	3569	interleukin 6 (interferon, beta 2)
INHBE	83729	inhibin, beta E
INSIG2	51141	insulin induced gene 2
INSR	3643	insulin receptor
IRGM	345611	immunity-related GTPase family, M
IRS1	3667	insulin receptor substrate 1
KCNMB3	27094	potassium large conductance calcium-activated channel, subfamily M beta member 3
KEAP1	9817	kelch-like ECH-associated protein 1
LAMP1	3916	lysosomal-associated membrane protein 1
LDLR	3949	low density lipoprotein receptor
LEPR	3953	leptin receptor
LEPROT	54741	leptin receptor overlapping transcript
LPL	4023	lipoprotein lipase
MANF	7873	mesencephalic astrocyte-derived neurotrophic factor
MAP1LC3A	84557	microtubule-associated protein 1 light chain 3 alpha
MAP1LC3B	81631	microtubule-associated protein 1 light chain 3 beta
MAPK1	5594	mitogen-activated protein kinase 1
MAPK14	1432	mitogen-activated protein kinase 14
MAPK8	5599	mitogen-activated protein kinase 8
MAPK9	5601	mitogen-activated protein kinase 9
MBTPS1	8720	membrane-bound transcription factor peptidase, site 1
MBTPS2	51360	membrane-bound transcription factor peptidase, site 2
MCM4	4173	minichromosome maintenance complex component 4
MLXIPL	51085	MLX interacting protein-like
MTOR	2475	mechanistic target of rapamycin (serine/threonine kinase)
MXD3	83463	MAX dimerization protein 3
NDUFB6	4712	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6, 17kDa
NFKB1	4790	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NPC1	4864	Niemann-Pick disease, type C1
NPLOC4	55666	nuclear protein localization 4 homolog (S. cerevisiae)

NQO1	1728	NAD(P)H dehydrogenase, quinone 1
NR1H2	7376	nuclear receptor subfamily 1, group H, member 2
NR1H3	10062	nuclear receptor subfamily 1, group H, member 3
NR1H4	9971	nuclear receptor subfamily 1, group H, member 4
NUCB1	4924	nucleobindin 1
OS9	10956	osteosarcoma amplified 9, endoplasmic reticulum associated protein
PCK2	5106	phosphoenolpyruvate carboxykinase 2 (mitochondrial)
PCNA	5111	proliferating cell nuclear antigen
PDIA3	2923	protein disulfide isomerase family A, member 3
PDIA4	9601	protein disulfide isomerase family A, member 4
PDK4	5166	pyruvate dehydrogenase kinase, isozyme 4
PFDN5	5204	prefoldin subunit 5
PIK3C3	5289	phosphoinositide-3-kinase, class 3
PIK3CA	5290	phosphoinositide-3-kinase, catalytic, alpha polypeptide
PIK3CG	5294	phosphoinositide-3-kinase, catalytic, gamma polypeptide
PIK3R1	5295	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)
PIK3R4	30849	phosphoinositide-3-kinase, regulatory subunit 4
PKLR	5313	pyruvate kinase, liver and RBC
PNPLA2	57104	patatin-like phospholipase domain containing 2
PNPLA3	80339	patatin-like phospholipase domain containing 3
PPA1	5464	pyrophosphatase (inorganic) 1
PPARA	5465	peroxisome proliferator-activated receptor alpha
PPARG	5468	peroxisome proliferator-activated receptor gamma
PPARGC1A	10891	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
PPIA	5478	peptidylprolyl isomerase A (cyclophilin A)
PPP1R15A	23645	protein phosphatase 1, regulatory (inhibitor) subunit 15A
PRKAA1	5562	protein kinase, AMP-activated, alpha 1 catalytic subunit
PRKCSH	5589	protein kinase C substrate 80K-H
PTEN	5728	phosphatase and tensin homolog
PTPN1	5770	protein tyrosine phosphatase, non-receptor type 1
RAB24	53917	RAB24, member RAS oncogene family
RB1	5925	retinoblastoma 1
RBP4	5950	retinol binding protein 4, plasma
RGS19	10287	regulator of G-protein signaling 19
RNF5	6048	ring finger protein 5
RNF5P1	286140	ring finger protein 5 pseudogene 1
RPLP0	6175	ribosomal protein lateral stalk subunit P0
RPN1	6184	ribophorin I
RPS6KB1	6198	ribosomal protein S6 kinase, 70kDa, polypeptide 1
RRM2	6241	ribonucleotide reductase M2 polypeptide
RXRA	6256	retinoid X receptor, alpha
SAMM50	25813	sorting and assembly machinery component 50 homolog (S. cerevisiae)
SCAP	22937	SREBF chaperone
SCD	6319	stearoyl-CoA desaturase (delta-9-desaturase)
SEC62	7095	SEC62 homolog (S. cerevisiae)
SEC63	11231	SEC63 homolog (S. cerevisiae)
SEL1L	6400	sel-1 suppressor of lin-12-like (C. elegans)
SERP1	27230	stress-associated endoplasmic reticulum protein 1
SERPINE1	5054	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
SIL1	64374	SIL1 homolog, endoplasmic reticulum chaperone (S. cerevisiae)
SLC17A2	10246	solute carrier family 17 (sodium phosphate), member 2
SLC27A5	10998	solute carrier family 27 (fatty acid transporter), member 5
SLC2A1	6513	solute carrier family 2 (facilitated glucose transporter), member 1
SLC2A2	6514	solute carrier family 2 (facilitated glucose transporter), member 2
SLC2A4	6517	solute carrier family 2 (facilitated glucose transporter), member 4
SNCA	6622	synuclein, alpha (non A4 component of amyloid precursor)
SNORA29	677812	small nucleolar RNA, H/ACA box 29
SNORD14C	85389	small nucleolar RNA, C/D box 14C
SNORD14D	85390	small nucleolar RNA, C/D box 14D
SOCS3	9021	suppressor of cytokine signaling 3
SQSTM1	8878	sequestosome 1
SREBF1	6720	sterol regulatory element binding transcription factor 1
SREBF2	6721	sterol regulatory element binding transcription factor 2
STAT3	6774	signal transducer and activator of transcription 3 (acute-phase response factor)
SUMO3	6612	small ubiquitin-like modifier 3
SYVN1	84447	synovial apoptosis inhibitor 1, synoviolin

TCP1	6950	t-complex 1
TFEB	7942	transcription factor EB
TGFB1	7040	transforming growth factor, beta 1
TGM2	7052	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)
TMEM74	157753	transmembrane protein 74
TNF	7124	tumor necrosis factor (TNF superfamily, member 2)
TNFSF10	8743	tumor necrosis factor (ligand) superfamily, member 10
TOR1A	1861	torsin family 1, member A (torsin A)
TP53	7157	tumor protein p53
TRIB3	57761	tribbles homolog 3 (Drosophila)
TYMS	7298	thymidylate synthetase
UBE2G2	7327	ubiquitin-conjugating enzyme E2G 2 (UBC7 homolog, yeast)
UBXN4	23190	UBX domain protein 4
UFD1L	7353	ubiquitin fusion degradation 1 like (yeast)
UGGT1	56886	UDP-glucose ceramide glucosyltransferase-like 1
UHRF1	29128	ubiquitin-like with PHD and ring finger domains 1
ULK1	8408	unc-51-like kinase 1 (C. elegans)
ULK2	9706	unc-51-like kinase 2 (C. elegans)
USP14	9097	ubiquitin specific peptidase 14 (tRNA-guanine transglycosylase)
UVRAG	7405	UV radiation resistance associated gene
VCP	7415	valosin-containing protein
VIMP	55829	VCP interacting membrane selenoprotein
WIPI1	55062	WD repeat domain, phosphoinositide interacting 1
XBP1	7494	X-box binding protein 1

Table S7.1 Selected genes of interest included in the analysis

	GeneID (Entrez)	Function
ACACA	31	Fatty acid synthesis
ACLY	47	Biosynthetic pahtways, including lipogenesis and cholesterogenesis
ACSL5	51703	Lipid biosynthesis and fatty acid degradation
ACTB	60	Cell motility, structure and integrity
ATF6	22926	Transcription factor, ER stress sensor
ATG4A	115201	Autophagy (biogenesis of autophagosomes)
CD36	948	Fatty acid transport
CEBPB	1051	Transcription factor; immune and inflammatory responses
CPT1A	1374	Mitochondrial transport fatty acids, beta oxidation
CREB3L3	84699	Transcription factor (ATF6-like properties)
CTSS	1520	Protein degradation, antigen presentation
CXCR4	7852	Chemokine receptor
ERN1	2081	ER stress sensor
FASN	2194	Fatty acid synthesis
FGF21	26291	Metabolic regulator
FOXO1	2308	Transcription factor
G6PC	2538	Gluconeogenesis and glycogenolysis
GABARAPL1	23710	Autophagy (biogenesis of autophagosomes)
GANC	2595	Glycogenmetabolism
CAPN3	825	Protease, function presently unknown
GCK	2645	Glycolysis
GK	2710	Glycerol uptake and metabolism
HPRT1	3251	Purine nucletoides synthesis
HSPA4L	22824	Chaperone (aggregated proteins)
IGF1	3479	Mimics insulin functions
IGFBP1	3484	Binds IGF, prolongs its half-life
INHBE	83729	Inhibitor hormone secretions
INSIG2	51141	Blocks processing SREBP
LDLR	3949	Receptor, uptake LDL
LEPR	3953	Receptor, regulation fat metabolism
LEPROT	54741	Receptor-mediated cell signaling; negative regulation growth hormone and leptin signaling
PDK4	5166	Regulation glucose metabolism

PKLR	5313	Glycolysis
PPA1	5464	Phosphate metabolism
PPARA	5465	Transcription factor; metabolism, immune and inflammation
PPARGC1A	10891	Transcriptional coactivator; energy metabolism
RB1	5925	Tumor suppressor
SCD	6319	Fatty acid biosynthesis
SERPINE1	5054	Inhibitor of fibrinolysis
SOCS3	9021	Negative regulator cytokine signaling
STAT3	6774	Transcription factor; in response to cytokines and growth factors
TGFB1	7040	Growth factor; proliferation and differentiation
USP14	9097	Deubiquination
WIPI1	55062	Assembly of multiprotein complexes

Table S7.2 Information on the genes that were significantly altered *The function of the genes as supplied by the NCBI of the genes that were significantly upregulated or repressed (>1.2 fold) in the different analyses. [accession date 25th of July 2016]*