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Mixed-meal challenge differentially modulates metabolic pathways in adipose tissue in healthy abdominally obese subjects with high versus low liver fat: a secondary analysis of a randomized clinical trial

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Abstract

Background Increased liver fat increases the risk of chronic metabolic diseases. This study is an exploratory secondary analysis aimed at (1) investigating whether transcriptomic responses of abdominal subcutaneous adipose tissue (SAT) to a high-fat-high-glucose meal challenge differ according to varying levels of liver fat accumulation and (2) identifying pathways in abdominal SAT metabolism that may be related to liver fat accumulation. We examined differences in abdominal SAT gene expression and pathway activity both at fasting and in response to a mixed-meal challenge, comparing individuals with varying levels of liver fat.

Method From the subset of 66 of 110 middle-aged participants of a previous intervention study, we grouped participants by tertiles of intrahepatic lipids (IHL) into high liver fat group ($n = 22$, IHL: 8.0%-32.6%), middle liver fat group ($n = 22$, IHL: 2.5%-8.0%) and low liver fat group ($n = 22$, IHL: 0.1%-2.5%). Participants received a high-fat-high-glucose mixed-meal challenge (3833 kJ). Abdominal SAT samples were collected before and 4 h after the challenge for microarray gene expression analysis.

Results At fasting, 87 gene sets were differently expressed (FDR < 0.25) between the high and the low liver fat group, and 66 gene sets were differently expressed between the high and middle liver fat group, pathways related to energy metabolism were lower expressed in the high compared to the low liver fat group. Postprandially, 17 gene sets responded differently to the mixed meal challenge, of which 7 changed within the high liver fat group, 2 changed within the middle liver fat group and 4 within the low liver fat group. The challenge increased the expression of genes involved in oxidative phosphorylation more in the high compared to the low liver fat group.

Conclusions Compared to individuals with low liver fat, individuals with high liver fat have lower gene expression but a higher response of energy-related pathways in abdominal SAT at fasting and after a high-fat-high-glucose

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challenge. Whether this is the cause or consequence of increased liver fat storage or an early stage of insulin resistance needs to be investigated.

Trial registration This trial was registered at clinicaltrials.gov as NCT02194504.

Keywords Obesity, Adipose tissue, Liver fat accumulation, Fat metabolism, Gene expression

Background

In the last decades, there has been a global rise in obesity making it prevalent among the world's population. Many health problems are caused or exacerbated by obesity [1], such as type 2 diabetes, metabolic dysfunction-associated steatite liver disease (MASLD), which was previously known as non-alcoholic fatty liver disease (NAFLD), and cardiovascular disease [2, 3]. Adipose tissue and the liver play significant roles in the regulation of whole-body energy homeostasis [4]. The metabolic activity of both adipose tissue and the liver is tightly controlled by insulin and other metabolic hormones [5]. The SAT is the most important energy storage depot in the body [6]. If the SAT is not able to accurately store the increased amount of energy in the form of fat because of adipose tissue dysfunction, the energy will partly be stored viscerally and at ectopic places such as the liver. Mitochondria also plays a key role in SAT by regulating energy metabolism, lipid oxidation, ATP production, and thermogenesis, and mitochondrial dysfunction in adipose tissue has been linked to metabolic disorders such as insulin resistance and obesity [7]. The increased accumulation of fat in the liver leads to MASLD, which may progress to metabolic dysfunction-associated steatohepatitis (MASH), which was previously known as non-alcoholic steatohepatitis (NASH), then to cirrhosis, and eventually to liver failure [4, 5, 8].

Examining the adipose tissue of people in the fasting state does however not reflect the metabolic capacity of the adipose tissue to respond to an energy load that needs to be stored after such as a meal. A widely used test to evaluate phenotypic flexibility i.e. the capacity of metabolic organs to respond to a challenge and the ability to maintain or regain homeostasis is by performing challenge tests [9, 10]. The oral glucose tolerance test is a well-known test that is used to measure how well the body can deal with a large amount of glucose [11]. In analogy to the metabolic function of OGTT, high-fat tolerance tests with high glucose, high fat or high protein have also been applied as a challenge to evaluate the metabolic adaptation capacity by evaluating the postprandial responses to a meal [12–14]. Most of these postprandial responses are described by measurements of plasma glucose, insulin and lipids. However, blood reflects the sum of all processes in metabolic organs and not of each organ individually. It is difficult to access the metabolic response of each organ individually as especially in relatively healthy people tissues and organs such as the liver are not easily accessible. A metabolic active tissue that is still accessible in volunteers is the SAT which plays a key role in the storage of excess of energy. The adipose tissue is critical for determining the fluxes of lipids to the liver in both the fasting and fed states, the liver can also in turn signal to the adipose tissue to modulate lipolysis [15]. To explore the potential mechanism in adipose tissue underlying liver fat accumulation in overweight subjects, we would like to explore how far the signaling response in the adipose tissue towards a meal high in fat and glucose is different between people with high versus low liver fat. Therefore, we compared the whole genome gene expression profile response of SAT, both in the fasting state and in response to a meal challenge in individuals with low and high liver fat.

Methods

Participants

The study population consisted of a subpopulation of a previously described study [16]. In this study, 110 participants were involved in a dietary intervention to explore the nutritional regulation of metabolic health. Of these 110 individuals, intrahepatic lipids (IHL) and adipose tissue microarray data were available for 66 persons. To select people with high, middle and low liver fat, the whole group were divided by tertiles. Subjects in the first tertile (0.1%–2.5%) were classified as the low liver fat group ($n = 22$), subjects in the middle tertile (2.5%–8.0%) were classified as the middle liver fat group ($n = 22$), and subjects in the last tertile (8.0%–32.6%) were classified as the high liver fat group ($n = 22$). All of the participants were abdominally obese and otherwise healthy at the time of recruitment. Inclusion factors were body mass index (BMI) > 27 kg/m^2 or a waist circumference > 88 cm in women or > 102 cm in men. Exclusion criteria were: (1) Earlier diagnosed with diabetes or diagnosed during our screening (OGTT, fasting glucose > 7mmol/L, after 2 h > 11.1mmol/L). (2) Daily alcohol intake of > 20 g (women) or 30 g (men). (3) Smoking (4) Unstable body weight (weight gain or loss of > 3 kg in the past three months). (5) Diagnosed with a long-term medical condition. (6) Using medication is known to interfere with glucose or lipid homeostasis. (7) Being allergic to fish oil or restricted to a vegetarian dietary regime.

Study design

This study was a sub-analysis of a previously conducted parallel-designed randomized intervention study conducted at Wageningen University, Netherlands [16]. For this analysis, we only included the baseline data before the intervention. A high-fat-high-glucose mixed meal shake was offered to subjects after an overnight fasting. The mixed meal shake was 3833 kJ and comprised 76.3 g of carbohydrates, 17.6 g of protein, and 60 g of fat. Before this day, they were asked to refrain from alcohol, avoid strenuous exercise, ensure a sufficient night's rest, and consume a low-fat standardized meal before 20:00, after which they had to fast for at least 12 h, the same with which were reported previously [16, 17]. Blood was drawn before and at 30, 60, 120, and 240 min after consumption of the mixed meal shake and analyzed for plasma glucose and at 60, 120, and 240 min for plasma insulin. Blood samples were collected before and at 120, 240 and 360 min after the mixed meal shake and analyzed for plasma triglycerides and free fatty acids (FFA). Adipose tissue biopsy samples of these participants were taken before and 4 h after this mixed meal. The 4-hour postprandial time point was chosen based on prior studies involving postprandial adipose tissue biopsies [18, 19]. In addition, our selection was guided by the hypothesis that both metabolic and transcriptional responses to the ingestion of a mixed meal might be initiated and become detectable around this time point.

Clinical measurements

Intrahepatic lipid values were measured by image-guided single-voxel spectroscopy, a quantitative version of 1 H-magnetic resonance spectroscopy (MRS), plasma glucose, insulin and triglycerides were analyzed photometrically (Cobas 8000, Roche Diagnostic Limited) by a center for medical diagnostics (Stichting Huisartsenlaboratorium Oost). Plasma free fatty acids were determined using an enzymatic assay (INstruchemie). Heart rate was assessed automatically (DINAMAP PRO100) for 10 min with a 3-minute interval, Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was measured using the formula fasting insulin × fasting glucose/22.5, with fasting insulin expressed in μU/ml and fasting glucose expressed in mmol/l, more details can be found in the previous paper [16]. Adipose tissue insulin resistance index (Adipo-IR), which was established to assess the insulin sensitivity of the adipose tissue, was calculated by multiplying the fasting insulin (mU/L) by the fasting FFA (mmol/L) [20, 21].

RNA isolation

Adipose tissue samples were taken before and 4 h after the mixed milkshake and were immediately frozen. The RNA of these adipose tissue samples were isolated by the Trizol method (Trizol-chloroform extraction; Thermo Fisher Scientific), purified by Qiagen Mini column kit according to the protocol of the manufacturer (Qiagen) and qualified by Nanodrop ND 1000 (Nanodrop Technologies, Wilmington, DE, USA). RNA integrity number was checked by an Agilent 2100 Bioanalyser with RNA 6000 microchips (Agilent Technologies, South Queensferry, UK). All RNA samples that were selected for gene expression analysis had an RNA integrity number (RIN) > 6.2.

Microarray processing

Total RNA was labelled using a 1-cycle cDNA labelling kit (MessageAmp™ II-Biotin Enhanced Kit; Ambion, Inc., Nieuwekerk a/d IJssel, Netherlands) and hybridized to GeneChip Human Gene 2.1 ST arrays (Affymetrix, Inc. Santa Clara, CA, USA; RRID: SCR_007817). Sample labelling, hybridization to chips, and image scanning were performed according to the manufacturer's instructions. Raw CEL files were normalized by using the Robust Multi-array Average algorithm [22], as implemented in the affyPLM R package [23]. We used a custom annotation based on reorganized oligonucleotide probes that combine all individual probes for a gene (MBNI Brainarray CDF file; ENTREZG v21) [24]. Genes with log2 signal > 3.5 in at least 20 arrays were defined as expressed and selected for further analysis.

Statistical analysis

In this study, fasting characteristics were compared between the liver fat groups, one-way ANOVA with Bonferroni post hoc pairwise comparisons was used for comparisons of numerical data (mean ± SD), and Fisher's exact test was used for categorical data (%) (software IBM SPSS Statistics version 28; RRID: SCR_016479). Principal component analysis (PCA) was performed for the expressed genes at fasting to explore the patterns of adipose tissue gene expression at fasting across liver fat groups. Differentially expressed genes were identified by using linear models that incorporate an empirical Bayes method to shrink probe-wise sample variances towards a common value (Bioconductor library limma; RRID: SCR_010943) [25]. To account for potential confounding by insulin sensitivity, HOMA-IR was included as a covariate. The expression of genes was defined to be significantly different liver fat groups when the moderated p-value was <0.05 To correct for multiple testing, the Benjamini-Hochberg method was used to calculate false discovery rates (FDR).

Changes in gene expression were related to biologically meaningful changes using gene set enrichment analysis (GSEA) [26]. GSEA evaluates gene expression at the level of gene sets that are based on prior biological knowledge, e.g. published information about biochemical pathways or signal transduction routes, allowing more reproducible

and interpretable analysis of gene expression data. As no gene selection step (fold change and/or p-value cut-off) is used, GSEA is an unbiased approach. GSEA was performed using the Bioconductor package clusterProfiler (RRID: SCR_016884) [27]. Genes were ranked by their moderated t-value. Gene sets were retrieved from the expert-curated Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database [28]. Only gene sets comprising more than 15 and fewer than 500 genes were taken into account. Gene sets with FDR < 0.25 were considered to be significantly changed.

Ingenuity pathway analysis

Outcomes from microarray analysis were also analyzed by Ingenuity Pathway Analysis (IPA; RRID: SCR_008653) system for upstream regulators. This analysis can determine likely upstream regulators that are connected to dataset genes through a set of direct or indirect relationships. An activation Z-score is defined by this analysis to find likely regulating molecules based on a statistically significant pattern match of up-and down-regulation and also to predict the activation state (either activated or inhibited) of a putative regulator [29]. Regulators with a P-value of overlap < 0.05 and a Z-score > 2 or < −2 between fasting (T = 0) and postprandial (T = 240 min) were set as activated or inhibited within the group. Regulators with the P-value of overlap < 0.05 and the Z-score > 2 or < −2 in the changes of gene expression between the high liver fat group and the low liver fat group were set as differently regulated between the two groups.

Results

Subject characteristics

The flow diagram of the selection of the 22 individuals with low liver fat and the 22 individuals with high liver fat is shown in Fig. 1.

The fasting characteristics of the study population according to liver fat groups are shown in Table 1. As expected, the HOMA-IR, Adipo-IR and fasting plasma

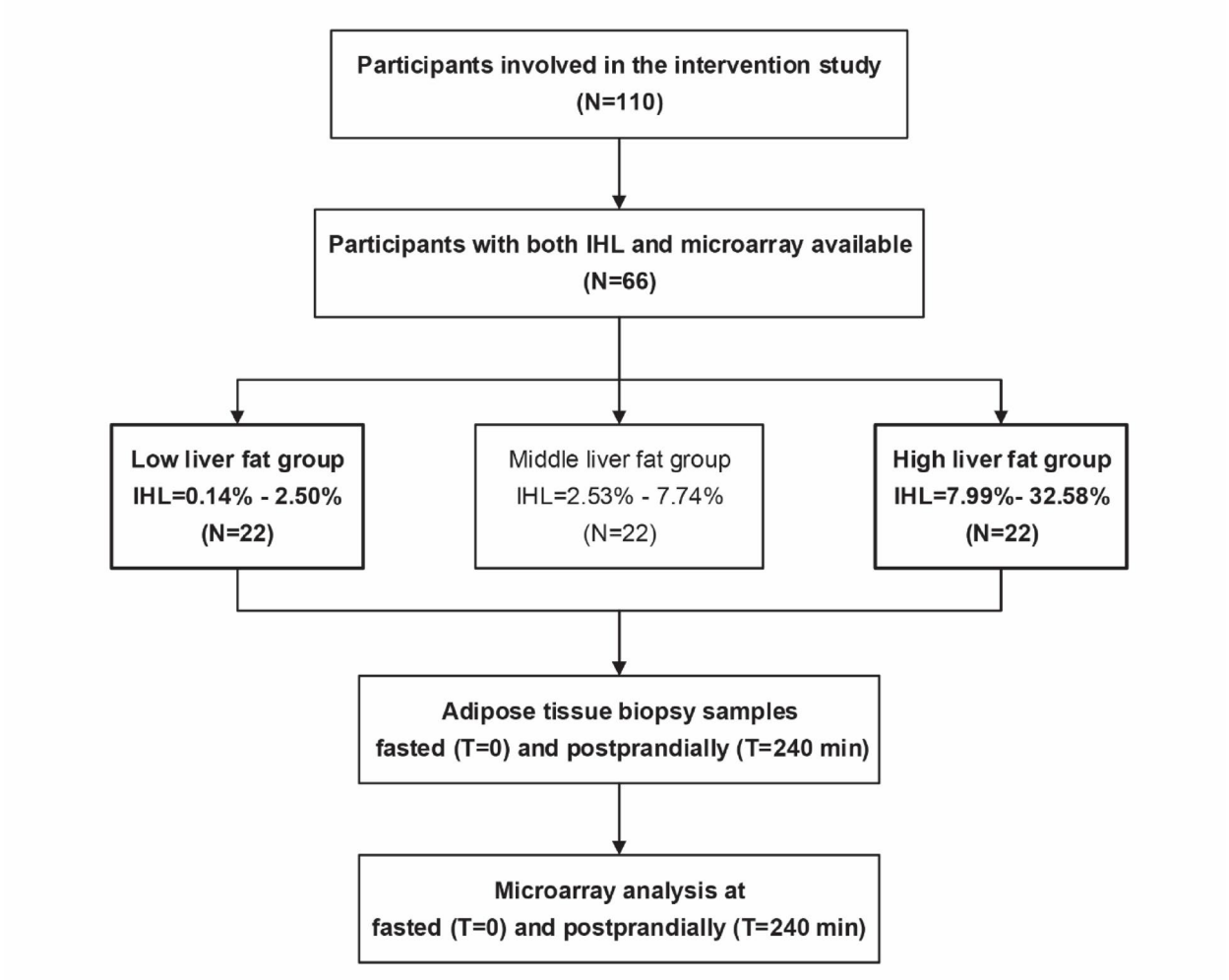

Fig. 1 Flow diagram of the selection of the 22 individuals with low liver fat and the 22 individuals with high liver fat. IHL: intrahepatic lipid

Table 1 Fasting characteristics of subjects according to liver fat groups

Low liver fat group ($n=22$)	Middle liver fat group ($n=22$)	High liver fat group ($n=22$)	*P*-Value
Age (years)	59 ± 8	58 ± 8	59 ± 8	0.77
Women, n (%)	12 (54.5%)	8 (36.4%)	10 (45.5%)	0.48
BMI (kg/m^2)	30.9 ± 3.6	31.5 ± 4.4	32.4 ± 2.7	0.39
Weight (kg)	92.0 ± 13.6	93.6 ± 14.6	95.7 ± 14.6	0.69
Waist circumference (cm)	107.7 ± 10.7	107.3 ± 8.8	109.1 ± 9.2	0.82
Heart rate	58 ± 7	63 ± 9	59 ± 9	0.14
Ratio VAT/SAT (%)	40 ± 30	45 ± 43	65 ± 2	0.21
Plasma FFA (mmol/L)	0.40 ± 0.11	0.5 ± 0.2	0.44 ± 0.22	0.07
Plasma glucose (mmol/L)	5.6 ± 0.5	5.5 ± 0.5	5.6 ± 0.7	0.76
Plasma insulin (mU/L)	9.5 ± 3.0^a	12.9 ± 5.5^b	19.7 ± 12.3^b	< 0.001
HOMA-IR	2.374 ± 0.83^a	3.2 ± 1.5^a	5.162 ± 3.758^b	< 0.001
Adipo-IR	3.889 ± 2.061^a	7.4 ± 5.6^a	8.879 ± 8.40^b	0.02

Data are presented as means ± 1 SD. Different letters (a and b) indicate significant post hoc differences ($P<0.05$) between liver fat groups. Differences between the liver fat groups were assessed using one-way ANOVA for numerical data (mean ± SD) and Fisher's exact test for categorical data (gender)

BMI, body mass index; HOMA-IR, homeostatic model assessment for insulin resistance; Adipo-IR, adipose tissue insulin resistance index; VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue; FFA, free fatty acids

insulin differed across different liver fat groups. HOMA-IR and Adipo-IR were significantly higher in the high and middle liver fat group compared to the low liver fat group, and fasting plasma insulin was higher in the high liver fat group compared to the middle and low liver fat group. All other variables were not different between both groups.

Metabolic response

Comparisons of the postprandial response between the high and low liver fat group of plasma glucose, insulin, triglycerides and FFA are shown in Fig. 2. Analyses by linear mixed-effects models showed that the high liver fat group responded with higher postprandial plasma glucose and insulin response at 60 and 120 min (Fig. 2A and B) and higher postprandial plasma triglycerides at 360 min, compared with the low liver fat group (Fig. 2C). Response in plasma FFA was not different between both groups (Fig. 2D). The postprandial responses of these clinical markers in the middle liver fat group were generally intermediate between those of the high and low liver fat groups (Fig. 2).

Gene expression changes in the adipose tissue

PCA of gene expression profiles at fasting showed that the high liver group diverged from the middle and low groups primarily along PC2 (Fig. 3A), while the middle and low liver fat groups overlapped substantially, reflecting lower variability in their adipose tissue gene expression patterns. The number of genes significantly differently expressed in the SAT between the high and the low liver fat groups at fasting and towards the mixed-meal challenge are shown in Fig. 3B. At fasting, 1733 genes were significantly differently expressed between the high and the low liver fat groups, 2657 genes were significantly differently expressed (moderated $p<0.05$) between the high and middle liver fat groups, and 1681 genes were significantly differently expressed between the middle and the low liver fat groups (moderated $p<0.05$). However, after the false discovery rate (FDR) correction, the number of significant genes was reduced to 108, 572 and 41, respectively. After the mixed meal challenge, 637 genes showed a significantly different response (T4-T0) between the high and the low liver fat groups, and 621 genes showed a significantly different response between the middle and the low liver fat groups. However, after FDR correction, no genes remained significantly different in their responses between either the high and low or the middle and low liver fat groups. Additionally, the top 10 differentially responsive genes between the high and low liver fat groups exhibited a similar pattern of response in the middle and high liver fat groups. However, a distinct response pattern was observed in the low liver fat group (Supplemental Fig. 1).

Gene set enrichment analysis (GSEA)

Although no genes remained significantly different in their responses between either the high and low or the middle and low liver fat groups after FDR correction, this does not rule out the possibility of subtle but coordinated changes in gene expression. To identify these changes, Gene set enrichment analysis (GSEA) was performed. At fasting 87 gene sets were significantly differently expressed between the high and the low liver fat group, 66 gene sets were significantly differently expressed between the high and middle liver fat group, and 93 gene sets were significantly differently expressed between the middle and low liver fat group. Additionally, 17 gene sets responded differently postprandially to the mixed meal, of which 7 changed significantly within the high liver fat group, 2 changed significantly within the middle liver fat group and 4 within the low liver fat group (Fig. 4).

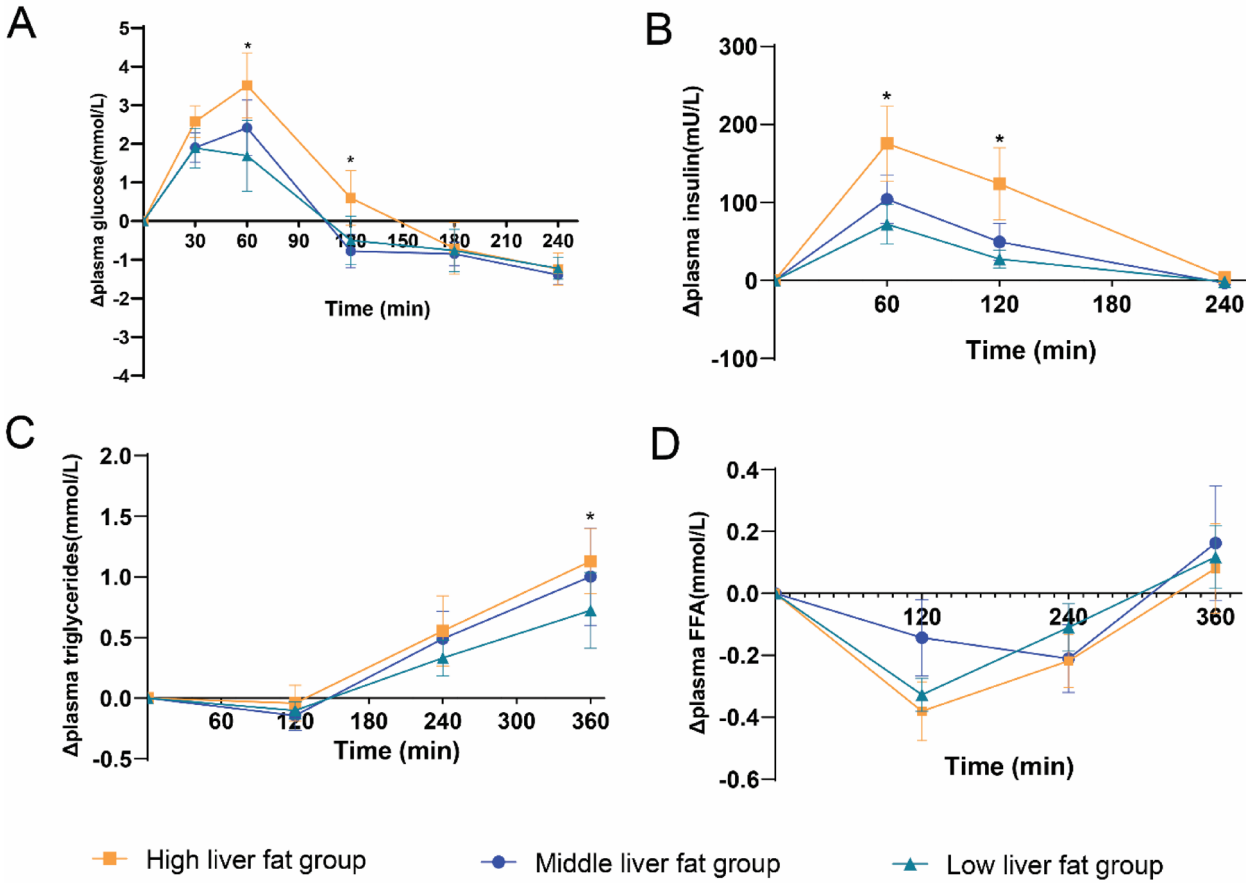

Fig. 2 Postprandial glucose, insulin, triglycerides and FFA responses to the mixed-meal challenge. Postprandial responses of plasma glucose (**A**), insulin (**B**), triglycerides (**C**) and FFA (**D**) to the mixed meal. Linear mixed model tests were used to compare the differences between the high and low liver fat groups. * P-value < 0.05 indicates posthoc analysis differences at that time point between the high and the low liver fat group. Error bars represent standard deviation (SD)

The top 20 significantly enriched gene sets derived from the comparison between the high and low liver fat groups at fasting are shown in Fig. 5, among the top 20 significantly differently expressed gene sets between the high and low liver fat groups at fasting, mitochondria-related gene sets exhibited a lower expression levels in the adipose tissue of the high compared to the low liver fat group, including gene sets related to *oxidative phosphorylation*, *thermogenesis* and *mitophagy*. In addition, pathways related to protein synthesis and processing, such as *ribosome*, *proteasome* and *N-Glycan biosynthesis*, also showed a lower expression in the adipose tissue of the high liver fat group compared to the low liver fat group. Conversely, immune-related pathways were higher in the adipose tissue of the high liver fat group compared to the low liver fat group. Notably, all of the lower expressed gene sets of these top 20 gene sets were also significantly enriched when comparing the high and middle liver fat groups, showing exactly the same direction of expression comparisons. The complete results of the GSEA at fasting are provided in Supplemental Table 1.

The list of the 17 gene sets that showed a significantly different postprandial response between the high and the low liver fat groups is shown in Fig. 6. Among these 17 gene sets, 7 of them changed significantly within the high liver fat group upon the mixed meal, including decreased *cGMP-PKG signaling pathway* and increased *oxidative phosphorylation*, 4 of them changed significantly within the low liver fat group upon the mixed meal, 2 of them changed significantly within the middle liver fat group upon the mixed meal. *Circadian rhythm* decreased in all liver fat groups after the consumption of the mixed meal, while the magnitude of reduction differed between the groups. Ferroptosis increased in both high and low liver fat groups upon the mixed meal, while the increase in the high liver fat group was greater. Notably, although the gene set *regulation of lipolysis* didn't change significantly upon the mixed meal change in any liver fat group, the response of this pathway remained significantly different between the high and low liver fat groups, as it decreased

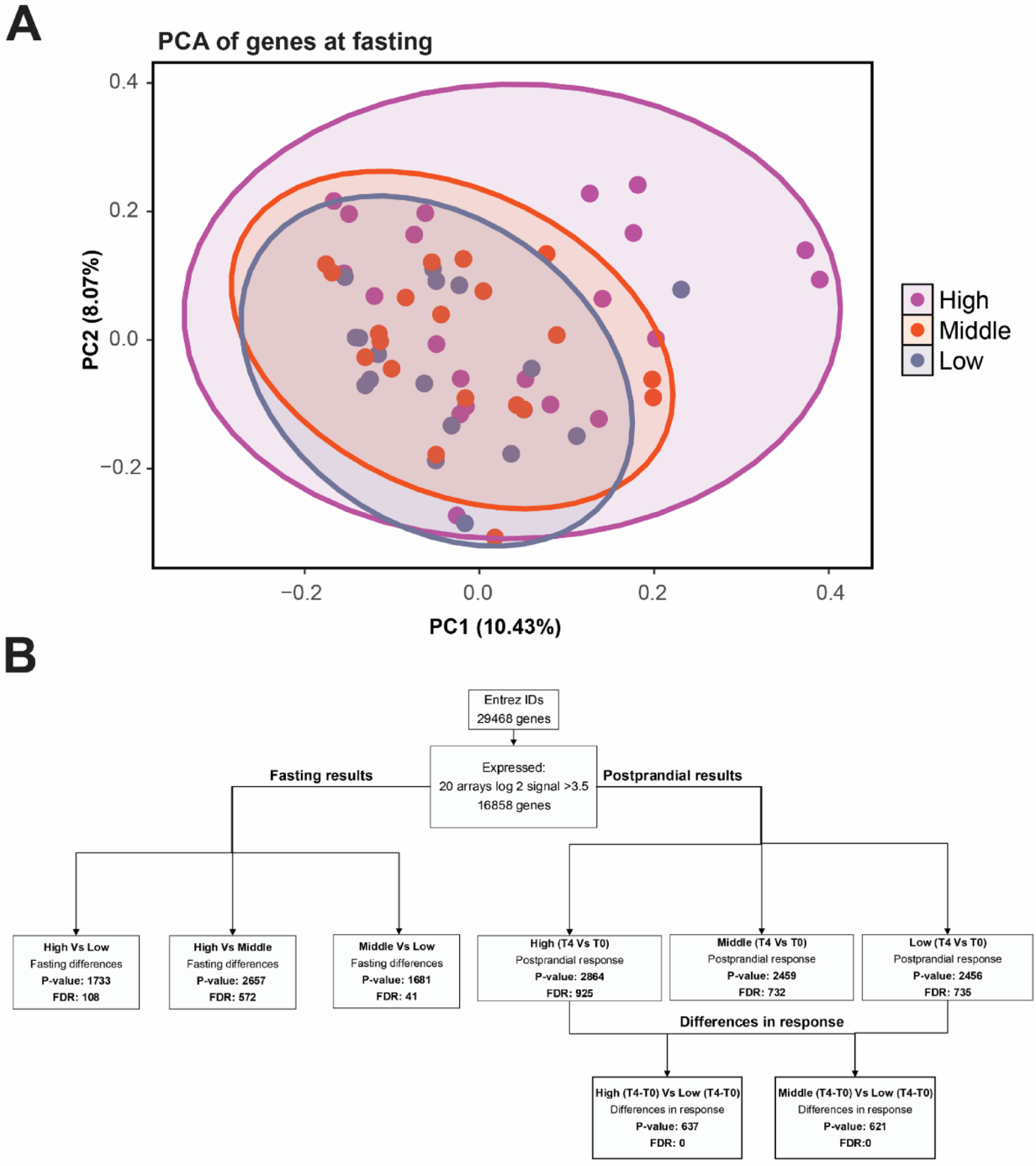

Fig. 3 PCA plot and flowchart and numbers of genes of which the expression changed in fasting and postprandial state. PCA of expressed genes at fasting (**A**), flowchart and numbers of genes of which the expression changed in fasting and postprandial state (**B**). The results were shown at both the levels of p-value < 0.05 and FDR < 0.05

in the high liver fat group and increased in the low liver fat group.

To explore how postprandial changes in leading-edge genes, which are a subset of genes that contribute most to the enrichment score, drive differences in the responses of pathways, *oxidative phosphorylation* and *regulation of lipolysis*. The heatmap of the individuals in the high and low liver fat groups have different directions in gene expression changes in these two pathways (Supplemental Fig. 2). On average the expression of genes in the oxidative phosphorylation pathway was significantly increased postprandially within the high liver fat group, whereas

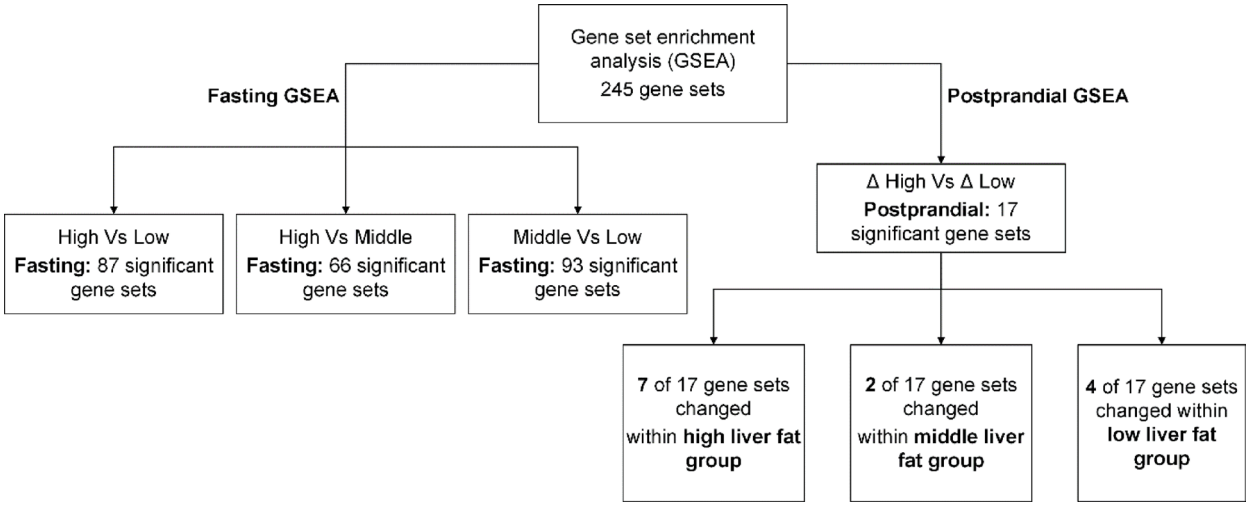

Fig. 4 Flow chart of GSEA Flow diagram shows the summary of the Gene set enrichment analysis (GSEA) on the number of gene sets enriched significantly differently between liver fat groups at fasting, and gene sets changed significantly differently within the liver fat groups after the high fat and high glucose mixed meal, as well as the gene sets changed significantly differently in response to the mixed meal between the high and low liver fat groups. Gene sets and changes were identified as significant when FDR < 0.25

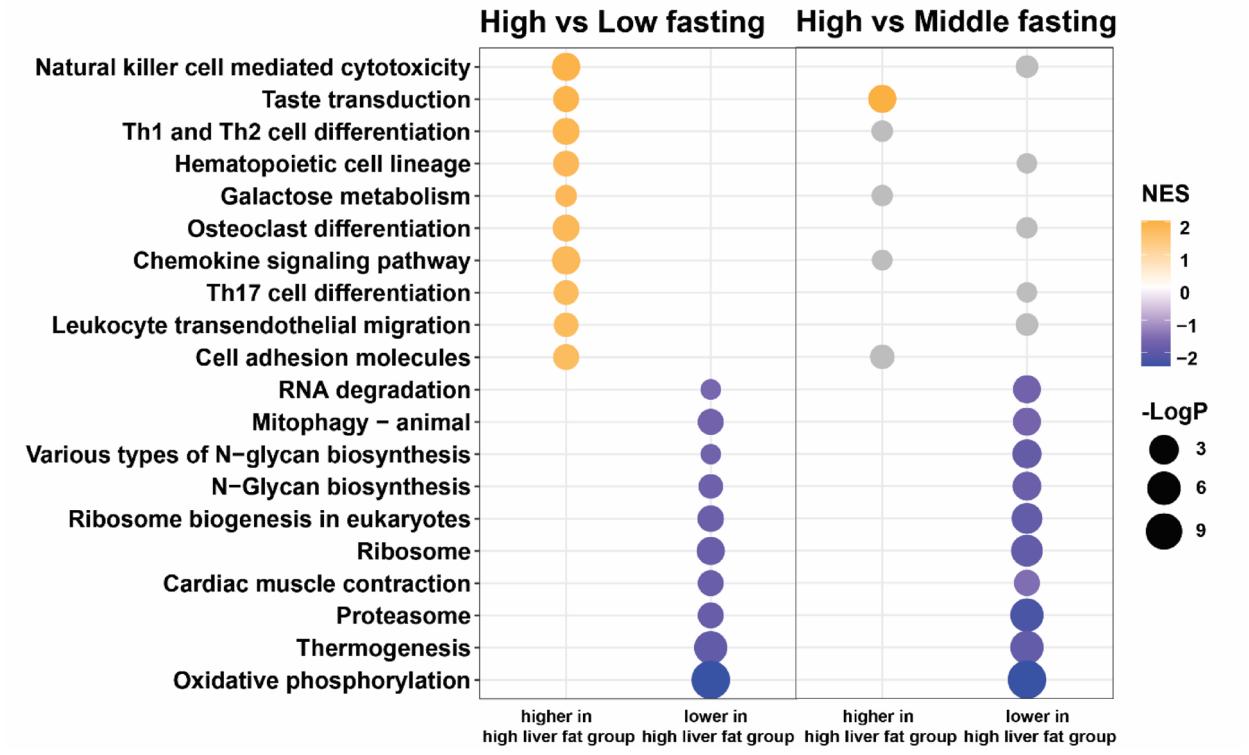

Fig. 5 Top 20 gene sets significantly different expressed between the low and high liver fat group at fasting. Top 20 gene sets expressed differently between the low and high liver fat group at fasting. and the results of these top 20 gene sets from the high and middle liver fat group comparison. NES means normalized enrichment score, yellow dots indicate they are higher expressed in the high liver fat group compared to the low or middle liver fat group, and the blue dots indicate they are lower expressed in the high liver fat group compared to the low or middle liver fat group, and the grey dots indicate they are not expressed significantly different. The size of dots reflects the negative log 10 of adjusted P value (FDR)

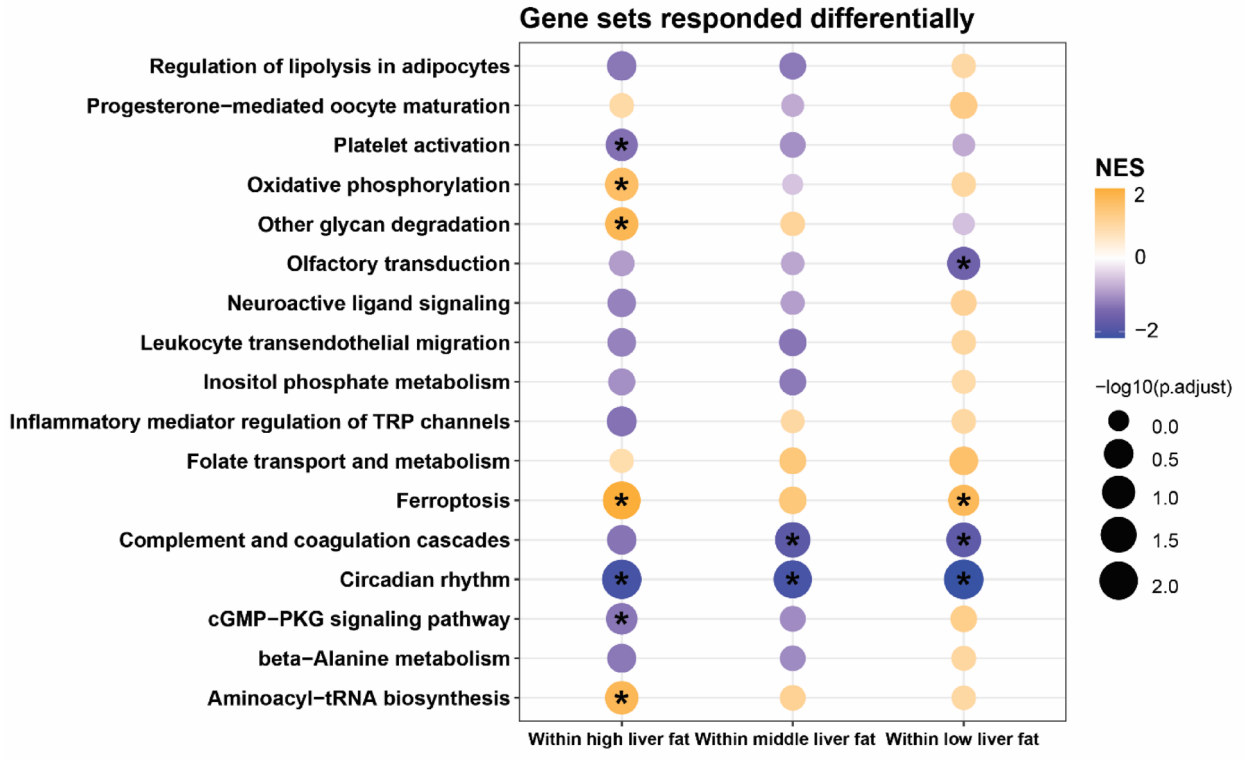

Fig. 6 Gene sets postprandially changed in adipose tissue in high, middle and low liver fat groups. Gene sets responded differentially to the mixed meal between the high and low liver fat groups. NES: normalized enrichment score. Yellow dots indicate upregulated postprandially within corresponding liver fat groups, the blue dots indicate downregulated postprandially within corresponding liver fat groups, and asterisks indicate the changes are significant (FDR < 0.25) within the corresponding liver fat groups. The size of the dots reflects the negative log 10 of FDR within the corresponding groups

not significantly change within the low liver fat group. In the lipolysis pathway, the average of the expression of genes was significantly decreased postprandially within the high liver fat group, whereas no significant change within the low liver fat group.

To explore whether the postprandial changes in the expression of these leading-edge genes correlate with intrahepatic lipids, HOMA IR, Adipo-IR and the fasting or postprandial changes in cardiometabolic markers, we performed correlation analyses. Postprandial changes in the expression of leading-edge genes of pathway oxidative phosphorylation and lipolysis correlated differently with cardiometabolic markers between the high and low liver fat group. Postprandial changes in the expression of genes involved in Oxidative phosphorylation (COX6C and ATM5MG) are positively correlated with IHL, HOMA IR, fasting glucose, fasting insulin and postprandial changes in insulin in the high liver fat group, but not in the low liver fat group. Postprandial changes in the expression of genes involved in the lipolysis pathway negatively correlated with Adipo IR (i.e. ACDY9) and fasting glucose (i.e. PIK3CB, PTGS1 and ADCY7) in the high liver fat group, but not in the low liver fat group (Supplemental Fig. 3).

Ingenuity pathway analysis (IPA)

To identify potential upstream regulators of the genes that were differently expressed between the groups, Ingenuity Pathway analysis (IPA) was performed. The software predicted 4 upstream transcription regulators for the differences in postprandial response (ΔHigh compared to ΔLow) between high and low liver fat. Table 2 shows these 4 upstream transcription regulators, i.e. retinoblastoma (RB1), nucleophosmin (NPM1), mothers against decapentaplegic homolog 4 (SMAD4) and RuvB-like 1(RUVBL1). RB1 was predicted to be lower postprandially activated in the adipose tissue of people in the high liver fat group compare to the low liver fat group. The total list of ingenuity pathway analyses is shown in Supplemental Table 2.

Discussion

In this secondary analysis, we aimed to investigate whether transcriptomic responses of abdominal SAT to a high-fat-high-glucose meal challenge differ according to varying levels of liver fat accumulation and identify pathways in subcutaneous fat metabolism that may be affected in individuals with increased liver fat compared to those with low liver fat which potentially may be affected and influence redirecting fat storage from

Table 2 Ingenuity pathway analysis

Upstream regulator	ΔHigh Vs ΔLow		High liver fat group (T4 Vs T0)		Low liver fat group (T4 Vs T0)		Fasting High Vs Low		Target molecules
	z-score	*p*-value	z-score	*p*-value	z-score	*p*-value	z-score	*p*-value	
RB1	−2.83	< 0.01	1.19	< 0.01	2.46	< 0.01	0.82	0.03	*CD274, GADD45A, GADD45B, JUNB, KLF10,LCK, PER1,RELB, ZFP36*
NPM1	2.64	0.02	1.23	< 0.01	0.17	< 0.01	0.69	0.03	*F3, JUNB, KLF10,PLOD1,SGK1,STC1,SYDE1, mir-204*
SMAD4	2.03	< 0.01	−0.53	< 0.01	−0.98	0.01	No prediction		*ACVR1C, ARHGEF2,CITED2,FSTL3,GADD45A, GADD45B, IER3,SGK1*
RUVBL1	2.00	< 0.01	No prediction		−1.41	< 0.01	No prediction		*CARS1, GADD45B, SOCS3,SPACA9*

the adipose tissue to the liver. Therefore, we explored differences in gene expression changes in SAT at fasting and in response to a mixed-meal challenge between individuals with high, middle and low liver fat. At fasting, adipose tissue gene expression profiles were comparable between individuals with middle and low liver fat, whereas individuals with high liver fat exhibited a more distinct gene expression pattern. This may explain the large overlapping differentially expressed gene sets observed when comparing the high liver fat group with both the low and middle liver fat groups. Adipose tissue of individuals with high liver fat had a lower expression of genes involved in mitochondria-related and protein synthesis and processing-related gene sets, such as oxidative phosphorylation, mitophagy, ribosome and proteasome, compared to subjects with middle and low liver fat. Postprandially, individuals with high liver fat showed an increased expression of genes in the oxidative phosphorylation pathway compared to individuals with low liver fat. Significantly expressed or changed gene sets were not consistent for fasting and postprandial states. The inconsistency of the fasting and postprandial gene sets presented suggests that the fasting and postprandial conditions activate distinct metabolic pathways, which also highlights the physiological relevance and importance of studying postprandial status/responses.

Pathways related to mitochondrial and energy metabolism were lower expressed at fasting in adipose tissue of the high compared to the low liver fat group, such as oxidative phosphorylation and mitophagy, while the pathway of oxidative phosphorylation increased upon a high-fat-high-glucose mixed meal challenge.

There are limited published studies on postprandial oxidative phosphorylation in human adipose tissue, but several are present on fasting. One human study showed that genes involved in mitochondrial oxidative phosphorylation were lower expressed in SAT of morbidly obese subjects compared with lean subjects, and a remarkable number of these genes were up-regulated after surgery-induced weight loss [30]. Multiple studies have consistently shown reduced expression of oxidative phosphorylation genes and proteins in the SAT of obese individuals compared to lean or non-obese co-twins [31, 32]. Typical for all these studies is that they compare people with a high and a low BMI, while our study subjects had the same BMI and VAT/SAT ratio and only differed in liver fat and HOMA-IR. This indicates that differences in mitochondrial oxidative phosphorylation may begin to emerge as liver fat content and insulin levels start to increase. In a human study comparing skeletal muscle between individuals with type 2 diabetes and insulin resistance and those with normal glucose tolerance, a lower expression of genes involved in the oxidative phosphorylation pathway was observed individuals with type 2 diabetes and insulin resistance, who have higher BMI [33]. In summary, people with a higher BMI or type2 diabetes seem to have a lower expression of genes in oxidative phosphorylation in metabolic active tissue like adipose tissue and muscle. Our study adds that we have already observed a lower gene expression of oxidative phosphorylation in people with increased liver fat and increased insulin levels, but with the same BMI, demonstrating that these effects are already present at an early stage.

A comparison of insulin-resistant and insulin-sensitive individuals' mitochondrial function in adipose tissue was conducted in proteomics analysis of subcutaneous adipocytes isolated from adipose tissue biopsy [34]. The analysis demonstrated lower mitochondrial function measured by the protein levels in the adipose tissue of the insulin-resistant group compared to the insulin-sensitive group. These findings are consistent with our results, which showed lower gene expression of oxidative phosphorylation in the high liver fat group, which also presents higher insulin compared to the low liver fat group. Association studies in cohorts, primarily aimed at investigating the mitochondrial biogenesis and function in human SAT, have found that lower oxidative phosphorylation, as measured by adipocyte mitochondrial DNA (mtDNA) copy number, is associated with increased BMI [35], insulin resistance [36] and type 2 diabetes [37]. In our study, we found lower gene expression levels of oxidative phosphorylation in subjects with high liver fat compared to those with low liver fat in the fasting state. Additionally,

the high liver fat group also exhibited higher HOMA-IR and Adipo-IR levels. These results point towards that people with a higher liver fat, HOMA IR and Adipo-IR may have a reduced function and reduced presence of the mitochondria of adipose tissue in the high liver fat compared to the low liver fat.

Interestingly, in the fasting state, we also observed a lower expression of genes involved in mitophagy in the high compared to the low and middle liver fat groups. Mitophagy plays a crucial role in maintaining mitochondrial quality and turnover, and reduced mitophagy is a known marker of impaired mitochondrial function [38, 39]. This supports the notion that mitochondrial dysfunction in adipose tissue may already be present at an early stage, even when BMI is similar. This may also explain the more pronounced increase in postprandial gene expression in the subjects with high liver fat in our study as it may need to compensate for a reduced number of mitochondria or a reduced Function requiring a higher activation of gene expression after consuming the same mixed meal challenge. Even though we could not find studies on postprandial gene expression changes, we previously showed that postprandial responses in PBMCs gene expression profiles also resulted in a lower induction of oxidative phosphorylation gene expression after the 20% energy restriction intervention in people with weight loss which was paralleled with a reduced liver fat compared to the weight-stable control group [40]. Furthermore, the correlation heatmap shows positive correlations between the cardiometabolic markers and the response of genes involved in oxidative phosphorylation in both groups.

From the difference in postprandial genes changed by the mixed meal challenge, RB1 is the upstream regulator predicted to be a reduced activation postprandially in the liver fat group, although the gene expression of RB1 was not affected. A study in RB1 KO muscle cells showed an increase in the expression of genes related to oxidative metabolism and glucose and fatty acid disposal compared with control cells [41]. This points towards an inhibitory regulation of RB1 on the gene expression of oxidative metabolism. People with high liver fat showed a more pronounced postprandial activation of oxidative phosphorylation, hence a reduced activation of RB1 might play a role in the adipose issue of people with high liver fat and insulin resistance compared to people with low liver fat.

Genes involved in lipolysis showed differential responses to the mixed meal challenge between the high and low liver fat groups. The most important function of adipose tissue is energy storage and lipid fuel release [42, 43]. In healthy conditions, the rise in insulin during the postprandial state suppresses lipolysis in adipose tissue [44]. Interestingly, genes involved in lipolysis decreased in the adipose tissue of the high liver fat group but increased in the low liver fat group, although the change was not significant within each group. This was opposite to what we expected because the subjects in the high liver fat group presented more insulin resistance compared to the low liver fat group. On the other hand, this finding is in line with a previous study that showed that compared to non-diabetic normal weight controls, both diabetic and non-diabetic subjects with obesity were characterized by decreased lipolysis measured by the lipoprotein lipase activity in both mRNA and enzyme levels at both fasting and postprandial states [45]. A possible explanation could be that after an overnight fast, the sensitivity of the antilipolytic effect of insulin is markedly enhanced in the high compared to the low liver fat group, which was also reported in subjects with obesity compared with controls previously [46]. However, such a hypothesis warrants further investigation.

There are several advantages and limitations of the current study. First, we had the opportunity to have a study in which we compared the whole genome gene expression response of SAT, the use of adipose tissue biopsies enables tissue-specific mechanistic insights that go beyond peripheral blood markers. Additionally, the inclusion of gene expression in both the static fasting state and in response to a meal challenge provides insight into metabolic flexibility and postprandial responses, which provide more information than only at fasting states. Furthermore, the various levels of liver fat were determined with a high-quality proton magnetic resonance spectroscopy which accurate liver fat content can be measured. However, the limitation is that in this secondary analysis and due to the distribution of people with high, middle and low liver fat the number of participants per subgroup is limited, which may limit generalizability and statistical power for detecting subtle differences. Additionally, although there is a meal challenge, the adipose tissue gene expression data is observational per group and cannot establish causality. Also, at the fasting state, HOMA-IR, Adipo-IR and plasma insulin levels are higher in the high liver fat group compared to the low liver fat group. This indicates that the findings cannot be attributed solely to the differences in liver fat but are likely also due to differences in insulin resistance and adipose tissue insulin resistance in the subjects. Furthermore, due to limitations in the availability of the original adipose tissue samples, we unfortunately were not able to measure the protein levels of the related genes. Future studies with integrated findings on protein levels and transcriptomic levels are warranted to further validate and extend these findings.

Conclusions

In conclusion, we demonstrated that individuals with increased liver fat respond with different gene expression changes in abdominal SAT to a high-fat-high-glucose meal than people with lower liver fat. The main differences were related to oxidative phosphorylation in which a lower level at fasting resulted in a higher induction of gene expression changes postprandially; this might be due to a reduced number or function of mitochondria leading to a reduced capacity of the oxidative phosphorylation pathway to deal with postprandial energy load in the adipose tissue, which need to be compensated for. Whether this is the cause, or the consequence of increased storage of liver fat or early stage of insulin resistance needs to be further investigated.

Abbreviations

ACDY9	Adenylate cyclase 9
ADCY7	Adenylate cyclase 7
Adipo-IR	Adipose tissue insulin resistance index
ATP5MG	ATP synthase membrane subunit g
BMI	Body mass index
COX6C	Cytochrome c oxidase subunit 6 C
ER	Energy-restricted
FFA	Free fatty acids
GSEA	Gene set enrichment analysis
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
iAUC	Incremental area under the curve
IHL	Intrahepatic lipids
IPA	Ingenuity Pathway analysis
MASH	Metabolic dysfunction-associated steatohepatitis
MASLD	Metabolic dysfunction-associated steatotic liver disease
NASH	Non-alcoholic steatohepatitis
NPM1	Nucleophosmin
OGTT	Oral glucose tolerance test
OXPHOS	Oxidative phosphorylation
PIK3CB	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Beta
PTGS1	Prostaglandin-endoperoxide synthase 1
RIN	RNA integrity number
RB1	Retinoblastoma
RUVBL1	RuvB-like 1
SMAD4	Mothers against decapentaplegic homolog 4
SAT	Subcutaneous adipose tissue

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

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Author contributions

Study design and execution: YF and LAA; Data analysis: YF and GH; Data interpretation: YF, LAA and GH; Original draft: YF; Writing, review and editing: YF, GH and LAA. All authors read and approved the final manuscript.

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Data availability

The datasets used during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of Wageningen University and registered at clinicaltrials.gov as NCT02194504. Informed consent was obtained from all participants.

Competing interests

The authors declare no competing interests.

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