Markers of adipose tissue fibrogenesis associate with clinically significant liver fibrosis and are unchanged by synbiotic treatment in patients with NAFLD.

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Background and Aims: Subcutaneous adipose tissue (SAT) dysfunction contributes to NAFLD pathogenesis and may be influenced by the gut microbiota. Whether transcript profiles of SAT are associated with liver fibrosis and are influenced by synbiotic treatment (that changes the gut microbiome) is unknown. We investigated: (a) whether the presence of clinically significant, ≥F2 liver fibrosis associated with adipose tissue (AT) dysfunction, differential gene expression in SAT, and/or a marker of tissue fibrosis (Composite collagen gene expression (CCGE)); and (b) whether synbiotic treatment modified markers of AT dysfunction and the SAT transcriptome.

Methods: Sixty-two patients with NAFLD (60% men) were studied before and after 12 months of treatment with synbiotic or placebo and provided SAT samples. Vibration-controlled transient elastography (VCTE)-validated thresholds were used to assess liver fibrosis. RNA-sequencing and histological analysis of SAT were performed to determine differential gene expression, CCGE and the presence of collagen fibres. Regression modelling and receiver operator characteristic curve analysis were used to test associations with, and risk prediction for, ≥F2 liver fibrosis.

Results: Patients with ≥F2 liver fibrosis (n=24) had altered markers of AT dysfunction and a SAT gene expression signature characterised by enrichment of inflammatory and extracellular matrix-associated genes, compared to those with <F2 fibrosis (n=38). Differences in transcript profiles between patients with vs without ≥F2 liver fibrosis were largely explained by adjusting for differences in HOMA-IR. Gut microbiome-modifying synbiotic treatment did not change SAT transcriptomic profiles or circulating inflammatory/adipokine markers. SAT CCGE values were independently associated with (8.38 (1.72-40.88), p=0.009), and were a good predictor of, ≥F2 fibrosis (AUROC 0.79, 95%CI 0.69-0.90). Associations between SAT transcriptomic profiles and ≥F2 fibrosis were reproduced using end-of-trial data.

Conclusion: A differential gene expression signature in SAT associates with ≥F2 liver fibrosis is explained by a measure of systemic insulin resistance and is not changed by synbiotic treatment. SAT CCGE values are a good predictor of ≥F2 liver fibrosis in NAFLD.

Keywords

Liver fibrosis, NAFLD, Adipose tissue, Transcriptome, Synbiotic, Gut microbiome.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a multisystem disease that increases the risk of developing cardiovascular disease (CVD), type 2 diabetes mellitus (T2DM) and various extra-hepatic cancers [1-3]. Within the spectrum of liver disease in NAFLD, the presence of clinically significant fibrosis (≥F2 stage) increases the risk of both all-cause and disease-specific mortality [4]. However, the underlying biological factors and/or processes contributing to the development and progression of liver fibrosis in NAFLD remain unclear. This is particularly relevant for ≥F2 fibrosis, which is amenable to potential new treatments that are currently being tested for NAFLD [5,6].

Obesity-associated dysfunction in adipose tissue (AT) contributes to the development of NAFLD [7-10]. 'Metabolic inflexibility' [11] and 'limited AT expandability' [9,12] are two main hypotheses that have been proposed to provide potential contextual explanations for how white AT (WAT) may contribute to the pathological accumulation of lipids in the liver. While these hypotheses may explain how alterations in lipid handling in WAT may contribute to early NAFL (i.e., hepatic steatosis), it is unclear whether alterations in WAT may also promote further disease development through the later stages of NAFLD (i.e., liver fibrosis). Circulating biomarkers of AT insulin resistance (IR) such as the AT IR index (AdipoIR) are associated with the presence and severity of NAFLD including fibrosis [13-15], thus indicating that AT dysfunction is potentially also involved in the promotion of hepatic fibrogenesis during later stages of NAFLD.

Previous studies have demonstrated a role of the gut-AT-liver axis in the pathogenesis of NAFLD [16,17], thereby raising the question of whether modifying the gut microbiota, (e.g. with a synbiotic treatment) is able to change AT function and thereby liver disease severity. Transcriptomic profiles in subcutaneous AT (SAT) in patients with NAFL and non-alcoholic steatohepatitis (NASH) indicate a more inflammatory profile compared to those without or with less severe NAFLD, suggesting that alterations in SAT may be important in early NAFLD progression [17-21]. Moreover, recent evidence suggests that SAT fibrogenesis is associated with the presence of hepatic steatosis [22]. However,

whether SAT transcriptomic profiles and markers of SAT fibrogenesis are associated with the presence of clinically significant liver fibrosis is currently unknown.

Therefore, the aims of this exploratory study were to test, in patients with NAFLD, whether: (a) biochemical markers of AT dysfunction, alterations in SAT transcriptomic profiles and a gene expression signature of SAT fibrogenesis are associated with the presence ≥F2 liver fibrosis; and (b) whether a synbiotic treatment (previously shown to have changed the gut microbiota in the **In**vestigation of **Sy**nbiotic **T**reatm**e**nt in NAFLD (INSYTE) trial [23]) modified (a) above.

2. Methods

2.1 - Patient cohort details

A subset of sixty-two patients with NAFLD (age range of 21-77 years), for whom RNA-sequencing data for SAT were available, were studied to perform this secondary analysis of data collected from patients recruited to the INSYTE randomised double-blind placebo-controlled trial (www.clinicaltrials.gov registered number NCT01680640). This provided baseline and end-of-trial tissue biopsies for SAT and assessments of liver fat and liver fibrosis. Details of patient recruitment, the INSYTE trial design (including inclusion and exclusion criteria) and intervention have been described in detail previously [23,24]. The trial design was approved by the Southampton and Southwest Hampshire research ethics committee (12/SC/0614). All patients gave their written informed consent.

2.2 - Anthropometric and biochemical measurements

Anthropometric and biochemical measurements were collected as previously described [23,24]. Body composition was assessed by dual-energy x-ray absorptiometry (DEXA). Details of abdominal magnetic resonance imaging (MRI), anthropometry and biochemical measurement methodology can be found in the supplementary material. Homeostasis model assessment-insulin resistance (HOMA-

IR) [25], AdipoIR [26], enhanced liver fibrosis (ELF) score [27], Fibrosis-4 (FIB-4) index [28] and the AST to platelet ratio index (APRI) [29] were calculated as previously described.

2.3 - Liver fat and vibration-controlled transient elastography

Liver fat and vibration-controlled transient elastography (VCTE)-derived kilopascal (kPa) measurements were collected as previously described [23,24]. Liver VCTE-derived kPa measurements were assessed as a clinically recognised proxy measure of liver stiffness using the Echosens (Waltham, MA) Fibroscan® by a trained clinician (ES). Data are expressed as the median (IQR) in kPa. Liver VCTE-derived kPa measurements of ≥8.2 kPa were used as a validated proxy threshold for identification of ≥F2 fibrosis as recently reported [30].

2.4 - SAT RNA extraction, sequencing, and analysis

Abdominal SAT biopsies were collected from the lower anterior abdominal wall (1 cm inferior and medial to anterior superior iliac spine) from patients with NAFLD. Prior to the incision, 2% Xylocaine with adrenaline (1:100000) was administered to the area of biopsy and a 1 cm incision was then made to expose the SAT. Fat lobules were excised from the wound and stored in RNAlater (QIAGEN, Hilden, Germany; Catalogue number 74804) at -80^{o}C as previously described [24]. RNA integrity (RIN) and quantity of extracted RNA were determined using an Agilent 2100 Bioanalyser and all samples had a RIN score of >7.0. Transcriptome sequencing was outsourced to Novogene Ltd (Cambridge, UK) and performed on a total of 124 SAT RNA samples (62 paired baseline and end-of-trial samples). Ribosomal RNA depletion was used during library-preparation and sequencing was performed using Illumina's Novaseq 6000 with approximately 50 million 150bp paired-end reads per sample. Alignment was performed with STAR[31], read counting with HTSeq[32] and differential expression evaluation with EdgeR [33]. For full details see Supplementary Methods. Composite collagen gene expression (CCGE) values were calculated for each sample as the average expression of 12 collagen gene isoforms 1A1, 1A2, 3A1, 5A1, 5A2, 5A3, 6A1, 6A2, 6A3, 12A1, 14A1, and 24A1 after converting

expression values (log2cpm) of each isoform to a Z-distribution as previously reported [22]. CCGE values are shown as z-scores.

2.5 - Statistical analysis

Non-transcriptomic data (other than CCGE values) were analysed using Statistical Package for the Social Sciences (SPSS) Version 26.0 (New York, USA). Data were first tested for normality using the Shapiro-Wilk and Kolmogorov-Smirnov tests and are presented as mean ± SD for normally distributed or median (IQR) for non-normally distributed variables. Comparisons of continuous variables between groups were performed with the unpaired Student *t*-test for normally and the Mann-Whitney U test for non-normally distributed variables. Differences in proportions were examined using the chi-squared test. Univariable associations were investigated using Pearson's linear correlations for normally distributed or Spearman's rank correlations for non-normally distributed variables. Multivariable linear regression modelling was used to explore the effects of the synbiotic treatment on changes in circulating concentrations of inflammatory markers, adipokines and SAT CCGE values. Binary logistic regression modelling was used to investigate whether AdipoIR and SAT CCGE values were independently associated with ≥F2 liver fibrosis. The goodness of fit for the models was tested with the Hosmer-Lemeshow test. Receiver-operator characteristic (ROC) curve analysis for CCGE values was performed to estimate areas under the receiver-operator characteristic curves (AUROCs) to distinguish patients with NAFLD with vs without ≥F2 fibrosis. The statistical significance of differences in the C-statistic for each model was compared as previously described [34]. A *P* value of <0.05 was considered statistically significant.

3. Results

3.1 - Biochemical markers of adipose tissue dysfunction associate with liver fibrosis severity in patients with NAFLD

Patients were stratified by the presence or absence of ≥F2 liver fibrosis using the previously validated liver VCTE threshold of ≥8.2 kPa [30]. Patients with NAFLD and ≥F2 fibrosis had higher serum AST and ALT concentrations and higher FIB-4 and APRI scores compared to patients with <F2 fibrosis (**Table 1**). Age (53.0 ± 13.4 and 54.2 ± 8.0 years) and sex (60.5% and 58.3% men) were similar between groups. There were also no differences in whole-body adiposity, truncal SAT and VAT depot volumes or mean SAT adipocyte size between groups (**Table 1**). However, HOMA-IR, and AdipoIR were higher, while adiponectin concentrations were lower, in patients with ≥F2 compared to those with <F2 fibrosis (**Table 1**). Patients with ≥F2 liver fibrosis also had elevated circulating concentrations of several inflammatory markers (GDF-15, TNFα, IL-6, IL-8, IL-10). There was a greater prevalence of T2DM and/or MetS in patients with vs without ≥F2 liver fibrosis (**Table 1**). In regression analysis, AdipoIR was found to be positively associated with the presence of ≥F2 fibrosis independently of potential confounding factors (OR 1.03, 95%CI 1.01-1.06, *P* = 0.02 (**Supplementary Table 1**). The positive association between AdipoIR and the presence of ≥F2 liver fibrosis remained significant after adjusting for BMI, total body, truncal adiposity or circulating leptin concentrations (data not shown). Conversely, AdipoIR was no longer significantly associated with the presence of ≥F2 liver fibrosis after including circulating concentrations of adiponectin (another established marker of adipose tissue insulin resistance) (data not shown).

3.2 - Synbiotic treatment does not alter circulating inflammatory markers, adipokines or SAT transcript profiles

Previously, we showed that synbiotic treatment during the INSYTE trial successfully modified the gut microbiota but did not improve liver fibrosis severity [23]. Consistent with this, no changes specific to the synbiotic treatment group in circulating inflammatory markers (IL-6, IL-8, IL-10, TNFα, MCP-1, hsCRP and GDF-15) or adipokines (leptin and adiponectin) were identified (**Supplementary Figure 1).** Moreover, regression analysis confirmed no significant effects of the synbiotic treatment on the

change in concentrations of inflammatory and adipokine markers (**Supplementary Table 2**). This finding was also observed following similar analysis of the larger INSYTE cohort (N=88, n=44 per treatment group) (**Supplementary Table 3).** We also investigated whether synbiotic treatment influenced SAT transcriptomic profiles in biopsies collected from patients in the INSYTE trial. Paired differential gene expression (DGE) analysis of baseline and end-of-trial SAT transcriptomes from patients who received synbiotic treatment (n=29) or placebo (n=33) did not identify any significant differentially expressed genes (DEGs) associated with either treatment arm (**Supplementary Figure 2**). This observation remained consistent even after only including those considered to be 'responders' [23] to the synbiotic treatment (**Supplementary Figure 2**). Collectively, these results suggest that the synbiotic treatment, previously shown to alter the gut microbiota [23], in patients with NAFLD did not affect circulating markers of inflammation, adipokines or SAT transcriptome.

3.3 – A differential gene expression signature in SAT associates with clinically significant liver fibrosis and is influenced by HOMA-IR but not by sex or adiposity

To explore potential differences in SAT gene expression that may be associated with ≥F2 liver fibrosis, DGE analysis was next performed comparing biopsies from patients with vs without ≥F2 fibrosis at baseline. A total of 229 (113 downregulated and 116 upregulated) DEGs were identified to be significantly (FDR< 0.05) different (**Figure 1a, Supplementary Figure 3a**). Of these 229 DEGs, only 11 genes exhibited an expression fold-change of 2 or more; eight were upregulated, while 3 were down-regulated (**Supplementary data file; 'Baseline fibrosis'**). We next explored whether the number of DEGs associated with ≥F2 liver fibrosis was altered by anthropometric variables known to influence both NAFLD severity and AT biology. Adjusting for sex or adiposity revealed 522 and 418 significant DEGs respectively. Despite the increase in DEGs, all 229 unadjusted DEGs observed in patients with ≥F2 fibrosis remained differentially expressed in patients with NAFLD and ≥F2 liver fibrosis (**Figure 1b**). In contrast, after controlling for HOMA-IR, there was a striking 96% reduction in the number of statistically significant DEGs (FDR <0.05) between patients with vs without ≥F2

fibrosis and only 8 DEGs remained (**Figure 1b).** Importantly, this reduction was not altered after removing patients who were receiving insulin treatment (Data not shown). Adjusting for BMI reduced the number of significant DEGs to 84, with 78 being common to DEGs in the unadjusted analysis (i.e., 34% of DEGs from the unadjusted analysis) (**Supplementary data file** and **Supplementary Figure 3B**). We also observed a strong positive association between HOMA-IR and BMI (r=0.42, P<0.0001), whereas a much weaker association was observed between HOMA-IR and total body adiposity (r=0.26, P=0.04) (**Supplementary Figure 3C-D**). That the synbiotic treatment did not alter SAT transcriptome afforded the opportunity to additionally explore whether differential gene expression in SAT between patients with vs without ≥F2 liver fibrosis was also present at the end-of-trial. In doing so, we observed that of the 229 DEGs observed at baseline, 101 (44.1%) were also differentially expressed (FDR <0.05) at end-of-trial (**Supplementary Figure 3E-F**). Collectively, these findings suggest that in patients with NAFLD and ≥F2 fibrosis, SAT DEGs are associated with systemic insulin resistance but not sex or adiposity.

3.4 - Differential SAT gene expression in patients with NAFLD and ≥F2 liver fibrosis implicates impaired oxidative metabolism and adipogenesis while increasing inflammation and ECM remodelling

To identify which biological processes were enriched in SAT and associated with ≥F2 liver fibrosis, a gene-set enrichment analysis (GSEA) was performed against the non-redundant mSigDB Hallmark gene sets. In patients with NAFLD and ≥F2 fibrosis, five hallmark gene sets were negatively enriched; these included oxidative phosphorylation (OXPHOS), adipogenesis, fatty and bile acid metabolism and KRAS signalling down (**Figure 1c**, **Supplementary Table 4 and Supplementary Figure 4a**). Conversely, sixteen Hallmark gene sets were positively enriched and represented several inflammatory and immune processes (i.e. inflammatory response, IL6-JAK-STAT3 signalling, TNFα signalling via NFκB, IL2-Stat5 signalling, Interferon-gamma response, and complement) (**Figure 1c**, **and Supplementary Table 4 and Supplementary Figure 4b**). For more granular information of

enriched biological and metabolic pathways, GSEA was also performed against the mSigDB Reactome and KEGG gene sets. This identified twelve Reactome and thirteen KEGG gene sets that were negatively enriched and included respiratory electron transport, OXPHOS and tricarboxylic acid cycle (**Supplementary Table 4).** In contrast, a total of forty Reactome gene sets and nineteen KEGG gene sets were positively enriched and included multiple sets related to inflammation and immune cell signalling (i.e., IL-10 signalling, immunoregulatory interactions between a lymphoid and non-lymphoid cell, other IL signalling, IL-12 family signalling, signalling by interleukins, IL-4 and IL-13 signalling and NOD-like receptor signalling pathway) (**Figure 1d** and **Supplementary Table 4**). Importantly, three Reactome-specific gene sets relating to extracellular matrix (ECM) remodelling (i.e., ECM organisation, ECM proteoglycans and degradation of ECM) were also positively enriched in patients with NAFLD and ≥F2 liver fibrosis (**Figure 1d, Supplementary Table 4, Supplementary Figures 4c-d**). We identified 12 genes that were also DEGs (FDR<0.05) and contributed to the positive enrichment of ECM-organisation and ECM proteoglycans (i.e., *COL6A1, COL6A2, FN1, LUM, CD44, CD47, CTSL, VCAN, TGFB3, P3H3 and MMP3*) in SAT of patients with NAFLD and ≥F2 liver fibrosis (**Figure 1e**). Moreover, enrichment of the Reactome ECM organisation gene set was confirmed following GSEA of the end-of-trial data (**Supplementary Table 5**) and this included at least four ECM-organisation-related genes (i.e., *COL6A1, COL6A2, FN1 and TGFB3*) and two additional collagen gene isoforms (*COL8A2 and COL18A1*) (**Supplementary data file;** '**End Fibrosis**'). Collectively, these data suggest that in patients with NAFLD, the presence of ≥F2 fibrosis is associated with altered SAT gene expression signatures linked to decreased mitochondrial oxidative metabolism and adipogenesis, and an increase in adipose tissue inflammation and ECM remodelling.

3.5 - Markers of SAT fibrogenesis associate with clinically significant liver fibrosis in patients with NAFLD

We next explored whether selective gene transcripts indicative of SAT fibrogenesis are also elevated and associated with the presence of ≥F2 liver fibrosis. Targeted assessment of genes encoding 12 collagen isoforms identified 7/12 were significantly increased in patients with ≥F2 liver fibrosis (*P* value <0.05 for all), however, only 2/7 of these were significant according to an FDR threshold of (<0.05) (**Supplementary Table 6**). Moreover, patients with NAFLD and ≥F2 fibrosis had significantly higher CCGE values (0.4 ± 0.5 vs -0.3 ± 0.6 for with vs without ≥F2 fibrosis respectively, P<0.0001) (**Figure 2a**). Consistent with this, the transcript expression of *TIMP1* and *FN1* was also lower in patients without vs with ≥F2 fibrosis: *FN1* (9.1 ± 0.5 vs 9.6 ± 0.7, *P*=0.002) and *TIMP1* (6.0 ± 0.5 vs 6.5 ± 0.5, *P*=0.001). In addition to differential expression, there was a positive linear association between CCGE values and the expression of both *TIMP1* and *FN1* (**Figure 2b-c).** As observed for SAT CCGE values, a significant positive linear association was also observed between and liver VCTE-derived kPa measurements and the expression of both *TIMP1* and *FN1* (**Figure 2d-f**). Interestingly, the expression of *HIF1α* was also positively and linearly associated with the expression of *TIMP1* and *FN1* (r = 0.54, *P*<0.00001 and r = 0.41, *P*=0.001 respectively) along with CCGE values (r = 0.43, *P*<0.001) (data not shown).

To explore whether CCGE values from SAT were associated with the presence of ≥F2 fibrosis independently of potential confounding factors, the univariate associations between CCGE values and other anthropometric and clinical variables were first explored. SAT CCGE values were positively associated with markers of insulin resistance (namely fasting insulin, HOMA-IR, and AdipoIR) and inversely associated with adiponectin concentrations (**Supplemental Table 7**). Additionally, SAT CCGE values were positively associated with circulating triglyceride (TAG), AST, IL-6 and IL-10 concentrations and inversely associated with HDL-cholesterol concentrations (**Supplementary Table 7**). Conversely, SAT CCGE values were not associated with age, BMI, total body fat, fasting glucose, liver fat content, FIB-4, ELF or APRI scores (**Supplementary Table 7**).

In a binary logistic regression model that included sex, age, T2DM status, SAT CCGE values, circulating GDF-15 and adiponectin concentrations as putative explanatory factors and the presence or absence of ≥F2 liver fibrosis as the outcome, only SAT CCGE values, GDF-15 and adiponectin concentrations were independently associated with the presence of ≥F2 fibrosis (**Table 2**). This regression model was statistically significant ($X^2(6) = 42.1$, $P<0.00001$) and explained 67.0% (Nagelkerke R^2) of the variance in the presence or absence of ≥F2 fibrosis. Re-analysis of this regression model (after the addition of HOMA-IR) revealed that the association between SAT CCGE values and the presence of ≥F2 fibrosis was not influenced (data not shown). SAT CCGE values also remained independently and positively associated with the presence of ≥F2 liver fibrosis after including BMI, total body adiposity and leptin concentrations as explanatory variables in separate regression models (data not shown). Similarly, re-analysis of the regression model without the inclusion of GDF-15 and adiponectin concentrations revealed that both SAT CCGE values and the presence of T2DM were independently associated with the presence of ≥F2 liver fibrosis (data not shown).

Stepwise analysis of the model shown in **Table 2** identified that SAT CCGE values alone explained 32.1% of the variance in the presence or absence of ≥F2 fibrosis. Additionally, GDF-15 and adiponectin concentrations explained a further 23.6% and 8.6% respectively of this variance. Furthermore, ROC curve analysis indicated that SAT CCGE values had a good ability to discriminate between the presence or absence of ≥F2 fibrosis in patients with NAFLD (AUROC = 0.79, 95%CI; 0.68-0.90, P<0.001) (**Figure 3a and 3b**). We identified that a CCGE value of 0.1 (Youden Index) provided optimal sensitivity (85.7%) and specificity (68.4%) and had negative and positive predictive values of 82.4% and 64.3% respectively for the predicting the presence of ≥F2 liver fibrosis. Although GDF-15 and adiponectin concentrations were also independently associated with ≥F2 fibrosis and explained a combined 32.2% of the variance in liver fibrosis status, the addition of either of these protein's concentrations to SAT CCGE values did not have a significant effect on AUROC for the prediction of ≥F2 fibrosis (data not shown). Importantly, at the end of the trail, CCGE values were positively

associated with liver stiffness measurements **(Supplementary Figure 5A)** and were significantly higher in patients with vs without ≥F2 fibrosis **(Supplementary Figure 5B).** Similarly, SAT CCGE values were positively associated with the presence of ≥F2 fibrosis although, in our fully adjusted model, this association did not reach conventional statistical significance (**Supplementary Table 8**). Conversely, SAT CCGE values at the end of the trial were significantly associated with the presence of ≥F2 fibrosis after removing adiponectin concentrations from the regression model (**Supplementary table 9**). Consistent with our results at baseline, SAT CCGE values had a good ability to distinguish NAFLD patients with, ≥F2 fibrosis **(Supplementary Figure 5C).** Moreover, the synbiotic treatment used within the INSYTE trial did not affect SAT CCGE values (**Supplementary Table 2**).

By assessing collagen protein deposition, we confirmed that SAT from patients with extremes of CCGE values also exhibited histologically visible differences but this was not reflected in altered Fibrosis score of adipose tissue (FAT) [35] which were all <FAT1 (**Supplementary Figure 6**). A general increase in the amount of pericellular collagen fibres imaged by second harmonic generation (SHG) coupled with two-photon fluorescence (TPF) microscopy was observed in samples with high CCGE values compared to those with lower CCGE values (**Figure 4 and Supplementary Figure 6**). This was less evident with polarised light imaging of picrosirius stained sections. The TPF signals from the pericellular regions also increased but they were not always colocalised with the SHG fibrillar collagen signals. Collectively, these data suggest that in patients with NAFLD, clinically significant liver fibrosis is positively associated with the expression of ECM genes and collagens indicative of increased fibrogenesis in SAT.

4. Discussion

This is the first study to explore the association between SAT gene expression signatures and the presence of ≥F2 liver fibrosis (using Fibroscan®) in patients with NAFLD. There are several key novel findings in this study. Firstly, in SAT from patients with NAFLD, a gene expression signature of increased inflammation, ECM remodelling and tissue fibrogenesis was associated with the presence

of ≥F2 liver fibrosis and was largely explained after adjusting for HOMA-IR. SAT CCGE values were positively and independently associated with ≥F2 fibrosis and explained a large proportion (32%) of the variance in ≥F2 fibrosis status. ROC curve analysis confirmed that SAT CCGE values were a good predictor of ≥F2 liver fibrosis.

We previously reported that in the INSYTE trial, synbiotic treatment affected the composition of gut microbiota by fostering the abundance of *Bifidobacterium* and *Faecalibacterium* at the expense of *Oscillibacter* and *Alistipes.* As previously discussed [23], such changes could have beneficial effects on systemic inflammatory markers. However, in the current study, we did not detect any effect of the synbiotic treatment on either circulating inflammatory markers and adipokines, or on SAT transcriptomic profiles (including CCGE values) even when analysis was carried out in the larger INSYTE cohort. Thus, these data could indicate that the synbiotic-associated alterations in these specific bacterial populations may not influence AT function in patients with NAFLD.

Our findings that the expression of genes associated with inflammation and immune cell signalling in SAT were increased in patients with vs without ≥F2 fibrosis are consistent with the results of previous studies carried out in individuals with less advanced stages of NAFLD (i.e. NAFL and NASH without fibrosis) [17-21,36,37]. Of these previous studies, only one considered the presence of liver fibrosis (>F2 fibrosis) in patients with NASH [20]. However, only 6 individuals with NASH and fibrosis were included and this prevented the option to stratify individuals by the presence of ≥F2 fibrosis. In our study, the reduced expression of genes implicated in OXPHOS, the ETC and adipogenesis in patients with ⩾F2 fibrosis is also consistent with the notion that the expression of these genes is reduced in WAT from individuals with obesity and/or obesity-associated metabolic dysfunction [38-43]. This may further support both the metabolic inflexibility and limited adipose tissue expandability hypotheses. Moreover, recent evidence using functional assays also indicated that the respiratory capacity of WAT was reduced in individuals with NAFLD compared to those without NAFLD [44,45]. Since previous studies observed similar findings in individuals with less severe NAFLD than those

352 explored in our study, it is plausible that the fibrosis-associated gene signature we have observed is
353 indicative of the continued presence and/or development of more severe metabolic dysfunction,
354 rather than liver fibrosis severity *per se*. Indeed, we show for the first time that, unlike sex and
355 adiposity, adjustment for HOMA-IR substantially reduced the number of DEGs associated with ≥F2
356 fibrosis in patients with NAFLD. This important observation may imply that the overarching
357 differences in SAT transcript profiles in patients with NAFLD and ≥F2 fibrosis vs without ≥F2 fibrosis
358 are intimately connected to systemic insulin resistance. Indeed, our findings are consistent with
359 observations made in other studies which observed a positive association between markers of
360 adipose tissue insulin resistance (including AdipoIR) and the presence and severity of liver fibrosis in
361 patients with NAFLD [15].

362 Our findings indicating that OXPHOS was a negatively enriched process in SAT in patients with vs
363 without ≥F2 liver fibrosis appear to contrast with other recent findings indicating that mitochondrial
364 respiration is decreased in VAT but not SAT in obese individuals with fatty liver disease [44]. However,
365 there are some important factors which should be considered when comparing our work to that of
366 Pafili et al [44]. Firstly, in our study, we only present OXPHOS-related data at the transcript level and
367 are thus unable to directly compare SAT mitochondrial enzymatic activity differences between
368 patients with vs without ≥F2 liver fibrosis. Similarly, there are important differences between our
369 cohort and the cohort studied by Pafili et al which may provide potential explanations for the
370 apparent contrasting results. In our study, both sexes were represented relatively evenly whereas
371 the participants in Pafili et al's study were predominantly women. Moreover, the women within our
372 study were post-menopausal whereas those reported in Pafili et al appear to be largely of a pre-
373 menopausal age. Given that sex and menopausal status are known to have substantial effects on
374 adipose tissue biology and function (including beiging), one should be cautious when comparing the
375 results of the present study with those observed in the study by Pafili et al. Moreover, whilst the
376 negative enrichment of OXPHOS and adipogenesis in patients with vs without ≥F2 liver fibrosis
377 appears to support the metabolic inflexibility and the limited adipose tissue expandability

hypotheses, the methods used in our study do not allow us to directly compare SAT expandability nor TAG synthesis between groups as others have done [22].

In our study, we found that the SAT CCGE values were independently and positively associated with the presence of ≥F2 fibrosis. Moreover, SAT CCGE values alone explained 32% of the variance in the presence or absence of this clinically important stage of liver disease severity. This is consistent with our finding that the expression of genes associated with ECM organisation, ECM proteoglycans and the degradation of ECM were positively enriched in SAT from patients with NAFLD and ≥F2 fibrosis. The formation and remodelling of the ECM are required during the expansion of AT in response to prolonged periods of caloric surplus to facilitate an increase in AT mass [46]. Indeed, the expression of genes encoding for components of the ECM in SAT is increased in individuals with obesity compared to those who are lean [47]. Results from a recent study indicated that markers of SAT fibrosis (including CCGE values in SAT) were further increased in individuals with obesity and hepatic steatosis compared to individuals with only obesity, indicating that SAT fibrosis is likely to be associated with hepatic steatosis independently of obesity *per se* [22]. Whilst, in the present cohort, SAT CCGE values were not associated with liver fat content, a strong positive association between these values and ≥F2 fibrosis was observed to be independent of sex, age, adiposity, T2DM status, circulating GDF-15 and adiponectin concentrations and HOMA-IR.

Given the cross-sectional nature of this study, the directionality of the association between SAT CCGE values and liver fibrosis severity cannot be determined. That said, a wealth of literature from pre-clinical models supports the role of AT fibrosis as a factor partly responsible for the development of systemic metabolic complications (as reviewed elsewhere [48,49]). It is well established that NAFLD is a multisystem disease which increases the risk of developing many extrahepatic diseases, including CVD and CKD [1-3]. The risk of these NAFLD-related extrahepatic complications is most strongly associated with the severity of liver fibrosis in NAFLD [2,3]. In line with advanced stages of NAFLD, a key feature of both CVD and CKD is the development of cardiac [50] and renal fibrosis [51], respectively. The

strong positive association between SAT CCGE values and the presence of ≥F2 liver fibrosis, which is independent of a range of potential confounders (i.e., age, sex, T2DM status, GDF-15 concentrations, adiponectin concentrations, HOMA-IR and total body adiposity). This suggests that the association is not dependent on these systemic metabolic factors or adiposity. Thus, the presence of liver fibrosis in NAFLD is likely linked to additional systemic pro-fibrogenic factors which drive the development of fibrosis in extra-hepatic tissues such as SAT. Moreover, this may extend to fibrosis in multiple other tissues implicated in NAFLD-associated comorbidities (e.g. kidney, and heart). Indeed our studies over the last decade have shown NAFLD to be a multisystem disease and is independently associated with incident CKD and incident heart failure[2,52,53]. Although this is currently a hypothesis that needs testing, increasing evidence suggests that the association between NAFLD and extra-hepatic incident disease is stronger with liver fibrosis than it is with liver fat [54]. In the context of NAFLD, hepatic and adipose tissue dysfunction and fibrosis may exacerbate systemic metabolic dysfunction, consequently forming a bidirectional relationship between adipose tissue and liver dysfunction. It is plausible that this bidirectional relationship between hepatic and adipose tissue function results in changes in the release of various pro-fibrogenic factors which contribute to the development of fibrosis in other tissues including the heart and kidney. Consequently, further studies are warranted to determine whether the full complement of fibrotic tissues may co-exist in patients with NAFLD and ≥F2 liver fibrosis.

Although the development of fibrosis is tissue-specific, it is known to involve the following key stages; tissue dysfunction/damage response, chronic inflammation, proliferation of pro-fibrotic (collagen-producing) cells and ECM reorganisation [55,56]. In obesity, AT fibrosis can occur during unhealthy tissue expansion following unresolved chronic inflammation and localised hypoxia [46]. Clinical studies have also suggested that chronic hypoxia in AT increases inflammation and is associated with an elevation in the expression of genes encoding for ECM proteins [57-59] Indeed, in the current study, the presence of ≥F2 fibrosis was associated with an increased expression of genes associated with all these stages, as well as HIF-1α. Moreover, the expression of *HIF-1α* gene was

positively associated with the expression of gene markers of fibrogenesis, including CCGE values in SAT. Collectively, these findings indicate that increased SAT fibrosis is observed in patients with NAFLD and ≥F2 fibrosis.

This study has numerous strengths. For example, we were able to undertake a randomised placebo-controlled trial with paired baseline and end-of-trial biopsies of SAT. Moreover, this is the largest study exploring SAT transcriptomic profiles in relation to ≥F2 liver fibrosis using data generated from a high depth of sequencing. Furthermore, prior to biopsy collection, patients were not subjected to calorie-restrictive diets that are typically utilised in individuals undergoing weight-loss bariatric surgery. That said, it is important to acknowledge that other studies exploring transcript profiles in VAT in the context of obesity and/or NAFLD also suggest increased inflammation and mitochondrial dysfunction with greater disease severity (i.e. NAFL vs NASH) [19,20,60]. Given the proximity of VAT to the liver and the gut (potentially indicating it is a more plausible target of intestinal dysbiosis), VAT dysfunction may be more strongly involved in the development and progression of NAFLD. However, access to VAT is challenging and requires a much more invasive procedure compared to that required to obtain a SAT biopsy.

The main limitation of this exploratory study is that the identification of NAFLD patients with ≥F2 fibrosis was determined using a previously validated VCTE-derived threshold of ≥8.2 kPa [30], rather than liver histology-diagnosed fibrosis. That said, growing evidence indicates that liver VCTE has good diagnostic accuracy for the non-invasive identification of liver fibrosis in patients with NAFLD [61]. Furthermore, a recent large study validated the use of a liver VCTE threshold of ≥8.2 kPa as a good diagnostic threshold for identifying ≥F2 fibrosis on histology (AUROC; 0.77, 95%CI; 0.72-0.82) [30]. Whilst our study is the largest to explore SAT transcriptome profiles in patients with NAFLD and ≥F2 liver fibrosis, it includes a relatively small number of patients which may mean that it lacks sufficient statistical power to detect differences between groups, and/or independent associations between a) some risk factors and the presence of ≥F2 liver fibrosis and b) the effects of the synbiotic treatment

on circulating inflammatory markers, adipokines and SAT transcript profiles. That said, we have improved confidence in our findings demonstrating that the key observations made with the baseline dataset were largely reproduced in paired biopsies at end-of-trial.

In conclusion, the results of this exploratory study show for the first time that in patients with NAFLD, the presence of ≥F2 fibrosis was associated with a specific SAT gene expression signature that indicated an increased expression of inflammatory genes and ECM remodelling and a decrease in adipogenic and oxidative metabolism genes. The observed differences in SAT DEGs were markedly influenced by insulin resistance (estimated by HOMA-IR) and, a gene expression marker of SAT fibrogenesis predicted and explained a large portion of variance in ≥F2 liver fibrosis. Furthermore, we showed that a synbiotic treatment that modified the gut microbiota did not significantly affect SAT gene expression profiles, inflammatory markers or adipokine concentrations. Future studies should further look to validate our findings in larger cohorts of patients with NAFLD and determine whether a similar gene signature of SAT fibrosis is a reliable marker of extra-hepatic tissue fibrosis. This is particularly important because NAFLD, not only affects the liver, but is also associated with an increased risk of developing several extra-hepatic diseases linked to tissue fibrosis such as heart failure and CKD [2,3].

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Author contribution statement

Study concept and design, CDB, JB, PCC, JKS; acquisition of data, CDB, JB, SM, JR, ES, JKS; analysis and interpretation of data, CDB, JB, SM, CJO, JR, JKS; drafting of the manuscript, CDB, JB, CJO, JKS; critical revision of the manuscript for important intellectual content, PRA, CDB, DB, JB, LB, PCC, JL, SM, CJO, JR, ES, JKS, GT; statistical analysis, CDB, JB, JKS; obtained funding, CB, DB, PCC, SM, JKS; administrative technical, or material support PRA, CDB, DB, JB, LB, PCC, JL, SM, CJO, JR, ES, JKS; study supervision CBD, DB, PCC, JL, SM, JKS.

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Data statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflicts of interest:

CDB has received an independent research grant from ECHOSENS. The authors declare no other competing interests.

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673 **Table 1 – Patient characteristics stratified by the presence or absence of ≥F2 liver fibrosis.**

Variables	<F2 fibrosis (n=38)	≥ F2 fibrosis (n=24)	*P* value
Age, years	53.0 ± 13.4	54.2 ± 8.0	0.67
Sex, male (%)†	23 (60.5)	14 (58.3)	0.86
Menopausal status, post-menopausal (%)†	15 (100.0)	8 (80.0)	0.07
BMI, kg/m^2	32.6 ± 5.6	34.8 ± 3.2	0.05
Total body fat, %	35.3 ± 7.5	35.8 ± 6.6	0.76
Truncal fat, %	36.2 ± 7.2	37.1 ± 6.2	0.58
Truncal subcutaneous fat, %	33.0 ± 10.1	32.8 ± 8.3	0.91
Truncal visceral fat, %	17.4 (7.5)	17.4 (5.1)	0.90
Truncal SAT : VAT mass	2.1 ± 1.1	2.1 ± 1.2	0.98
SAT adipocyte area (μm^2)[b]	5815 ± 858	6069 ± 913	0.34
MetS, yes (%)†	28 (73.7)	23 (95.8)	**0.03**
T2DM, yes (%)	14 (36.8)	16 (66.7)	**0.02**
Glucose, mmol/L	6.0 (2.0)	7.1 (4.3)	0.05
HbA1c, mmol/mol	39.5 (13.0)	54.5 (30.3)	0.06
Oral antihyperglycemic treatment, yes (%)	10 (26.3)	15 (62.5)	0.005
Insulin treatment, yes (%)	1 (3.8)	3 (6.2)	0.16
Insulin, mIU/L	10.1 (8.5)	16.5 (16.3)	**0.001**
HOMA-IR	3.4 (2.3)	6.3 (4.9)	**<0.0001**
AdipoIR[a]	19.3 (33.4)	46.5 (51.8)	**0.006**
NEFA/body fat, mmol/L/kg[a]	0.01 ± 0.01	0.01 ± 0.01	0.44
NEFA, mmol/L[a]	0.4 (0.3)	0.4 (0.2)	0.20
Cholesterol, mmol/L	5.2 ± 1.4	4.5 ± 1.1	**0.05**
HDL-C, mmol/L	1.3 ± 0.3	1.1 ± 0.2	**0.007**
TAG, mmol/L	1.7 (0.8)	1.8 (1.4)	0.22
Liver fat content, %	21.0 (27.0)	30.5 (23.2)	0.16
AST, IU/L	29.0 (16.8)	44.0 (32.5)	**0.02**
ALT, IU/L	51.0 (28.1)	64.3 (29.4)	**0.045**
Liver VCTE, kPa	5.8 (1.8)	11.6 (4.5)	**<0.0001**

FIB4 score	0.9 (0.5)	1.3 (0.8)	**0.001**
ELF score	6.9 ± 0.4	7.0 ± 0.4	0.43
APRI score	0.9 (0.5)	1.3 (0.8)	**0.003**
GDF-15, pg/ml	752.6 (481.1)	1315.2 (1340.4)	**<0.001**
TNFα, pg/ml	10.0 (4.8)	13.3 (5.2)	**0.02**
IL-6, pg/ml	2.5 (1.5)	3.2 (1.4)	**0.02**
MCP-1, pg/ml	282.6 (142.2)	277.5 (151.1)	0.75
IL-8, pg/ml	13.2 (9.9)	18.2 (9.8)	**0.003**
IL-10, pg/ml	0.7 (0.4)	1.0 (0.9)	**0.008**
hs-CRP, mg/l	2.7 (3.3)	3.0 (4.8)	0.50
Leptin, ng/ml	22.0 (32.2)	26.2 (29.8)	0.53
Adiponectin, µg/ml	5.0 (3.8)	3.5 (1.5)	**0.002**

Data are presented as means ± SD or medians (IQR) for normally and non-normally distributed variables respectively. Variables with dichotomised variables are labelled with †. Of those patients with NAFLD and T2DM, 23/30 (77%) were receiving antihyperglycemic treatment. The following indicates numbers where data was not available for all participants: [a] n=37 vs 21, [b]n=31 vs 18.

Abbreviations: BMI, body mass index; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; MetS, metabolic syndrome; T2DM, type 2 diabetes mellitus; HbA1c, haemoglobin A1c; HOMA-IR, homeostatic model assessment for insulin resistance; AdipoIR, adipose tissue insulin resistance index; NEFA, non-esterified fatty acid; HDL-C, high density lipoprotein cholesterol; TAG, triacylglyceride; AST, aspartate aminotransferase; ALT, alanine aminotransferase; VCTE, vibration-controlled transient elastography; FIB4, fibrosis-4; ELF, enhanced liver fibrosis score; APRI, AST to platelet ratio index; GDF-15, growth differentiation factor-15; TNFα, tumour necrosis factor alpha; IL, interleukin; hs-CRP, high-sensitivity C reactive protein.

Table 2 - Factors independently associated with the presence of ≥F2 liver fibrosis at baseline.

Variables	OR (95% CI)	P value
Sex (M vs. F)	1.64 (0.32 – 8.35)	0.55
Age (years)	1.04 (0.96 – 1.12)	0.39
T2DM status (yes)	0.44 (0.06 – 3.22)	0.42
SAT CCGE (z-scores)	8.37 (1.72 – 40.88)	**0.009**
GDF-15 (pg/ml)	1.003 (1.001 – 1.006)	**0.006**
Adiponectin (µg/ml)	0.50 (0.29 – 0.85)	**0.01**

Dependent variable was liver VCTE measurements <8.2 vs. ≥8.2 kPa (0 and 1, respectively) as a proxy threshold for the non-invasive identification of ≥F2 fibrosis. Binary logistic regression exploring the effects of sex, age, SAT CCGE values, circulating adiponectin concentrations and T2DM status on the likelihood that patients have ≥F2 fibrosis. This regression model was statistically significant (X2(6) = 22.1, P<0.001) and explained 67.0% (Nagelkerke R2) of the variance in the outcome variable. Hosmer and Lemeshow Test P=0.95. Sample size n=62. SAT CCGE values remained independently and positively associated with the presence of ≥F2 liver fibrosis after the inclusion of BMI, total body adiposity, leptin concentrations, HOMA-IR or fasting insulin concentrations (when these additional exposures entered in separate regression models) and none of these additional exposures were associated with the presence of ≥F2 liver fibrosis independently of the other factors within the model. Re-analysis of this regression model without the inclusion of GDF-15 and adiponectin concentrations revealed that both SAT CCGE values and the presence of T2DM were both independently associated with the presence of ≥F2 liver fibrosis.

Abbreviations: T2DM, type 2 diabetes mellitus; SAT, subcutaneous adipose tissue; CCGE, composite collagen gene expression; GDF-15, growth differentiation factor-15.

Figure Legends

Figure 1: Differentially expressed genes in SAT of patients with ≥F2 liver fibrosis are influenced by HOMA-IR and enriched for gene sets linked to increased inflammation and extracellular matrix. A) Volcano plot of unadjusted differentially expressed genes in SAT associate ≥F2 liver fibrosis at baseline. **B)** Venn diagram showing that the vast majority of SAT DEGs associated with >F2 liver fibrosis (FDR≤0.05) at baseline are unaffected after adjusting for sex or adiposity but are reduced after adjusting for HOMA-IR. **C-D)** GSEA against the hallmark **(C)** and Reactome **(D)** gene sets showing significantly enriched gene sets in patients with NAFLD and ≥F2 fibrosis. **E)** Heat map of DEGs (FDR≤0.05) represented in "Reactome Extracellular matrix organisation" gene set. n=62

Figure 2: SAT CCGE is increased in patients with NAFLD and ≥F2 fibrosis and associates with FN1, TIMP1 and liver VCTE-derived kPa measurements. A) Bar chart comparing SAT CCGE values between NAFLD patients with vs without ≥F2 fibrosis. Data are expressed as means ± SD – Note a retrospective power calculation indicated that we had a power of 96.6% to detect the observed difference in CCGE between groups. Scatter plots of univariable correlation analysis between the SAT CCGE values and the expression of **B**) *TIMP1* and **C**) *FN1* (log2cpm) in SAT. Scatter plots of univariable correlations analysis between liver VCTE-derived kPa measurements; and **D**) SAT CCGE and the expression of **E**) *TIMP1* and **F**) *FN1* in SAT (log2cpm). n=62

Figure 3: ROC curve of SAT CCGE for ≥F2 fibrosis. A) ROC curve of SAT CCGE values for the prediction of ≥F2 fibrosis in patients with NAFLD. **B)** Histogram showing the distribution of CCGE values (z-scores) at baseline with mean ± SD for each group and the Youden index cutoff (J) for the identification of ≥F2 fibrosis. n=62

Figure 4: **Histological imaging demonstrates presence of pericellular collagen fibres in SAT from patients with ≥F2 fibrosis and highest CCGE z scores.** Representative SAT regions of interest were selected from patients **A**) with <F2 liver fibrosis and the lowest CCGE value (-1.15) and **B**) with ≥F2 liver fibrosis and the highest CCGE value (1.35). Paraffin embedded serial sections (5µm) were either stained with Picrosirius red (sirus red) or left unstained. Images were acquired with polarised light (PL) and bright field microscopy or with multiphoton second harmonic generation (SHG), two-photon autofluorescence (TPF) and bright-field microscopy as detailed in supplemental methods. All images were taken at 10x magnification, scale bar: 100 µm.

Abbreviations

AdipoIR - Adipose tissue insulin resistance index

ALT - Alanine aminotransferase

APRI - AST to platelet ratio Index

AST - Aspartate aminotransferase

AT - Adipose tissue

AUROC - Area under the receiver-operator characteristic

BMI - Body mass index

CCGE - Composite collagen gene expression

CKD - Chronic kidney disease

CVD - Cardiovascular disease

DEG - Differentially expressed gene

DEXA - Dual-energy x-ray absorptiometry

DGE - Differential gene expression

ECM - Extracellular matrix

ELF - Enhanced liver fibrosis

ETC - Electron transport chain

FDR - False discovery rate

FIB-4 - Fibrosis-4

FSH - Follicle-stimulating hormone

GDF-15 - Growth differentiation factor-15

GLM - Generalised linear model

GSEA - Gene-set enrichment analysis

HA - Hyaluronic acid

HbA1c - Haemoglobin A1c

HDL - High-density lipoprotein

HIF-1α - Hypoxia-inducible factor 1-alpha

HOMA-IR - Homeostasis model assessment-insulin resistance

hs-CRP - High-sensitivity C-reactive protein

IL- Interleukin

INSYTE - Investigation of synbiotic treatment in NAFLD

IR - insulin resistance

KEGG - Kyoto Encyclopaedia of Genes and Genomes

kPa - Kilopascal

KRAS - Kirsten rat sarcoma virus

MCP-1 - Monocyte chemoattractant protein-1

MetS - Metabolic syndrome

MRI - Magnetic resonance imaging

MRS - Magnetic resonance spectroscopy

NAFL - Non-alcoholic fatty liver

NAFLD - Non-alcoholic fatty liver disease

NASH - Non-alcoholic steatohepatitis

NEFA - Non-esterified fatty acid

NFκB - Nuclear factor kappa B

OR - Odds ratio

OXPHOS - Oxidative phosphorylation

RIN - RNA integrity

RNA - Ribonucleic acid

RNAseq - Ribonucleic acid sequencing

ROC - Receiver-operator characteristic

SAT - Subcutaneous adipose tissue

SHG – Second harmonic generation

SPSS - Statistical Package for the Social Sciences

T2DM - Type 2 diabetes mellitus

TAG - Triacylglyceride

TIMP-1 - Tissue inhibitor of metallo-proteinase-1

TNFα - Tumour necrosis factor alpha

TPF – Two-photon fluorescence

VAT - Visceral adipose tissue

VCTE - vibration-controlled transient elastography

WAT - White adipose tissue