Identification of novel targets in adipose tissue involved in non-alcoholic fatty liver disease progression

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ABSTRACT

Obesity is a major risk factor for the development of Nonalcoholic fatty liver disease (NAFLD). We hypothesize that a dysfunctional subcutaneous white adipose tissue (scWAT) may lead to an accumulation of ectopic fat in liver. Our aim was to investigate the molecular mechanisms involved in the causative role of scWAT in NALFD progression. We performed a RNA-sequencing analysis in a discovery cohort (n=45) to identify genes in scWAT correlated with fatty liver index, a qualitative marker of liver steatosis. We then validated those targets in a second cohort (n=47) of obese patients who had liver biopsies available. Finally, we obtained scWAT mesenchymal stem cells (MSCs) from 13 obese patients at different stages of NAFLD and established *in vitro* models of human MSC (hMSC)-derived adipocytes. We observed impaired adipogenesis in hMSC-derived adipocytes as liver steatosis increased, suggesting that an impaired adipogenic capacity is a critical event in the development of NAFLD. Four genes showed a differential expression pattern in both scWAT and hMSC-derived adipocytes, where their expression paralleled steatosis degree: *SOCS3, DUSP1, SIK1* and *GADD45B*. We propose these genes as key players in NAFLD progression. They could eventually constitute potential new targets for future therapies against liver steatosis.

Key words: adipose tissue, fatty liver, obesity, mesenchymal stem cell

1. INTRODUCTION

White adipose tissue (WAT) is a metabolic organ of paramount importance in the regulation of whole-body energy homeostasis. WAT functions as a key energy reservoir and secretes various cytokines (adipokines) and other metabolites to control systemic energy expenditure [1]. Subcutaneous white adipose tissue (scWAT) is located under the skin and is the largest storage site for excess lipids. It is endowed with a high plasticity, being able to contract and expand in response to changes in energy balance. The expansion of scWAT is determined by two main processes which include the differentiation of new mature adipocytes from their precursors, known as mesenchymal stem cells (MSC), and the ability to enlarge from those already formed adipocytes [2].

It is well established that each individual possesses an intrinsic limit on their capacity to store lipids in scWAT (i.e. the adipose tissue expandability hypothesis [3]). Subcutaneous adipocytes are the preferable storage fat depot, they can enlarge as result of a prolonged positive energy balance. When scWAT reaches its maximal storage capacity, adipose tissue fails to store lipids appropriately redirecting this lipid flux to other organs where it is accumulated as ectopic fat causing insulin resistance through lipotoxic- and inflammation-related mechanisms [4]. This ectopic accumulation occurs primarily in the visceral adipose tissue (visWAT) and liver, leading to visceral obesity and liver steatosis respectively [5].

Nonalcoholic fatty liver disease (NAFLD) is a general term for a variety of liver conditions caused by hepatic fat accumulation in people who drink little or no alcohol [6]. It is rapidly becoming one of the most common liver diseases worldwide [7]. Steatosis represents the first step of the NAFLD progression and is mostly benign and remains clinically silent. However, in several cases (≈ 30%) these complications ultimately lead

to steatohepatitis (NASH), fibrosis, cirrhosis or even hepatocarcinoma, where liver functions are severely altered [8].

Current evidence undoubtedly demonstrates a complex and dynamic interrelationship between adipose tissue and liver, where adipose tissue seems to play a major causative role governing hepatosteatosis [9]. However, the key factors determining ectopic liver fat accumulation are not fully understood. We hypothesize that gene modifications at scWAT level may represent a rational approach to specifically reduce ectopic liver fat accumulation.

Considering that, the main objective of this study was to obtain new insights into the molecular mechanisms underlying the causative role of scWAT in NAFLD. This aim was addressed by: 1) identifying targetable upregulated genes in scWAT potentially involved in the development and progression of NAFLD and, 2) establishing a functionally relevant *in vitro* model of human MSC (hMSC)-derived adipocytes throughout the progression of NAFLD. To this end, we sequentially combined a global RNA-sequencing analysis from scWAT from a discovery cohort with specific qPCR validation in a second independent cohort where paired adipose/liver biopsies and hMSC-derived adipocytes were available.

The scientific impact of our findings relies on the fact that subsequent modifications (e.g., gene edition) of these newly identified genes could represent a promising strategy to prevent or correct the development of NAFLD.

2. MATERIALS AND METHODS

2.1. Human samples and cohort description

Human abdominal scWAT and liver biopsies were obtained from well-characterized patients who underwent elective surgical procedures at the Miguel Servet University Hospital (HUMS, Zaragoza, Spain) as described previously [10]. Importantly, patients were screened to exclude chronic viral hepatitis or autoimmune hepatitis, and drug- or alcohol-related hepatotoxicity.

For this study, three different cohorts were recruited: 1) The discovery cohort included 45 patients with different adiposity levels with or without other metabolic conditions such as dyslipidemia, diabetes or hypertension. 2) The validation cohort encompassed 47 patients from whom scWAT and laparoscopic liver biopsies had been obtained in the same surgical procedure. 3) The hMSC cohort included 13 participants who donated a sufficient amount of scWAT (>5g) to allow the isolation and establishment of hMSC cell lines. Study design and workflow are depicted in Figure 1.

All subjects were categorized as dyslipidemic, diabetic or hypertensive following National Cholesterol Education Program-Adult Treatment Panel III (NCEP–ATPIII) criteria [11]. All patients provided written consent and the study was approved by the Regional Institutional Review Board of Ethics at Aragón, Spain (CEIC-A). Samples and data from patients included in this study were provided by the Biobank of the Aragon Health System (PT20/00112), integrated in the Spanish National Biobanks Network and they were processed following standard operating procedures with the appropriate approval of the Ethics and Scientific Committees.

2.2. Biochemical assays

Plasma biochemistries were performed at the Clinical Biochemistry department at the HUMS using state of the art analyzers. All analyses are in compliance with the requirements for quality and competence (ISO 15189:2012) for medical laboratories.

The fatty liver index (FLI) is a formula that includes waist circumference, body mass index (BMI), triglycerides, and gamma-glutamyl-transferase (GGT) for prediction of fatty liver [12].

2.3. Histological examination

Liver biopsies were deemed adequate when they were 16 mm in length and included at least 6 portal tracts. Paraffin sections of livers were stained with hematoxylin-eosin (H&E). Pathological features of steatosis (0–3), lobular inflammation (0–3), hepatocellular ballooning (0–2), and fibrosis (0–4) were scored by an experienced pathologist, according to criteria established by the Nonalcoholic Steatohepatitis Clinical Research Network [13]. Total NAFLD Activity Score (NAS) (ranging from 0-8) was calculated by adding the scores for steatosis, lobular inflammation, and hepatocellular ballooning as described [13].

2.4. Isolation of hMSC from human adipose tissue

hMSC were isolated from scWAT obtained from 13 subjects as previously described [14]. In brief, minced adipose tissue was digested with collagenase (Sigma-Aldrich). The digestate was then spun and the cell pellet was resuspended in 10% fetal bovine serum (FBS)-low-glucose DMEM (Gibco) and seeded in six-well plates.

2.5. hMSC culture and differentiation

hMSC cultures were maintained in 10% FBS-low-glucose DMEM under hypoxic conditions (5% O_2, 5% CO_2) and 37°C. Two days after confluence, hMSC were differentiated into adipocytes using an adipogenic cocktail in normoxic conditions for additional 5 days. The standard adipogenic cocktail consisted of 10% FBS–high-glucose DMEM plus (Gibco), 1.5 µM insulin (Novo Nordisk), 1 µM dexamethasone (Sigma), 500 µM 3-isobutyl-1-methylxanthine (Sigma), and 2 µM rosiglitazone (Sigma). hMSC-derived adipocytes were maintained in 10% FBS–high-glucose DMEM and were considered to have reached mature phenotype at 8 days post-differentiation. Intracellular lipids were stained with Oil Red O and then the dye was extracted with isopropanol and quantified at 500 nm absorbance as previously described [15].

2.6. RNA isolation

Total RNA was isolated from frozen biopsies of scWAT using TRIzol (Sigma) according to the manufacturer's protocol. All the RNA samples were treated with RNase-Free DNase (Life Technologies) to remove genomic DNA.

2.7. AmpliSeq human transcriptome analysis.

RNA samples were quantitated using the Qubit RNA high-sensitivity (HS) assay kit (#Q32855, ThermoFisher) and the Qubit 3.0 fluorometer (ThermoFisher). RNA integrity was checked by the 2200 TapeStation system (Agilent) with the High Sensitivity RNA ScreenTape (5067–5579, Agilent). All samples had an RNA integrity number (RIN) > 7. RNA (20 ng) was converted to cDNA using the SuperScript IV VILO Master Mix (#11756050, ThermoFisher) and automated library preparation was carried out with the

Ion AmpliSeq transcriptome human gene expression panel Chef-ready kit (#A31446, ThermoFisher) on the Ion Chef Prep station, chef package version IC.5.4.0 (ThermoFisher).

Sequencing was performed *per* the manufacturer's protocols using the Ion 540 Kit-Chef (# A30011, ThermoFisher) on the Ion Torrent S5-XL (ThermoFisher). Eight samples *per* 540 chip were sequenced obtaining 7.5 to 10 million reads/sample.

Next, bam files were further processed using the Ion AmpliSeq RNA plugin v5.4.01 on the Torrent Server (Torrent Suite Software, v5.4, ThermoFisher) to obtain normalized reads *per* kilobase of transcript *per* million reads mapped (RPKM). A principal component analysis (PCA) method was adopted to identify potential outliers among the samples.

Differentially expressed genes (DEGs) were subsequently determined by correlation analysis (Spearman) between the normalized RPKMs for each single gene and the fatty liver index. The false discovery rate (FDR) adjustment for multiple testing was accounted for using q-values.

2.8. Construction of weighted gene co-expression networks and identification of modules associated with the fatty liver index

Gene co-expression network analysis was performed with the R package WGCNA [16] using RNA-seq and clinical data from the discovery cohort. Genes with low variance (<25%) or low levels of expression (average < 5 RPKM) were excluded from the analysis. Sample clustering was also performed to detect outliers.

Scale independence and mean connectivity analysis of modules with different power values were performed to determine the soft threshold of module analysis. The power value was determined when the scale independence value was >0.9. Then, the adjacency matrix was calculated using the determined power value to ensure an unsigned scale-free network. Minimal module size and merge cut height were set at 30 and 0.25 respectively. A clustering dendrogram of genes, with dissimilarity based on topological overlap, was generated. Next, we identified modules that were significantly associated with the measured clinical trait by correlating eigengenes with the fatty liver index.

To understand the functional significance of genes from modules of interest, the gene ontology (GO) enrichment analysis-biological processes (BP) was used. We determined the over-representative GO categories in a module, based on the Fisher's exact test with a Bonferroni correction for multiple testing.

2.9. RT-qPCR

RNA was reverse-transcribed using PrimeScript Reverse Transcriptase (Takara Bio). Real-time PCR was performed using the StepOnePlus system (Applied Biosystems). 2 μl of the cDNA product was amplified using gene-specific primers (Table 1) in a total volume of 15 μl *per* reaction with SYBR Select Master Mix (Applied Biosystems). Relative gene expression was normalized to *β-ACTIN* or *FABP4* expression using the $2^{-\triangle\triangle Ct}$ method.

2.10. Western blot analysis

For analysis of proteins expression, aliquots of cellular lysates were subjected to reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using 12% polyacrylamide gels. Electrophoresis was carried out at 100 volts for 2 hours. Proteins were then transferred to polyvinylidene difluoride membranes (PVDF), that were blocked with 5% dry skimmed milk for 2 hours and probed with primary antibody against different proteins overnight at 4 ºC with mild shaking. The results were visualized using peroxidase–conjugated secondary antibodies, that were incubated 1 hour at r.t. with mild shaking. Antibody used were: β-Actin (Santa Cruz #SC-47778) diluted 1:1000, AFABP (Santa Cruz #SC-271529) diluted 1:1000, SOCS3 (Santa Cruz #SC-51699) diluted 1:500, GADD45B (Biorbyt #ORB215494) diluted 1:500, SIK1 (Proteintec #51045-1-AP) diluted 1:500, mouse secondary antibody (Santa Cruz #sc-525409) diluted 1:20000, rabbit secondary antibody (Santa Cruz #sc-2357) diluted 1:5000.

2.11. Statistical analysis

Results are expressed as either mean ± standard error of the mean (SEM) or median [interquartile range]. Pairwise group comparisons were calculated using Student's t test for Gaussian-distributed variables and Mann–Whitney U test for non-Gaussian-distributed data. The p value for trend was computed from the Pearson test to investigate whether or not the NAS systematically increases or decreases over the levels of the variables of interest. The statistical analysis was performed using R version 4.0.3 (http:// www.r-project.org) and the appropriate packages, and the level of significance was set at 0.05.

3. RESULTS

3.1. Identification of candidate genes potentially involved in adipose tissue – liver crosstalk

To obtain new insight into the molecular mechanisms underlying NAFLD progression, a global gene expression analysis from scWAT biopsies was carried out in 45 individuals. Table 2 describes phenotypical characteristics (clinical and biochemical) of selected patients (discovery cohort). This cohort encompasses both female (60%) and male patients (40%) with different BMI levels (7% normoweight, 7% overweight and 86% obese) and considerable presence of general descriptors of metabolic syndrome features.

In the discovery phase of this study, we used the Fatty Liver Index (FLI) as a proxy for the severity of liver steatosis [12]. To explore the broad molecular mechanisms behind the liver fat accumulation, we performed a weighted correlation network analysis (WGCNA). This systems biology method is useful for finding and summarizing clusters of highly correlated genes (modules) [16]. The mRNA levels of 11651 genes expressed in scWAT biopsies with clinical information were included in the analysis and a power of $\beta = 5$ was selected to ensure a scale-free network (Figure 2A). We detected 9 gene modules (8 co-expression modules and 1 module with the remaining uncorrelated genes), among which the number of genes ranged from 89 to 3501 (Figure 2B). We then calculated associations of each module with the FLI. Two modules (Pink and Red) were positively associated with FLI while 3 modules (Brown, Yellow, and Grey) showed a negative correlation with FLI (Figure 2C). Gene Ontology enrichment analysis showed that the Pink module consisted of genes involved in white blood cell migration and chemotaxis processes, while most of the genes in the Red modules engaged in immune responses (Figure 2D). On the other hand, the biological processes-enrichment analysis also showed that the Brown module was significantly enriched for genes involved in the regulation of

transcription, while genes in the Yellow module were associated with sensory perception, which might reflect the cross-talk between the nervous system and the adipose tissue. Lastly, the genes included in the Grey module were connected with regulation of molecular functions.

For a more focused candidate-gene approach, we applied Spearman correlation to identify a set of genes whose expression profiles varied with the severity of the fatty liver. Out of 17747genes, we found 475 genes strongly correlated with the FLI score (rho> 0.5 or rho< -0.5, $p<0.0001$) (Figure 3A). Those candidate genes were distributed over all 22 autosomes and the X chromosome. None were found on the Y chromosome (Figure 3B). Using a false discovery rate (FDR) threshold of $q<0.003$, we observed 81 differentially expressed genes (DEG) associated with FLI (Supplementary Figure 1). In the next step, we selected only positively correlated genes ($n = 30$), since our main objective was to find targets susceptible of being knocked-down.

Among those transcripts which displayed this positive association, a thorough bibliographic analysis was carried out to rank these genes according to their potential relevance and whose function had been previously linked to fatty acid metabolism and/or liver function. We finally selected 8 genes: *SOCS3, DUSP1, SIK1, GADD45B, S100A9, S100A12, CTGF* and *CDKN1A* (Figure 3C). It should be noted that all these genes were included in the Red module delimited by the WCNA, except for *S100A9* and *S100A12* which appeared in the Pink module. These candidate genes were further considered for subsequent analysis.

3.2. Validation of candidate genes in scWAT biopsies from a second (validation) cohort

To validate those candidate genes obtained from previous RNA sequencing analysis, their mRNA expression was determined in scWAT from a second validation cohort. This validation cohort included 47 subjects with a similar age and sex distribution to the discovery cohort. This validation cohort encompassed only obese patients (BMI>30 kg/m^2) who presented increased values of triglycerides, GGT, and leptin compared to their discovery cohort counterparts (Table 2).

Subsequently, mRNA levels of selected genes were quantified in scWAT by real-time qRT-PCR. All candidates showed mRNA levels within widely accepted range of Ct values indicative of a constitutive adipose tissue expression (Ct ≈ 16-25). Proving that the strength of this validation cohort is that it encompasses both scWAT and liver biopsies from the same patient, subjects were categorized according to their liver biopsies NAFLD activity score (NAS) into 3 groups: NAS = 0 - 1 (n = 26), NAS = 2 - 3 (n = 14), NAS 4 - 5 (n = 7). Consistent with the previous RNA-seq data in scWAT, mRNA levels of *SOCS3, DUSP1, SIK1, GADD45B, S100A9* and *S100A12* were also positively associated ($p<0.05$) with hepatic steatosis (NAS) in this second validation cohort (Figure 4A-F, Supplementary Table 1). *CDKN1A* and *CTGF* did not replicate that association and were excluded from subsequent analyses (Figure 4G-H, Supplementary Table 1).

Furthermore, expression of selected genes was also analyzed in paired visWAT biopsies (n = 47) from this second validation cohort. The association observed in scWAT was replicated in visWAT for *S100A9* and *S100A12*, but not for *SOCS3, DUSP1, SIK1, GADD45B, CDKN1A* and *CTGF*, suggesting a specific gene regulation of these candidates in scWAT fat depot (Supplementary Table 1).

3.3.Lipid content of adipocyte differentiated hMSC and their corresponding liver biopsies

Next, we sought to further characterize the specific role of adipocytes in fatty liver progression. To this end, hMSCs were isolated from scWAT biopsies and differentiated into adipocytes [14]. Thirteen different donors were included in this new *in vitro* hMSC cohort. Importantly, we observed no significant differences in any relevant clinical or biochemical parameter when comparing the hMSC donors and the discovery cohort (Table 2).

In order to test the functional feasibility of our hMSC cohort, adipogenic differentiation capacity of the selected 13 hMSC cell lines was compared by 1) quantifying oil red O (ORO) staining of lipid droplets in adipocytes and 2) by analyzing mRNA expression of a well-established adipocyte-related transcript, fatty acid binding protein 4 (*FABP4*) [17]. At day 8 post-differentiation, we observed a strong correlation between these two differentiation markers ($r = 0.45$, $p = 0.012$) which demonstrated that both are reliable indicators of the degree of adipocyte differentiation (Supplementary Figure 2).

To elucidate whether hMSC-derived adipocyte differentiation potential was associated with the liver phenotype of the donor, those 13 cell lines were grouped according to the NAS of the patient (NAS 0 – 1, n = 4; NAS 2 – 3, n = 5; NAS 4 – 5, n = 4). We observed an increased differentiation capacity, measured by both ORO and *FABP4* mRNA expression, from first group (NAS 0 - 1) compared to the third group (NAS 4 - 5) which had the lowest differentiation potential (Figure 5).

These results suggest that a lower lipid storage capacity of differentiated subcutaneous adipocytes *in vitro* could be related to an increased fat accumulation in liver. Therefore,

these data validate our model and cohort of hMSC derived-adipocyte as a reliable tool to further characterize fatty liver disease progression.

3.4. Expression of selected genes in differentiated hMSC of patients with different degrees of liver steatosis

To verify whether the previously observed differences in gene expression at scWAT level were adipocyte-specific or occurred in other cell types within the scWAT, we next investigated the expression levels of our candidate genes in previously characterized hMSC-derived adipocytes.

We observed a wide variation in the degree of differentiation of hMSC into adipocytes depending of the cell line (Figure 5). As mRNA levels refer to gene expression in the whole culture (including differentiated and undifferentiated cells), *FABP4* was used to normalize differences in adipocyte differentiation across the cell lines. We found high levels of mRNA expression of *SOCS3, DUSP1, SIK1* and *GADD45B*, but no expression of *S100A9* or *S100A12* was observed in any of the cell lines (data not shown). Moreover, a statistical significant increase was observed in mRNA levels of *SOCS3* (p=0.015), *DUSP1* (p=0.05), *SIK1* (p=0.009) and *GADD45B* (p=0.016) as donor's NAS increased (Figure 6A-D, Supplementary Table 2). These results confirm that these four genes have a differential regulation pattern in adipocytes, that correlates with the degree of liver steatosis.

To further characterize the role of these genes, we performed a protein expression analysis in four different cell lines, two of them obtained from scWAT of patients without liver steatosis (NAS = 0) and the other two from patients with high degree of hepatosteatosis (NAS = 4). Consistent with mRNA data, AFABP protein expression was significantly

upregulated in cell lines of patients with lower steatosis, showing the higher adipogenic capacity of these lines. To reduce differences in adipocyte differentiation across the cell lines, candidates' protein expression was normalized to AFABP. We observed a significant increase of SOCS3, SIK1 and GADD45B protein expression as steatosis increases (Figure 6E, F), following a concordant regulatory pattern between gene and protein expression. However, we could not replicate *DUSP1* mRNA expression outcomes at the protein level.

4. DISCUSSION

The main objective of this study was to clarify the physiological role that impaired scWAT expansion plays in the development and progression of NAFLD. To this end, we have analyzed scWAT biopsies to reveal genes associated with NAFLD progression and we have also established and validated an *in vitro* model to study adipose tissue in NAFLD based on hMSC-derived adipocytes isolated from patients with this condition. Our main outcome is that we identify four genes whose expression increases accordingly to the degree of hepatic steatosis both in scWAT and hMSC-derived adipocytes: *SOCS3, DUSP1, SIK1* and *GADD45B.*

The first objective of our study was to uncover novel genes whose regulation in scWAT could be essential in the progression of NAFLD. We initially performed a high throughput gene expression analysis from scWAT of patients with different degrees of liver steatosis and correlated these results with FLI. Although previous studies have proposed this biochemical parameter as a strong surrogate biomarker of liver steatosis [18], FLI calculation is built upon anthropometric and biological variables that are altered in other metabolic disturbances, therefore these associations might be due to other non-liver-related diseases [19–21]. However, we excluded this possibility by testing our candidate genes in a second cohort with similar prevalence of obesity-associated conditions (i.e. hypertension, dyslipidemia, and diabetes) and more importantly, where subjects were categorized according to their NAS. This histological score obtained from liver biopsies is, to this date, the most accurate method to diagnose liver steatosis [13]. This two-step validation of our findings in two independent cohorts of patients highlights the relevance of our experimental design as a cornerstone in addressing our research purpose.

The second objective of this study was to establish an *in vitro* model of hMSC-derived adipocytes that could reflect the different stages of NAFLD. Several studies have shown

that hMSC isolated from scWAT of patients with metabolic disorders like obesity and diabetes are defective in various functionalities and properties [22] including differentiation [23], multipotent state [24,25], metabolism [26,27] and immunomodulation [28]. Considering that, we tried to address if there were also a correlation between the physiological status of hMSC and the degree of liver steatosis in order to directly link adipose tissue expandability capacity to dysfunctional liver crosstalk.

As observed in hMSC from morbid obese patients [29], lipid accumulation in hMSC-derived adipocytes from NAFLD patients was decreased, indicating impaired adipogenesis of these pre-adipocytes along with NAFLD severity. This negative association between the adipogenic potential of hMSC and the degree of liver steatosis suggests that impaired hMSC differentiation relates to altered WAT expandability and thus, could be a crucial step in development and progression of NAFLD. Indeed, a lower lipid storage capacity in adipocytes may eventually promote fat accumulation in the liver, which is in agreement with previous reports [30] and consistent with our WAT expandability hypothesis. It can be argued that 5 out of 13 patients are diabetic in our hMSC cohort and impaired adipogenesis is also associated with diabetes [23]. However, we observed the same inverse association between markers of adipogenic differentiation (Oil Red O and FABP4) and NAFLD severity in both diabetic and non-diabetic patients (Supplementary Figure 3). This lends more plausibility to the relationship between adipogenesis and NAFLD progression.

These data validate our *in vitro* model and cohort of hMSC-derived adipocytes as a reliable and robust tool to further characterize the implication of scWAT in fatty liver disease progression. This model could overcome the possible limitations found in other *in vitro* approaches [31], providing that our model not only discriminates between healthy and unhealthy patients but also distinguishes between different stages of the disease.

Eventually, this "proposed" model (clinical data and associated hMSC-derived adipocytes) could be also of interest to address other research questions related to obesity-associated disorders such as diabetes, insulin resistance or cardiovascular diseases.

Taking advantage of the two main points underlined in this study, we further corroborated that the differential regulation of these four genes observed at scWAT level actually come from single adipocytes and not from other non-adipose cell types within this tissue. *SOCS3, DUSP1, SIK1* and *GADD45B* showed the same regulatory pattern in hMSC-derived adipocytes as observed in scWAT, reinforcing the idea that these genes are directly involved in NAFLD progression by exerting regulatory actions specifically in adipocytes (f. i., re-directing lipid flux from adipose tissue to the liver) thus, limiting scWAT fat accumulation capacity and lipid metabolism.

Interestingly, the regulatory pattern observed at mRNA level was translated to protein level for SOCS3, SIK1 and GADD45B, but we did not observe the presence of DUSP1 at the protein level in fully differentiated hMSC-derived adipocytes. Ferguson et al. have shown that this protein is only produced by 3T3L1 cells in the early phase of adipocyte differentiation [32]. Therefore, we hypothesize that DUSP1 may be playing an important role in scWAT *in vivo*, where there are hMSC at different stages of differentiation. Alternatively, DUSP1 mRNA could have regulatory effects in the early phase of adipocyte differentiation without being translated into protein.

On the other hand, the expression of these genes in visWAT do not correlate with liver steatosis, suggesting a specific regulation of *SOCS3, DUSP1, SIK1* and *GADD45B* in scWAT, that differs from visWAT. However, genes which are not expressed in adipocytes, *S100A9* and *S100A12*, showed the same regulatory pattern in both fat depots. These results suggest that the expression of *S100A9* and *S100A12* in WAT is also related with NAFLD but it comes from other cellular type(s) within the WAT, probably immune

system cells [33]. This hypothesis is supported by our bioinformatic analysis which found that the *S100A9* and *S100A12* genes included in the cluster enriched for genes related to white blood cell metabolism.

Previous studies support our findings, reporting a critical role of these newly identified genes in lipid metabolism (including lipid synthesis, breakdown and oxidation) in several models. For instance, liver-specific *SOCS3* deletion in mice enhances hepatic insulin sensitivity and lipogenesis resulting in fatty liver and obesity [34,35], while its overexpression in scWAT causes local insulin resistance [36,37]. However, whether these results are translated to humans remains still elusive. Other studies have also reported *DUSP1* overexpression in scWAT and peripheral blood mononuclear cells of obese humans [38], while its liver-specific overexpression in mice is linked to the promotion of hepatosteatosis [39]. Moreover, *DUSP1* regulates signaling during early 3T3-L1 adipocytes differentiation [32]. On the other hand, *SIK1* is involved in regulation of hepatic lipogenesis by controlling SREBP-1c phosphorylation [40] and promotes insulin resistance in obesity in mouse models [41], whereas *GADD45B* has been shown to play a role in coordination of liver fatty acid uptake and metabolic health [42] and regulates hepatic gluconeogenesis in mice [43].

This evidence points out the relevance of these genes in NAFLD progression and future functional studies will contribute to decipher their actions in humans and within metabolic disease context. However, important questions also arise from our current findings: for instance, how the temporal expression dynamics and the potential interaction of these candidates affect the clinical phenotype (NAFLD development and its severity). Indeed, these newly identified genes could be also considered as potential biomarkers of disease progression (alone or combined with other parameters) although some limitations exist and should be also considered with caution (scWAT biopsies availability). Besides, our

results concern mainly to individuals with obesity and whether these changes fully translate to non-obese also warrants further investigations.

In conclusion, our results constitute a proof-of-evidence of the paramount importance of adipose-liver crosstalk and suggest that impaired adipogenic capacity of hMSC is a critical event in promoting the development of NAFLD. Restoration of fully functional hMSC phenotype (such as through specific gene(s) modulation) might be an additional strategy to address NAFLD and its associated complications. We propose four genes from scWAT: *SOCS3, DUSP1, SIK1,* and *GADD45B* as key players in NAFLD progression. Although more functional interventions are warranted to test the cause and effect relationship, these genes could constitute potential new targets for future therapies.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Author Contributions

M.L.-Y., S.L.-C., and J.M.A.-M. designed the study. M.L.-Y., R.d.M.-B. and M.P.G.-S. performed research, V. B.-M., C.H., C.C. and A. S.-P. provided and analyzed samples, M.L.-Y., S.L.-C. and J.M.A.-M. analyzed data and wrote the manuscript; R.d.M.-B., M.P.G.-S., V. B.-M., C.H., C.C. and A. S.-P. reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

Gene expression and patient data is held by the Aragón Institute of Health Sciences (IACS). Access may be granted to those who meet pre-specified criteria for confidential access, available at https://www.iacs.es/instituto-aragones-ciencias-la-salud/oficina-virtual/solicitud-de-acceso-a-datos-para-realizacion-de-un-proyecto-de-investigacion-rpi01-3a and with prior authorization by the Ethics Committee of Aragon (CEIC-A) which can be obtained at https://www.iacs.es/investigacion/comite-de-etica-de-la-investigacion-de-aragon-ceica/ceica-evaluaciones-y-otras-presentaciones/.

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Table 1. Sequences of primers used in the study.

Gene ID		**Sequence (5'→ 3')**
ACTIN	Forward	CATGTACGTTGCTATCCAGGC
	Reverse	CTCCTTAATGTCACGCACGAT
FABP4	Forward	ACTGGGCCAGGAATTTGACG
	Reverse	CTCGTGGAAGTGACGCCTT
SOCS3	Forward	GTCCCCCCAGAAGAGCCTATTA
	Reverse	TTGACGGTCTTCCGACAGAGAT
DUSP1	Forward	TTCTTCCTCAAAGGAGGATACG
	Reverse	GTGGGGTACTGCAGGAACTG
SIK1	Forward	GCTTCTGAACCATCCACACAT
	Reverse	GTGCCCGTTGGAAGTCAAATA
GADD45B	Forward	TGCTGTGACAACGACATCAAC
	Reverse	GTGAGGGTTCGTGACCAGG
S100A9	Forward	TCGGCTTTGACAGAGTGCAAGA
	Reverse	TGCCCCAGCTTCACAGAGTA
S100A12	Forward	TTCCTGTGCATTGAGGGGTTA
	Reverse	TTAATGCCCCTTCCGAACTGAG
CTGF	Forward	CTTGCGAAGCTGACCTGGAAG
	Reverse	CCGTCGGTACATACTCCACAGA
CDKN1A	Forward	ACATCGCCAAGGAAAAACGC
	Reverse	GTCTGTTTCGGTACTGTCATCC

Table 2. Clinical and biochemical characteristics of the three cohorts and their statistical differences. Data are presented as number (%) or median [interquartile range]. Subjects were categorized as hypertensive, dyslipemic or diabetic following ATPIII criteria [11]. Differences between groups were tested with the Student's t-test; BMI, body mass index (kg/m^2); GGT, gamma-glutamyl transpeptidase; AST, aspartate transaminase; ALT, alanine transaminase.

	Cohort			Comparison		
	Discovery	**Validation**	**hMSC**	**p.Discovery vs Validation**	**p.Discovery vs hMSC**	**p.Validation vs hMSC**
	N = 45	N = 47	N = 13			
Sex:				0.870	0.853	0.853
Male	18 (40.0%)	17 (36.2%)	7 (53.8%)			
Female	27 (60.0%)	30 (63.8%)	6 (46.2%)			
Age (years)	48 [41.0; 54.0]	45 [40.5; 50.0]	47 [42.0; 51.0]	0.716	0.716	0.716
BMI	39.9 [35.9;44.6]	47.3 [42.8;50.7]	44.1 [41.7;50.3]	<0.001	0.003	0.306
BMI Category				0.023	1.000	1.000
Normoweight	3 (6.67%)	0 (0.00%)	0 (0.00%)			
Overweight	3 (6.67%)	0 (0.00%)	0 (0.00%)			
Obesity	39 (86.7%)	47 (100%)	13 (100%)			
Hypertension	19 (42.2%)	25 (53.2%)	6 (46.2%)	1.000	1.000	1.000
Dyslipidemia	13 (28.9%)	17 (36.2%)	7 (53.8%)	0.601	0.334	0.601
Diabetes	10 (22.2%)	17 (36.2%)	5 (38.5%)	0.432	0.432	1.000
Glucose (mg/dl)	96.5 [85.5;108]	103 [89.5;117]	117 [102;134]	0.119	0.040	0.119
Triglycerides (mg/dl)	98.0 [88.0;124]	127 [95.0;180]	144 [110;165]	0.049	0.049	0.862
Cholesterol (mg/dl)	191 [175;226]	188 [165;214]	188 [145;206]	0.596	0.596	0.927
GGT (U/l)	24.0 [18.0;40.0]	35.0 [23.5;44.0]	37.5 [25.0;48.5]	0.042	0.061	0.589
AST (U/l)	28.5 [22.0;35.2]	22.0 [18.5;31.0]	25.0 [22.8;28.8]	0.053	0.555	0.201
ALT (U/l)	28.0 [18.0;36.5]	25.0 [20.0;41.0]	40.0 [24.2;43.2]	0.699	0.268	0.337
Leptin (ng/mL)	22.1 [8.95;36.5]	51.3 [33.9;80.6]	50.5 [27.2;72.9]	<0.001	0.003	0.684
Glycated hemoglobin (mmol/mol)	5.70 [5.40;6.00]	5.80 [5.60;6.70]	5.60 [5.45;6.70]	0.211	0.690	0.669

FIGURE LEGENDS

Figure 1. Study design and workflow.

Figure 2. Weighted gene co-expression network analysis (WGCNA). (A) Determination of soft-thresholding power. (B) Hierarchical clustering of identified co-expressed genes in modules. Colored rows represent a color-coded module which contains a group of highly connected genes. (C) Heatmap of the correlation between module eigengenes (ME) and the fatty liver index. Each cell contains the correlation coefficient and the associated p-value. (D) Top five enrichment results of GO-Biological process for significant modules. The number of genes in the selected co-expression modules were 79 (Pink), 397 (Red), 1632 (Brown), 1610 (Yellow), and 1270 (Grey).

Figure 3. Identification of candidate genes potentially involved in adipose tissue – liver crosstalk. (A) Workflow of RNA-Seq analysis. (B) Chromosomal distribution of genes whose expression profiles varied with the severity of the NAFLD. (C) Expression of selected genes according to fatty liver index (FLI); male in red and female in blue; RPKM (Reads *per* kilo base *per* million mapped reads).

Figure 4. mRNA expression of selected genes in scWAT of a validation cohort. mRNA expression of *SOCS3* (A), *DUSP1* (B), *SIK1* (C), *GADD45B* (D), *S100A9* (E), *S100A12* (F), *CDKN1A* (G) and *CTGF* (H) in scWAT of patients from the validation cohort grouped according to their Nonalcoholic fatty liver disease Activity Score (NAS) (NAS = 0 - 1, n = 26; NAS = 2 - 3, n = 14; NAS 4 - 5, n = 7). Relative gene expression was normalized to *β-ACTIN* and values are expressed as mean ± SEM. P: p-value for the trend as detailed research design and methods section.

Figure 5. Fat content of adipocyte differentiated hMSC and their corresponding liver biopsies. (A). Oil Red O staining of hMSC-derived adipocyte (top panel) and liver histological examination stained with H&E (lower panel) from the same patient, each one representative of the overall subgroup. Quantification (absorbance at 500 nm) of Oil Red O staining (B) and *FABP4* mRNA expression (C) in 13 lines of hMSC-derived adipocytes grouped according to the Nonalcoholic fatty liver disease Activity Score (NAS) of the donor (NAS = 0-1, n=4; NAS = 2- 3, n=5; NAS = 4-5, n=4). Representative images were randomly selected from the overall image collection. Relative mRNA expression was normalized to β-*ACTIN* and values are expressed as mean ± SEM. *, $P < 0.05$.

Figure 6. Expression of selected genes in hMSC-derived adipocytes of patients with different degrees of liver steatosis. (A-D) mRNA expression of *SOCS3* (A), *DUSP1* (B), *SIK1* (C) and *GADD45B* (D) in 13 cell lines of hMSC-derived adipocytes of patients grouped according to their Nonalcoholic fatty liver disease Activity Score (NAS) (NAS = 0-1, n=4; NAS = 2-3, n=5; NAS = 4-5, n=4). Relative gene expression was normalized to *FABP4* mRNA levels. (E-F) Western blot protein expression analysis of AFABP, SOCS3, SIK1 and GADD45B in four different hMSC-derived adipocytes cell lines, two of them (CL1, CL2) obtained from scWAT of patients without liver steatosis (NAS = 0) and the other two (CL3, CL4) from patients with hepatosteatosis (NAS = 4). 20 μg of cellular lysate were loaded for SOCS3 detection (E) and 80 μg for SIK1 and GADD45B (F). Both Actin and AFABP were used as internal controls. Densitometry quantification was performed normalizing levels of protein expression to AFABP. Results show mean values of 3 independent experiments. Values are expressed as mean ± SEM. *, $P < 0.05$. CL, cell line.