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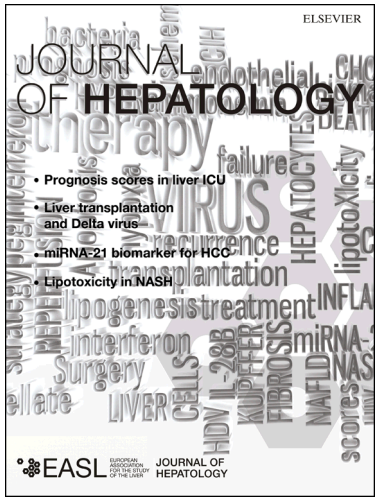

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TITLE PAGE

Title:

PPARα gene expression correlates with severity and histological treatment response in patients with Non-alcoholic Steatohepatitis.

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List of abbreviations:

ABT	Aminopyrine Breath Test
AFP	Alpha-fetoprotein
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
BMI	Body Mass Index
CPT1A	Carnitine Palmitoyl Transferase 1 Liver Isoform
CK 18	Cytokeratin 18
CT	Computed Tomography
FGF21	Fibroblast Growth Factor 21
GGT	Gamma Glutamyl Transpeptidase
HBA1c	Glycosylated Haemoglobin
HDL	High Density Lipoprotein
HOMA	Homeostasis Model Assessment
IDF	International Diabetes Federation
LDH	Lactate dehydrogenase

MABP	Mean Arterial Blood Pressure
NAFLD	Non-Alcoholic Fatty Liver Disease
NAS	NAFLD Activity Score
NASH	Non-Alcoholic Steatohepatitis
NASH CRN	NASH Clinical Research Network
NCEP-ATP III	US Third Adult Treatment Panel of the National Cholesterol Education Program
PDK4	pyruvate dehydrogenase kinase isoenzyme 4
PNPLA3	Patatin Like Phospholipase Domain-containing Protein 3
PPAR	Peroxisome Proliferator-Activated Receptor
PPRE	Peroxisome Proliferator Response Element
QUICKI	Quantitative Assessment Check Index
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
Suppl	Supplementary Materials
TG	Triglycerides
ULN	Upper Limit of Normal
USS	Ultrasound Steatosis Score
WHR	Waist-To-Hip Ratio

Key words
Non-alcoholic steatohepatitis; peroxisome proliferator-activated receptor; follow-up study; pathophysiology; metabolic syndrome

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Authors contributions:

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ABSTRACT

Background and aims: Peroxisome proliferator-activated receptors (PPARs) have been implicated in NASH pathogenesis, mainly based on animal data. Gene expression data in NASH patients are scarce. We studied liver PPARα, β/δ and γ expression in a large cohort of obese patients assessed for presence of NAFLD at baseline and 1 year follow-up. *Methods:* Patients presenting to the obesity clinic underwent a hepatic work-up. If NAFLD was suspected, liver biopsy was performed. Gene expression was studied by mRNA quantification. Patients were reassessed after 1 year. *Results:* 125 patients were consecutively included with follow-up including liver biopsy in n=85. Liver PPARα expression negatively correlated with presence of NASH (p=0.001) and with severity of steatosis (p=0.003), ballooning (p=0.001), NASH activity score (p=0.008) and fibrosis (p=0.003). PPARα expression was positively correlated to adiponectin (R^2=0.345, p=0.010) and inversely correlated to visceral fat (R^2=-0.343, p<0.001), HOMA IR (R^2=-0.411, p<0.001) and CK18 (R^2=-0.233, p=0.012). Liver PPARβ/δ and PPARγ expression did not correlate with any histological feature nor with glucose metabolism or serum lipids. At 1 year, correlation of PPARα expression with liver histology was confirmed. In longitudinal analysis, an increase in expression of PPARα and its target genes was significantly associated with histological improvement (p=0.008). *Conclusion:* Human liver PPARα gene expression negatively correlates with NASH severity, visceral adiposity and insulin resistance and positively with adiponectin. Histological improvement is associated with an increase in expression of PPARα and its target genes. These data might suggest that PPARα is a potential therapeutic target in NASH.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), the hallmark of which is the accumulation of fat in the liver in the absence of alcohol consumption or other causes of secondary steatosis, is highly prevalent in Western societies. Non-alcoholic steatohepatitis (NASH), the subtype of NAFLD where steatosis is accompanied by inflammation and hepatocellular damage, is increasingly recognized as an important cause of liver and non-liver-related morbidity and mortality with an increasing impact on health-care resources[1, 2].

The pathophysiology of NAFLD remains, however, largely unknown. Based mainly on epidemiological data, NAFLD and NASH are intimately linked to visceral adiposity, insulin resistance and diabetes, and the metabolic syndrome[2].

As insulin resistance and fatty acid metabolism have shown to impact on NAFLD, drugs targeting glucose or lipid metabolism have been evaluated for NASH treatment[3]. Several molecules targeting hepatic metabolic processes are currently under investigation. The most promising clinical results were up to now obtained with the thiazolidinediones, which are peroxisome proliferator-activated receptor (PPAR) gamma agonists[4]. Recently, hepatoprotective effects of a dual PPARα/δ agonist were reported in rodent NASH-models[5].

PPARs belong to the nuclear receptor superfamily[6]. After binding to their ligands, PPARs form heterodimers with retinoid X receptors and these heterodimers regulate transcription of various target genes[6]. PPARα is expressed in metabolically active tissues and regulates genes involved in fatty acid β-oxidation, although it also impacts on gluconeogenesis and inflammatory responses[7]. PPARβ/δ also seems to be an important metabolic regulator,

183 which may also act on Kupffer cells to modulate NASH[8]. PPARγ is predominantly
184 expressed in adipose tissue, where it controls adipocyte differentiation, but to a lesser extent
185 also in the liver. PPARγ activation improves insulin sensitivity and glucose homeostasis[9].
186
187 Although numerous data on the role of altered expression of PPARs in the development of
188 NAFLD and NASH in preclinical animal models are available, comparable human data are
189 scarce[6, 10-12].
190
191 The aim of our study was to analyse the liver tissue gene expression pattern of the PPARs in
192 relation to the histological severity of NAFLD and to analyse longitudinally the relation
193 between the changes in PPAR expression and in liver histology in drug-naive overweight
194 individuals.
195

PATIENTS AND METHODS

Metabolic work-up

Patients visiting the obesity clinic of the Antwerp University Hospital (a tertiary referral facility) for a problem of overweight (BMI≥25-29.9 kg/m²) or obesity (BMI≥30 kg/m²) were prospectively recruited. They underwent a metabolic and a liver-specific program as previously reported[13] (see supplementary materials, suppl.).

Patients were excluded from further analysis in case of significant alcohol consumption (>20 g/day), history of bariatric surgery, diagnosis of another liver disease, pre-existing diabetes (as diabetics constitute a specific risk group for NAFLD and anti-diabetic treatments (diet, drugs) were considered potential confounders) or treatment by fibrates (that have PPAR-agonistic properties). Patients who were, however, diagnosed with *de novo* diabetes at baseline or at follow-up were not excluded.

Suspicion of NAFLD was defined by abnormal liver tests [aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) and/or gamma glutamyl transpeptidase (GGT) and/or alkaline phosphatase (ALP)] and/or liver ultrasound abnormality [steatotic liver (Ultrasound steatosis score (USS) ≥ 1)].

Liver biopsy

When one or more of these criteria were met, a liver biopsy was proposed, as this is still the gold standard to accurately diagnose the different NAFLD subtypes and severity and reliable non-invasive tools are insufficiently validated in this specific population. This protocol is part

of the HEPADIP protocol (Belgian registration number B30020071389) that was approved by the Ethics Committee of the Antwerp University Hospital (reference 6/25/125).

For patients going to bariatric surgery, a liver biopsy was proposed regardless of the criteria. Liver biopsy was performed (after additional informed consent) percutaneously (16G Menghini) or peri-operatively (14G Tru-Cut).

Haematoxylin-eosin stain, Sirius red stain, reticulin stain and Perls' iron stain were routinely performed on all biopsies and analyzed by two experienced pathologists blinded for any clinical data. The different histological features of NAFLD were assessed using the NASH Clinical Research Network (NASH CRN) Scoring System[14].

The presence of NASH was defined according to Chalasani *et al* necessitating the combined presence of some degree of steatosis, some degree of ballooning and some degree of lobular inflammation[15]. We also looked at the NAFLD Activity Score (NAS)-based distribution of no NASH (NAS< 3), borderline or probable NASH (NAS=3 or 4) or definite NASH (NAS≥ 5) to make a subclassification of the overall population.

RNA extraction and Real Time Quantitative PCR

RNA was isolated from human liver by guanidinium thiocyanate/phenol/chloroform extraction. Reverse transcription was performed using the High Capacity Reverse Transcription kit (Applied Biosystems, Life Technologies, Carlsbad,USA). PCR was performed with Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies,Santa Clara,USA) on a Stratagene Mx3005P system (Agilent Technologies) using specific primers. mRNA levels were subsequently normalized to those of cyclophilin and relative changes in

gene expression were then determined using the $2^{-\Delta\Delta Ct}$ method using normal livers (NAS = 0 and no fibrosis) as the reference population set to 1. ΔC_t refers to the cycle number at which the transcripts were detectable (C_t) normalized to the cycle number of cyclophilin. To assess whether the observed changes in PPARα expression were functionally relevant, gene expression of well-characterized liver PPARα target genes was studied by RNA array as previously described[16].

Follow-up

Following their baseline assessment, patients preferentially entered a weight management program with emphasis on life style modification and without prescription of specific anti-obesity drugs. Patients who met the reimbursement criteria for bariatric surgery in Belgium (BMI $\geq$ 40 kg/m^2 or BMI $\geq$ 35 kg/m^2 + comorbidity) and who had a positive evaluation by the multidisciplinary team of the obesity clinic (our centre is an European Association for the Study of Obesity-accreditated Collaborating Centre for Obesity Management) could ask for bariatric surgery. After 1 year of follow-up, the baseline assessment was repeated in all patients, including liver biopsy if the patient consented. This protocol is part of the HEPADIP protocol (Belgian registration number B30020071389) that was approved by the Ethics Committee of the Antwerp University Hospital (reference 6/25/125).

Statistical analysis

Values were expressed as mean ± standard deviation (SD) whenever applicable. The results were analyzed with an independent samples t-test (normally distributed continuous variables), Mann Whitney U test or Kruskall-Wallis test (non-normally distributed continuous variables, categorical variables, scores) and Chi square test (prevalences) for the comparison of different groups. As most of the parameters were not normally distributed, Spearman rank correlations were calculated for the continuous variables. Binary or linear logistic regression was used to establish the relationship with categorical variables. Univariate linear regression was performed to identify factors independently predicting HOMA IR. Factors were reported by the adjusted coefficient of determination (R^2) and the significance (p-value). All variables significantly associated with HOMA IR in univariate linear regression analysis were included in multivariate forward conditional analyses to identify variables that where independently associated. Baseline and follow-up data were compared using paired samples t-test, Wilcoxon

277 signed rank test or McNemar test (dichotomous variables) as appropriate. Calculations were
278 made using SPSS 18.0 for Windows. A p value <0.05 was considered statistically significant.
279

RESULTS

Main characteristics

Between October 2005 and October 2008, 245 patients were screened. Seven patients were excluded from further analysis because of the discovery of a formerly unknown chronic liver disease (1 primary biliary cirrhosis, 1 haemochromatosis, 1 chronic hepatitis C, 2 chronic hepatitis B) or because of alcohol consumption disclosed upon repeated interrogation (2 patients). From the remaining 238 patients, 47 (19.7%) had none of the criteria to propose a liver biopsy. In the other 191 patients at least one criterion was met. Liver biopsy was performed in 128/191 of those patients (67.0 %, or 52.2% of the overall cohort of 245 patients). None of these patients were taking antidiabetic drugs; 28 were on statin treatment; 3 were on fibrates and excluded from further analysis. Gene expression studies were performed on spare frozen liver tissue samples of the 125 remaining patients. Their main characteristics are listed in Table 1.

Histology

Thirty-seven (29.6%) biopsies were performed during bariatric surgery. Mean biopsy length for lightmicroscopic evaluation was 17.2±7.9 mm (range 9-45) and the mean number of portal tracts was 8.7±2.9 (range 4–20). The most important histological features are listed in Table 2.

Gene expression and correlation with histology

Expression of PPARα decreased significantly with increasing grade of steatosis (p=0.003), the score for ballooning (p=0.001), the presence of NASH (p=0.001) (Fig. 1A), the NAS-based classification of no NASH/borderline NASH/definite NASH (p=0.002), the NAS (p=0.008) (Fig. 1B), as well as with fibrosis (p=0.003) (Fig. 1C) (Chi Square or Kruskal

Wallis non-parametric testing). PPARα expression was consistently lower with increasing severity for each parameter. PPARα expression also tended to be lower with increasing inflammation (p=0.084).

No significant correlations were found between PPARβ/δ or PPARγ expression and any of the histological features.

Gene expression and correlation with anthropometrical, biochemical and metabolic parameters

The anthropometrical, biochemical and metabolic parameters that significantly correlated with PPAR gene expression levels are listed in Table 3.

Predictors of HOMA IR

Insulin resistance is one of the key components of the metabolic syndrome. Factors that significantly correlate with HOMA IR in a univariate and multivariate analysis are listed in Suppl. Table 1 (Suppl.). In a multivariate analysis, PPARα expression remains an independent and negative predictor of HOMA IR, even if histological parameters are included. PPARα expression, waist and NAS have a global R^2 of 0.391 for the prediction of HOMA IR.

Follow-up

A complete follow-up dataset including liver biopsy was available in 85 patients, 50/80 (58.8%) had a weight management programme. Their main characteristics are listed in Table 1. In comparison to baseline, there is a significant improvement in anthropometric parameters, serum lipids, parameters of glucose metabolism and liver enzymes (Table 1).

Mean length of follow-up biopsy was 22.1±8.2 mm (range 12-42) and the mean number of portal tracts was 9.8±5.8 (range 4-24). The histological lesions are summarized in Table 2. In comparison to baseline, all histological parameters overall improved, including fibrosis. Although overall fibrosis regressed, a few patients showed fibrosis progression and 3 developed cirrhosis.

In a cross-sectional analysis on the 1-year follow-up biopsies, PPARβ/δ and PPARγ didn't correlate with histology. By contrast, expression of PPARα was significantly different according to the grade of steatosis (p=0.015), of inflammation (p=0.017), of ballooning (p=0.005), the presence of NASH (p=0.015), the NAS-based classification of no NASH/borderline NASH/definite NASH (p=0.005) and the NAS (p=0.030), reproducing the results of the cross-sectional analysis at baseline (Chi Square or Kruskal Wallis non-parametric testing). In contrast to baseline, there was no significant correlation of PPARα expression with fibrosis (p=0.321). PPARα expression was consistently lower with increasing severity for each parameter.

Overall PPARα expression significantly improved from 0.321±0.209 at baseline to 0.514±0.209 (p<0.001, paired samples Wilcoxon signed ranks test) (Fig. 2). In the 24/34 patients with NASH at baseline who did not have NASH at 1 year follow-up, PPARα expression highly significantly increased from 0.280±0.072 to 0.527±0.165 (p=0.004), whereas in the 10/34 who maintained the diagnosis of NASH PPARα expression was unchanged (0.384±0.138 vs. 0.392±0.143, p=0.983) (Fig. 3 and Suppl. Fig. 1).

As it was technically unfeasible to measure liver PPARα protein levels due to the lack of additional spare biopsy samples in the non-bariatric surgery patients and due to the

notoriously low quality of the available antibodies, the expression of well-known peroxisome proliferator response element (PPRE)-driven liver PPARα target genes was measured as an estimate of changes in PPARα activity. Carnitine palmitoyl transferase 1 liver isoform (CPT1A)[17], fibroblast growth factor 21 (FGF21)[18] and pyruvate dehydrogenase kinase isoenzyme 4 (PDK4) expression all significantly increased in parallel with PPARα in the 24/34 patients with NASH at baseline and no more NASH at 1 year, whereas their expression was unchanged in the 10/34 patients who maintained the diagnosis of NASH at 1 year (Fig. 3 and Suppl. Fig. 1). The evolution of several other genes related to metabolism and inflammation are shown in Fig. 3. The upregulation of PPARα and its target genes in patients with NASH at baseline but with resolution of NASH at 1 year follow-up was paralleled by a significant downregulation of inflammatory response genes as well as a number of genes involved in lipogenesis and lipid-droplet formation (Fig. 3).

In table 4 baseline and follow-up data are presented separately for the patients with a weight management program and the patients who underwent bariatric surgery. Although changes are more marked in the bariatric surgery group, overall results are comparable, with a significant improvement in PPARα expression and liver histology also when analysis is restricted to the weight management group.

The parameters that were associated with a decrease in NAS in univariate and multivariate analysis are listed in Suppl. Table 2 (Suppl.). The change in PPARα was related significantly to the decrease in NAS in univariate but not multivariate analysis.

DISCUSSION

Whereas several studies in animal models are available on metabolic pathways implicated in the pathogenesis of NASH, data on altered expression of genes involved in lipid and glucose metabolism in human liver tissue in NASH are scarce. We report on gene expression of the PPARs in human liver tissue at baseline and at 1 year of follow-up in a large series of patients with a wide range of histological severity of NAFLD. We show that the gene expression of PPARα is significantly lower with increasing severity of NASH, whereas the expression of the other PPARs is unaltered. In cross-sectional analysis at 1 year of follow-up, the inverse correlation between PPARα expression and histological severity of NASH was confirmed. In longitudinal analysis increase in expression of PPARα and its target genes was associated with histological improvement. This offers a rationale for drugs targeting PPARα in the treatment of NASH.

Before discussing the results, it should be emphasized that human studies on NAFLD are hampered by various methodological issues. Liver biopsy is still the gold standard for accurate assessment of NAFLD and NASH, but remains an invasive procedure, limiting the number of included patients as well as the availability of spare liver samples for additional analysis[19]. Most NASH studies are retrospective. Patients are mostly highly selected, as they are referred to specialized hepatology clinics because of elevated transaminases or, in case of bariatric surgery series, only represent one extreme of the spectrum. This results in patient series with relative high prevalence of more advanced disease. In some studies results are compared with a matched control group, the selection of which is also subject to bias and in which histological data are usually lacking.

403 The two-step approach of our study aimed at overcoming some of these methodological
404 issues. In a first step, all patients presenting to the obesity clinic were assessed, regardless of
405 any a priori suspicion of liver disease. They underwent a series of tests aiming at detecting
406 any sign of liver disease and if there was, a liver biopsy was proposed, representing the
407 second step. We estimate that this two-step prospective approach avoids some mechanisms of
408 bias and allows confidently applying the obtained results to the overall population of
409 overweight patients. Furthermore this approach resulted in a patient series containing the
410 whole NAFLD spectrum (which is by the design of the study inevitably different from the
411 patient population seen at our hepatology clinics). The patients who ultimately appeared to
412 have a normal liver histology were used as an internal control group, avoiding the need for an
413 external group of matched controls[20].
414
415
416 In our study, which is to our knowledge the first to report on PPAR gene expression in a large
417 cohort of well-documented NAFLD patients, PPARα expression was clearly decreased when
418 steatosis and steatohepatitis become more severe. As we are directly assessing the tissue
419 expression of the gene, these data provide strong evidence for the intimate link between
420 PPARα expression and NASH severity.
421
422 In the majority of the patients, a one year follow-up biopsy was available. Outside the setting
423 of bariatric surgery or clinical trials, series with paired liver biopsies in NAFLD patients are
424 rare. The largest series to date looking at the effects of lifestyle modification on liver
425 histology in NASH by Promrat *et al* included 28 patients with paired biopsies[21]. In our
426 series, 50 non-surgical patients were histologically re-evaluated after 1 year, making it the
427 largest reported series in this setting. The beneficial effect of life style modification on liver

histology could be confirmed, with loss of NASH diagnosis in about 30% of cases, in relation with loss of weight and waist circumference and improvement of insulin sensitivity.

The follow-up assessment of the patients first allowed for a second cross-sectional analysis, which confirmed the inverse correlation between PPARα expression and histological severity of NASH. Furthermore, longitudinal analysis convincingly showed that histological improvement is strongly associated with an increase in PPARα expression. As the changes were significant both in the non-bariatric as in the bariatric surgery group (although more pronounced in the latter, in relation to the more pronounced improvement in weight and metabolic parameters), the effect on PPARα expression could not be attributed to a specific metabolic effect of bariatric surgery.

The close association between PPARα expression and NASH severity both in cross-sectional analysis and in follow-up can, however, not resolve the issue of causality. A relevant end-point in the treatment of NASH-patients is the loss of the diagnosis of NASH, as this is the subtype at risk for progressive liver disease[22, 23]. Of the patients with the diagnosis of NASH at baseline, those who had no more NASH at follow-up had a significant increase in PPARα expression, whereas those who did not resolve their NASH also had no improvement in PPARα expression, further supporting the link between PPARα expression and NASH severity.

We did not study the protein expression, which was technically unfeasible due to the limited size of the spare biopsy sample (that was mostly entirely used for RNA extraction) and the notoriously low quality of the existing commercial antibodies. Therefore, to further assess the functional relevance of the changes in PPARα expression, we also studied the expression of

CPT1A, FGF21 and PDK4, well-known PPRE-driven PPARα target genes[17, 18]. This approach is relevant since previous studies in animal models revealed gene-dosage effects of PPARα gene expression on target gene regulation and function[24]. The expression of these target genes parallels the changes in PPARα, and they increase in relation with NASH disappearance, whereas NASH persists in association with unchanged expression of these target genes. These data support the functional relevance of the observed changes in PPARα gene expression.

Nevertheless the possibility that the observed changes in PPARα expression are secondary to the improvement in histology cannot be excluded by the current study design. Our data are, however, in line with convincing animal data that show a beneficial role for PPARα in NASH both in prevention and treatment[5]. These studies, including PPARα knock-out models and the use of several PPARα agonist, provide evidence that PPARα is protective against NASH and that modulation of its expression can improve liver histology, supporting the hypothesis that the observed improvement in our study is at least in part mediated by increased PPARα expression[10, 12, 25]. Few clinical pilot studies were performed to assess the impact of fibrates, which are PPARα agonists, on the evolution of NASH with conflicting results[26-29]. Preliminary results of GFT505, a dual PPARα and β/δ agonist with hepatotrophic activity, on parameters of glucose metabolism and on liver enzymes are prompting further research[30].

Interestingly, PPARα expression also correlates with parameters of glucose metabolism, adiponectin levels and visceral fat accumulation. The link between increased visceral fat accumulation, decreased adiponectin levels, increased insulin resistance (and impaired

glucose metabolism) and more severe NASH is well established [2, 31]. Adiponectin is protective against NASH, whereas insulin resistance is deleterious. This is also in line with recent data on the regulatory effect of adiponectin on hepatic PPARα expression and activity [9]. Our study shows that all these alterations are in parallel with a reduced PPARα expression in the liver and further support the hypothesis that reduced PPARα expression is a key phenomenon in progressing NASH.

Other PPARs have also been implicated in the pathogenesis and treatment of NASH. PPARγ-regulated genes stimulate adipogenesis and lipid uptake by fat cells and regulate fatty acid storage and glucose metabolism[6]. [32]. PPARγ is most abundantly expressed in adipose tissue[6]. Several animal studies show a beneficial role of PPARγ in NASH[33].

Little data exist on PPARγ expression in human NAFLD. Pettinelli *et al* reported PPARγ expression in 22 patients who underwent bariatric surgery (compared to 16 cholecystectomy controls) and found the highest expression in those with steatohepatitis[34]. The latter, however, represented only 6 patients. Lima-Cabello *et al* reported on 43 NAFLD patients compared to 44 hepatitis C and 22 cholecystectomy controls, showing higher levels in steatosis and NASH compared to controls, but PPARγ expression was not different comparing simple steatosis with NASH[35].

In our study PPARγ expression was not altered in relation to the presence or severity of NAFLD, leading to the hypothesis that the observed effect of glitazones on liver fat and inflammation[36] is mostly secondary to the improvement in extrahepatic metabolic features

and adiponectin, and not by a primary hepatic effect. This is also in line with the limited expression of PPARγ in the liver in comparison with adipose tissue[6]. It is also interesting to note that in our study liver PPARγ gene expression was not correlated to parameters of glucose metabolism or serum lipids, again suggesting that the direct effects of PPARγ agonist are predominantly extrahepatic. This might also explain why no further improvement in liver histology is observed beyond one year of treatment, although metabolic parameters further improve[37]. Finally, the number of patients included, the prospective assessment of the patients and the presence of the whole range of the NAFLD spectrum quite evenly distributed (allowing to compose a control group of patients with normal liver histology within the study population) are strengths of our study design and might explain why previous reports on enhanced PPARγ expression in human NAFLD samples could not be reproduced.

Finally we could not demonstrate a relation between PPARβ/δ and NAFLD severity. Recent experimental data point towards a role for Kupffer cell PPARβ/δ in NAFLD[38]. It might be that changes in Kupffer cell PPARβ/δ could not be detected by the methods we used due to the low contribution of Kupffer cell to the overall hepatic mRNA pool, to which parenchymal cells mainly contribute.

In conclusion, in line with experimental data on the role of PPARα in the pathogenesis of NASH, we demonstrated that liver PPARα expression decreases with progressing histological severity of NAFLD and increases in association with NASH improvement, whereas the expression of the other PPARs remain unaltered.

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LEGEND TO THE FIGURES

Fig. 1. **PPARα expression.** Bar chart showing the relative PPARα expression compared to normal liver tissue between patients without and with NASH **(1A)**, in relation to the NAS **(1B)** and in relation to stage of fibrosis **(1C)**. PPARα = peroxisome proliferator-activated receptor alpha; NAS = non-alcoholic fatty liver disease activity score; n = number of patients; * = significant difference with $p < 0.05$.

Fig. 2. **PPARα expression at baseline and follow-up.** Bar chart showing the relative PPARα expression compared to normal liver tissue at baseline and follow-up in the 85 patients with paired liver biopsies. PPARα = peroxisome proliferator-activated receptor alpha; * = significant difference with $p < 0.05$.

Fig. 3. **Changes in gene expression between baseline and follow-up of PPARα, PPARα target genes and related genes involved in fatty acid uptake, metabolism and inflammation in NASH patients with resolution of NASH on follow-up.** Significant upregulation upon follow-up is marked in green, downregulation is marked in red. * significant with $p < 0.05$; ** significant with $p < 0.01$; NC = no change.

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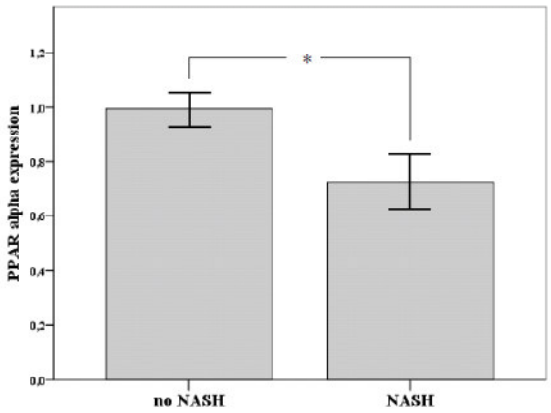

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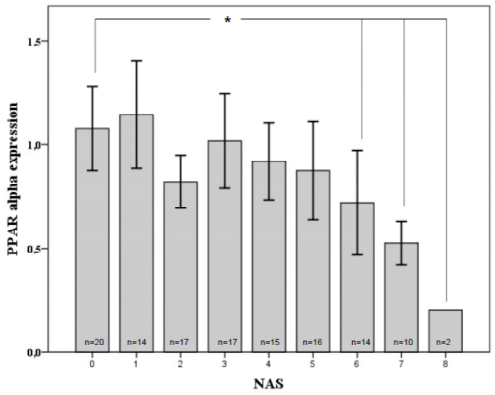

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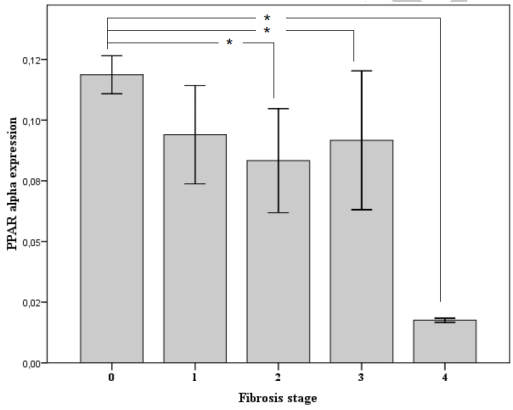

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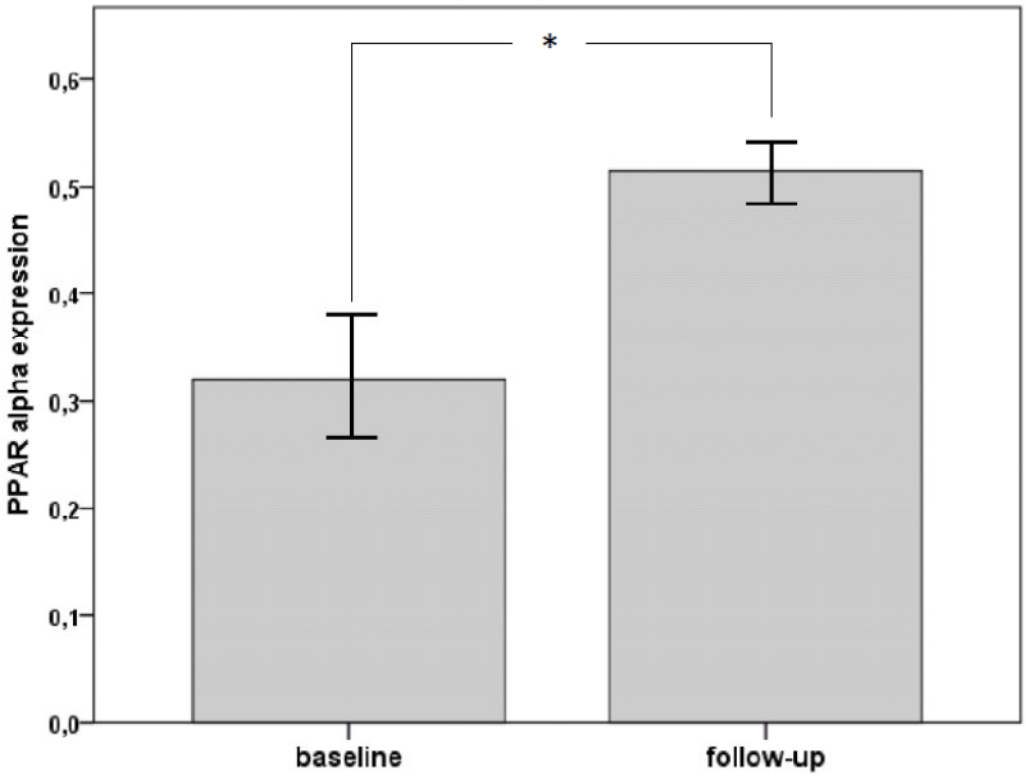

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	Gene symbol	NASH at baseline no NASH at follow-up = responder	NASH at baseline NASH at follow-up = non-responder
PPARA and PPARA target genes	*PPARA*	1.34*	NC
	CPT1A	1.61*	NC
	FGF21	1.39*	NC
	PDK4	1.21*	NC
FA uptake	*CD36/FAT*	1.27*	NC
	FATP2	NC	NC
	FATP5	NC	NC
Metabolism	*G6PC*	1.47*	NC
	ACL	NC	NC
	ACC	NC	NC
	FASN	NC	1.95*
	SCD1	1.28*	NC
	MOGAT1	1.48**	NC
	DGAT2	NC	NC
	PLIN2	1.7*	NC
	LPIN2	1.49*	NC
	LDL-R	1.7*	NC
	PRKAA1	NC	NC
	PRKAB2	NC	NC
Inflammation	*JUN*	2.53**	NC
	JUND	1.53**	NC
	JUNB	1.48*	NC
	FOS	1.21*	NC
	REL	1.21*	NC
	NFKBIA	NC	NC
	TLR4	1.22*	NC
	GADD45B	2.18*	NC
	CRP	3.68**	NC
	SAA1	1.69**	NC

Upregulated in follow-up patients

Downregulated in follow-up patients

NC No Change

*, p<0,05; **, p<0,01

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676 **Table 1. Main characteristics of the patient cohort at baseline (n = 125) and follow-up (n**
677 **= 85).**

		Baseline (n = 125)		Follow-up (n = 85)		p
	Units		**range**		**range**	
Gender M/F	%/%	32.0/68.0		40.0/60.0		
Age	y	46.9 ± 12.7	17 - 74	47.7 ± 12.7	21-75	
BMI	kg/m²	38.7 ± 6.7	28.5 - 69.1	32.2 ± 6.1	23.6 - 63.2	< 0.001
Waist	cm	116.7 ± 12.4	91 - 160	103.6 ± 13.4	75 - 132	< 0.001
WHR		0.967 ± 0.103	0.69 - 1.25	0.942 ± 0.096	0.70 – 1.25	< 0.001
Visceral adipose tissue	cm²	203.4 ± 94.9	43 - 622	143 ± 81	23 - 441	< 0.001
MABP	mm Hg	96.97 ± 9.86	71.3 - 133.3	91.84 ± 5.21	69.4 - 128.2	0.005
LDH	U/L	572.5 ± 100.5	381 - 1089	422.3 ± 171.8	159 - 848	< 0.001
AST	U/L	33.1 ± 17.6	17 - 142	25.8 ± 10.4	6 -74	< 0.001
ALT	U/L	47.1 ± 22.9	16 - 144	31.4 ± 17.3	13-158	< 0.001
AST/ALT		0.73 ± 0.16	0.43 - 1.31	0.86 ± 0.25	0.39 – 2.2	0.012
GGT	U/L	36.9 ± 26.1	10 - 160	29.9 ± 17.4	9 – 101	< 0.001
Total Cholesterol	mg/dL	202.9 ± 36.4	132 - 355	190.8 ± 41.5	51 - 298	< 0.001
HDL-cholesterol	mg/dL	49.9 ± 14.3	25 - 102	53.6 ± 15.9	27 - 95	< 0.001
TG	mg/dL	151.8 ± 83.8	42 - 594	125.2 ± 66.6	34 - 415	< 0.001
Fasting glucose	mg/dL	86.8 ± 18.1	67 - 206	81.6 ± 9.2	65 - 116	0.023
Fasting insulin	µU/mL	17.13 ± 10.96	3.5 - 55.6	10.04 ± 6.09	0.2 – 30.7	< 0.001
Fasting C-peptide	nmol/L	1.13 ± 0.42	0.47 - 2.57	0.85 ± 0.30	0.34 - 1.73	<

						0.001
HBA1c	%	5.67 ± 0.61	4.9 - 10.2	5.43 ± 0.39	4.9 - 7.9	< 0.001
HOMA IR		3.84 ± 3.12	0.71 - 20.33	2.06 ± 1.40	0.04 - 7.01	0.021
USS		2.2 ± 1.0	0 - 3	1.1 ± 1.0	0-3	< 0.001
Diabetes absent/present	%/%	91.1/8.9		79.7/20.3		ns
ABT peak excretion	%	9.03 ± 4.96	0.7 - 34.4	11.19 ± 4.90	2.1 - 23.1	< 0.001
ABT cumulative excretion	%	12.84 ± 6.60	1.1 - 39.6	16.51 ± 6.81	3.3 - 33.4	< 0.001
Number criteria Metabolic Syndrome NCEP ATP III		2.8 ± 1.0	0 - 5	1.6 ± 1.0	0 - 4	< 0.001
Number criteria Metabolic Syndrome IDF		2.9 ± 1.0	1 - 5	1.7 ± 0.8	0 - 4	0.004
Metabolic syndrome NCEP ATP III absent/present	%/%	41.7/58.3		75.5/24.5		0.001
Metabolic Syndrome IDF absent/present	%/%	37.5/62.5		69.4/30.6		0.003
PNPLA3 polymorphism (CC/CG/GG)	n (%)	73 (58.2)/47 (37.7)/ 5 (4.1)		40 (48.8)/39 (47.6)/3 (3.7)		ns
CK 18	U/L	218.5 ± 24.4	45.1 - 1781.0	NA		
Serum adiponectin	ng/mL	10348.0 ± 4849.9	4203 - 23251	15076.3 ± 7569.2	5137 - 35091	< 0.001
Serum leptin	ng/mL	55.25 ± 15.84	26.5 - 108.7	33.66 ± 8.64	19.1 - 60.8	< 0.001

678 Continuous variables are presented as mean ± standard deviation. p = p-value of the
679 comparison between baseline and 1 year follow-up (paired samples t-test, Wilcoxon signed
680 ranks test or McNemar as appropriate on n = 85). M = male; F= female; WHR = waist-to-hip
681 ratio; MABP = mean arterial blood pressure; LDH = lactate dehydrogenase; AST = aspartate
682 aminotransferase; ALT = alanine aminotransferase; GGT = gamma glutamyl transpeptidase;
683 HDL = high density lipoprotein; TG = triglycerides; HBA1c = glycosylated haemoglobin;
684 HOMA IR = homeostasis model of assessment insulin resistance; USS = ultrasound steatosis

score; ABT = aminopyrine breath test; y/n = yes or no; NCEP ATP III = US Third Adult Treatment Panel of the National Cholesterol Education Program; IDF = International Diabetes Federation; CK = cytokeratin, PNPLA3 = patatin like phospholipase domain-containing protein 3; NA = not available; ns = not significant

691 **Table 2. Histological characteristics of the patient cohort at baseline (n = 125) and**

692 **follow-up (n = 85).**

		Baseline n = 125			Baseline n = 85		Follow-up n = 85		p
Histological feature	**Range**	**n**	**%**		**n**	**%**	**n**	**%**	
Steatosis	0	38	30.4		15	17.6	56	65.9	< 0.001
	1	34	27.2		31	36.5	17	20.0	
	2	32	25.6		18	21.2	11	12.9	
	3	21	16.8		21	24.7	1	1.2	
NAS	0	20	16.0		8	9.4	49	57.6	< 0.001
	1	14	11.2		5	5.9	5	5.9	
	2	17	13.6		11	12.9	7	8.2	
	3	17	13.6		11	12.9	8	9.4	
	4	15	12.0		16	18.8	5	5.9	
	5	16	12.8		9	10.6	5	5.9	
	6	14	11.2		17	20.0	5	5.9	
	7	10	8.0		7	8.2	1	1.2	
	8	2	1.6		1	1.2	0	0	
NASH according to Chalasani	No NASH	63	50.4		51	60	73	86.9	< 0.001 *
	NASH	62	49.6		34	40	11	13.1	
NAS-based classification	No NASH	51	40.8		24	28.2	61	71.8	< 0.001
	Borderline NASH	32	25.6		27	31.8	13	15.3	
	Definite NASH	42	33.6		34	40.0	11	12.9	
Fibrosis stage	0	75	60.0		49	57.6	65	76.5	0.011
	1	21	16.8		17	20.0	10	11.8	
	2	19	15.2		11	12.9	5	5.9	
	3	9	7.2		8	9.4	2	2.4	
	4	1	0.8		0	0	3	3.5	

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695 The distribution of the patients according to the degree of steatosis, the stage of fibrosis, the
696 NAS, the presence of NASH according to Chalasani *et al* and the classification as no
697 NASH/borderline NASH/definite NASH based on the NAS as proposed by Kleiner *et al* are
698 listed as absolute numbers (n) and percentages (%) for the overall baseline cohort (n = 125)
699 and for the cohort of patients with paired baseline and 1 year follow-up biopsy (n = 85).
700 Baseline and follow-up data were compared using non-parametric paired samples Wilcoxon
701 signed ranks test or McNemar (*) test. NAS = NAFLD actvity score; NASH = non-alcoholic
702 steatohepatitis, p = p-value of the comparison between baseline and follow-up.

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705 **Table 3. Factors that significantly correlate with PPAR expression in liver tissue at**

706 **baseline (n = 125).**

	PPARα	**PPARβδ**	**PPARγ**
	R^2 (p)	R^2 (p)	R^2 (p)
BMI	-0.238 (0.008)		
Waist	-0.381 (< 0.001)		
WHR	-0.343 (<0.001)		
Total adipose tissue	-0.237 (0.008)		
Visceral adipose tissue	-0.334 (<0.001)		
Total Cholesterol	0.179 (0.046)		
TG	-0.181 (0.047)		
LDH			0.290 (0.033)
AST			0.312 (0.019)
GGT	-0.207 (0.020)		
Bilirubine total			0.292 (0.030)
Thrombocytes			-0.321 (0.018)
INR			0.289 (0.032)
PAI1			0.325 (0.016)
HBA1c	-0.181 (0.045)		
Fasting insulin	-0.466 (< 0.001		
Fasting C-peptide	-0.441 (p < 0.001)		
Quicki index	0.462 (< 0.001)		
HOMA	-0.411 (p < 0.001)		
USS	-0.261 (0.007)		
ABT peak excretion	0.209 (0.022)		
ABT cumulative excretion	0.209 (0.009)		
Adiponectin	0.345 (0.010)	0.374 (0.005)	
CK18	-0.233		
Gender			0.377 (0.004)

PNPLA3		0.253 (0.005)	

The different antropometrical, biochemical or other factors (excluding histology) that significantly correlate with the expression of PPARα, PPARβ/δ and PPARγ in liver tissue in the baseline cohort (n = 125) are listed. R^2 = Spearman correlation coëfficiënt (continous variables) or adjusted R square of the lineary logistic regression (catogorical variables); p = p-value of the correlation or univariate linear regression; BMI = body mass index; WHR = waist-to-hip ratio; MS = metabolic syndrome; LDH = lactate dehydrogenase; AST = aspartate aminotransferase; ALT = alanine aminotransferase; GGT = gamma glutamyl transpeptidase; HDL = high density lipoprotein; TG = triglycerides; LDL = low density lipoprotein; HBA1c = glycosylated haemoglobin; INR = international normalized ratio; PAI-1 = tissue plasminogen activator inhibitor 1; QUICKI = quantitative assessment check index calculated as 1/[log(fasting insulin) + log(fasting glucose)]; HOMA IR = homeostasis model of assessment insulin resistance calculated as [fasting insulin (mU/L) x fasting glucose (mmol/L)]/22.5; USS = ultrasound steatosis score; ABT = aminopyrine breath test; NCEP ATP III = US Third Adult Treatment Panel of the National Cholesterol Education Program; CK18 = cytokeratin 18; PNPLA3 = patatin like phospholipase domain-containing protein 3; PPAR = peroxisome proliferator activator receptor.

727 **Table 4. Main baseline and 1 y follow-up characteristics of the patients with paired liver**

728 **biopsies according to the type of treatment.**

		Weight management program (n = 50)			Bariatric Surgery (n = 35)		
	Units	Baseline	Follow-up	p	Baseline	Follow-up	p
Gender M/F	%/%	40/60			40/60		
Age	Y	49.3±12.8			42.5±1.9		
BMI	kg/m²	37.6±0.9	33.9±0.9		41.7±5.3	29.8±4.1	< 0.001*
Waist	Cm	115.5±1.5	107.4±1.5	< 0.001*	124.1±2.2	97.8±2.3	< 0.001*
WHR		0.97±1.5	0.95±1.7	< 0.001*	0.99±0.02	0.92±0.02	< 0.001*
Visceral adipose tissue	cm²	217.1±10.4	171.4±10.5	< 0.001*	219.2±15.9	93.6±8.2	< 0.001*
LDH	U/L	572.8±14.4	490.14±20.3	< 0.001*	560.2±17.7	321.5±28.5	< 0.001*
AST	U/L	35.3±2.8	28.2±1.6	0.009*	32.8±2.7	22.6±1.4	0.001*
ALT	U/L	49.3±5.4	33.5±3.0	< 0.001*	46.7±4.6	28.5±1.6	< 0.001*
GGT	U/L	41.5±4.5	30.6±3.7	< 0.001*	55.8±9.7	28.9±2.8	0.002*
Total Cholesterol	mg/dL	204.8±6.1	199.7±6.4	0.360	201.5±9.2	179.0±6.4	< 0.001*
HDL-cholesterol	mg/dL	48.9±2.5	51.3±2.6	0.113	48.4±2.4	56.6±2.3	< 0.001*
TG	mg/dL	165.4±14.5	138.7±10.9	0.026*	148.1±11.8	105.4±7.9	< 0.001*
Fasting glucose	mg/dL	85.6±2.6	83.4±1.4	0.392	86.3±2.5	79.0±1.3	0.010*
Fasting insulin	µU/mL	13.9±1.1	11.9±0.9	0.058	18.2±1.6	7.3±0.6	< 0.001*
HBA1c	%	5.72±0.11	5.52±0.06	0.039*	5.73±0.10	5.28±0.05	< 0.001*

HOMA IR		2.97±0.26	2.50±0.23	0.075	4.04±0.44	1.42±0.12	<0.001*
USS		2.13±0.15	1.43±0.15	<0.001*	2.21±0.15	0.50±0.12	<0.001*
ABT peak excretion	%	9.28±0.58	12.21±0.81	<0.001*	8.43±0.61	9.83±0.73	0.035*
ABT cumulative excretion	%	13.34±0.79	17.72±1.14	<0.001*	12.12±0.97	14.9±1.05	0.004*
Number criteria Metabolic syndrome NCEP ATP III		2.35±0.18	1.85±0.19	0.006*	2.65±0.24	1.24±0.24	<0.001*
Metabolic syndrome NCEP ATP III absent/present	%/%	49/51	68/32	0.58	45.5/54.5	88.9/11.1	0.002*
PNPLA3 polymorphism (CC/CG/GG)	n (%)	24(48)/23(46)/3(6)			16(48.5)/16(48.5)/1(3)		
Serum adiponectin	ng/mL	9892.3±1034.2	14339.9±1581.0	<0.001*	9683.8±2508.6	19494.3±2516.6	0.016*
PPARα expression		0.295±0.041	0.375±0.047	0.039*	0.331±0.027	0.66±0.0.473	<0.001*
Steatosis	0/1/2/3	7/19/11/13	21/17/11/1	<0.001*	8/12/7/8	35/0/0/0	<0.001*
Lobular inflammation	0/1/2/3	11/24/10/5	26/14/9/1	<0.001*	10/11/13/1	33/2/0/0	<0.001*
Ballooning	0/1/2	8/18/24	21/18/11	<0.001*	11/15/9	31/2/2	<0.001*
NAS		4.1±0.3	2.3±0.3	<0.001*	3.51±0.38	0.23±0.12	<0.001*
Fibrosis	0/1/2/	37/7/4/0/2	34/8/4/4/0	0.41	15/9/7/4/0	28/3/1/2/1	0.00

stage	3/4			7			9*
No NASH/NASH		16/34	27/23	0.006*	15/20	35	< 0.001*

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731 The main characteristics of the patients, including the histological characteristics and the
732 presence of NASH according to Chalasani *et al* are listed for the cohort of patients with paired
733 baseline and 1 year follow-up biopsy (n = 85) according to their treatment. Continuous
734 variables are presented as mean ± standard error of the mean. Histological characteristics are
735 presented as distributions, except for NAS. p = p-value of the comparison between baseline
736 and 1 year follow-up (paired samples t-test, Wilcoxon signed ranks test or McNemar as
737 appropriate). M = male; F= female; WHR = waist-to-hip ratio; LDH = lactate dehydrogenase;
738 AST = aspartate aminotransferase; ALT = alanine aminotransferase; GGT = gamma glutamyl
739 transpeptidase; HDL = high density lipoprotein; TG = triglycerides; HBA1c = glycosylated
740 haemoglobin; HOMA IR = homeostasis model of assessment insulin resistance; USS =
741 ultrasound steatosis score; ABT = aminopyrine breath test; y/n = yes or no; NCEP ATP III =
742 US Third Adult Treatment Panel of the National Cholesterol Education Program; PNPLA3 =
743 patatin like phospholipase domain-containing protein 3; PPAR = peroxisome proliferator
744 activator receptor; NAS = NAFLD activity Score; NASH = Non-Alcoholic Steatohepatitis; *
745 = statistically significant ($p < 0.05$)

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