Research Article
NAFLD and Alcohol-Related Liver Diseases

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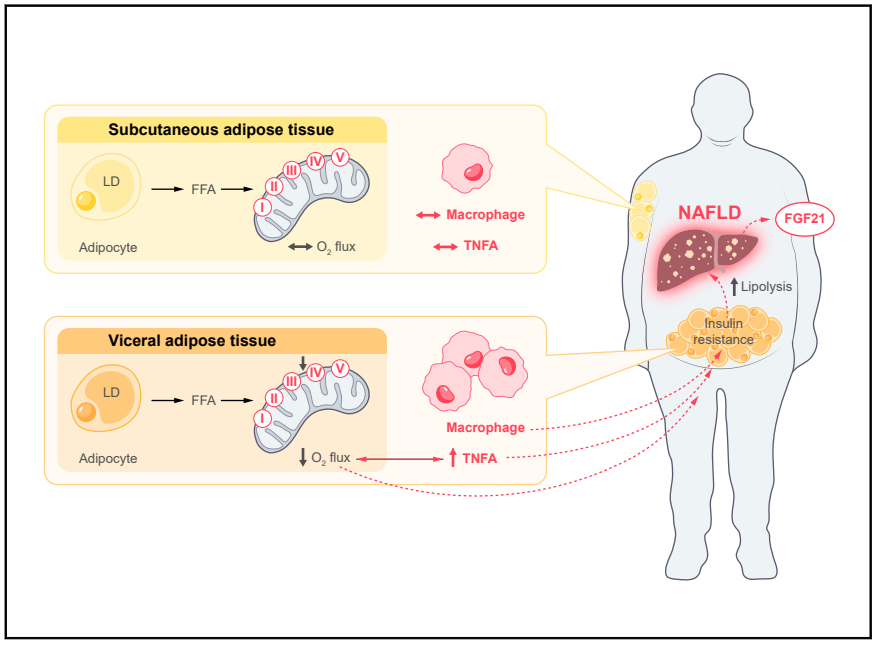
Mitochondrial respiration is decreased in visceral but not subcutaneous adipose tissue in obese individuals with fatty liver disease

Graphical abstract



Highlights

- Differences in mitochondrial features are observed between SAT and VAT in human obesity.
- VAT respiration is downregulated in obese humans with fatty liver.
- VAT respiration is also decreased in obese humans with non-alcoholic steatohepatitis.
- Lower VAT respiration is coupled with lower adipose tissue insulin sensitivity.

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Lay summary

Adipose tissue (commonly called body fat) can be found under the skin (subcutaneous) or around internal organs (visceral). Dysfunction of adipose tissue can cause insulin resistance and lead to excess delivery of fat to other organs such as the liver. Herein, we show that dysfunction specifically in visceral adipose tissue was associated with fatty liver disease.

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Mitochondrial respiration is decreased in visceral but not subcutaneous adipose tissue in obese individuals with fatty liver disease

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Background & Aims: Adipose tissue dysfunction is involved in the development of insulin resistance and is responsible for excessive lipid delivery to other organs such as the liver. We tested the hypothesis that impaired mitochondrial function is a common feature of subcutaneous (SAT) and visceral adipose tissue (VAT), but may differently contribute to adipose tissue insulin resistance (IR) in obesity, non-alcoholic fatty liver (NAFL) and steatohepatitis (NASH).

Methods: In this cross-sectional study, we analyzed tissue-specific insulin sensitivity using stable isotope dilution and hyperinsulinemic-normoglycemic clamp tests. We also assessed mitochondrial respiration, mRNA and protein expression, and tissue morphology in biopsies of SAT and VAT from obese humans without NAFL, with NAFL or with NASH (n = 22/group).

Results: Compared to individuals without liver disease, persons with NAFL and NASH had about 30% (p = 0.010) and 33% (p = 0.002) lower maximal mitochondrial respiration, respectively, in VAT, but not in SAT. The lower maximal mitochondrial respiration of VAT was associated with lower adipose tissue insulin sensitivity (β = 0.985, p = 0.041) and with increased VAT protein expression of tumor necrosis factor *A* across all groups (β = −0.085, p = 0.040). VAT from individuals with NASH was characterized by lower expression of oxidative phosphorylation complex IV (p = 0.042) and higher mRNA expression of the macrophage marker *CD68* (p = 0.002) than VAT from participants without NAFL.

Conclusions: Humans with non-alcoholic fatty liver disease have distinct abnormalities of VAT energy metabolism, which correlate with adipose tissue dysfunction and may favor progression of NAFL to NASH.

Lay summary: Adipose tissue (commonly called body fat) can be found under the skin (subcutaneous) or around internal organs (visceral). Dysfunction of adipose tissue can cause insulin resistance and lead to excess delivery of fat to other organs such as the liver. Herein, we show that dysfunction specifically in visceral adipose tissue was associated with fatty liver disease.

Clinical trial number: NCT01477957.

Introduction

Adipose tissue (AT) contributes to the orchestration of whole-body metabolic homeostasis via release of free fatty acids (FFAs), hormones, cytokines and exosomes.[1,2] During development of obesity, AT dysfunction in association with local insulin resistance (IR) and inflammation favors ectopic fat deposition, whole-body IR and ultimately type 2 diabetes (T2D).[3] In the context of IR and fasting hypertriglyceridemia, circulating FFAs derived from AT lipolysis contribute to about 60% of hepatic triglyceride synthesis in overweight/obese humans with non-alcoholic fatty liver disease (NAFLD).[4] Recent studies suggest that AT dysfunction with increased lipogenic substrate flux may be key to NAFLD development.[5]

White AT dysfunction is characterized by abnormal adipokine release and progressive fibrosis, but may also include altered mitochondrial function.[6] Although subcutaneous AT (SAT) represents the main source of circulating FFAs and can exhibit lower

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expression of genes regulating the mitochondrial respiratory chain in obesity and T2D,[2] lower citrate synthase activity (CSA) has also been reported in the visceral AT (VAT) of obese humans.[7] VAT can also secrete hormones and proinflammatory cytokines that interact with resident liver macrophages and other immune cells involved in inflammation-induced IR.[8] Secretome analyses showed that proteins involved in the regulation of cellular processes, in inflammatory responses or in extracellular matrix organization are released prominently and abundantly by human VAT.[9,10] A recent study also identified distinct properties of VAT stem cells, promoting fibrotic remodeling under obesogenic stimuli in mammals.[11] Collectively, there is evidence that VAT could contribute to hepatic dysregulation in metabolic diseases. However, little is known about tissue-specific differences between SAT and VAT parameters contributing to mitochondrial functionality, oxidative and endoplasmic reticulum (ER) stress.

NAFLD, ranging from fatty liver (NAFL), steatohepatitis (NASH) to fibrosis and cirrhosis, correlates tightly with IR.[12] However, hepatic mitochondrial respiration is not uniformly impaired in obese humans with NAFLD. Indeed, hepatic mitochondrial respiration is even higher in obese humans with NAFL compared to those with NASH.[13] As VAT has been considered a major culprit in the development of NAFLD,[14] it is interesting to examine whether oxidative capacity in VAT shows similar alterations to that observed in the liver.

We tested the hypothesis that (i) impaired mitochondrial function is a common feature of SAT and VAT, but (ii) may differently contribute to AT IR in obesity-related NAFL and NASH. Specifically, VAT could present with abnormal mitochondrial respiration in NAFL (OBE-NAFL) and even more so in NASH (OBE-NASH) when compared to obese humans without NAFL (OBE-CON).

Materials and methods

Study cohort

This cross-sectional study included all consecutive obese volunteers undergoing bariatric surgery within the BARIA_DDZ cohort (supplementary CTAT table), recruited between March 2015 and February 2020, who had a complete data set for mitochondrial respiration in SAT and VAT (Fig. S1). Based on liver histology, participants were stratified into three groups: OBE without (OBE-CON, n = 22) or with NAFL (OBE-NAFL, n = 22) or NASH (OBE-NASH, n = 22), with similar age, sex, body weight and BMI. All volunteers showing at least grade 1 steatosis were classified as having NAFL and those with at least grade 1 steatosis plus at least grade 1 ballooning and at least grade 1 lobular inflammation were classified as having NASH.[15] All participants with NASH exhibited profound steatosis, liver cell ballooning and lobular inflammation (Table S1, Fig. S2). Specific causes of liver disease were excluded based on medical history, lab tests and histological features. Some participants from the OBE-CON (n = 2), OBE-NAFL (n = 5) and OBE-NASH (n = 9) groups had T2D with near-normoglycemic control by lifestyle modification or antihyperglycemic medication (Table S2).

Before inclusion, all participants gave written informed consent to the protocol, which was approved by the institutional review board of Heinrich-Heine-University Düsseldorf and conducted in accordance with the ethical standards as set down in the 1964 Declaration of Helsinki and its last amendments of 2013.

Statistical analyses

Statistical analyses are described in the supplementary materials and methods.

For further details regarding the materials and methods used, please refer to the CTAT table and supplementary information.

Results

Individuals with NASH exhibit greater AT IR

All three groups had comparable age, sex and body composition as well as circulating FFA levels (Table 1, Fig. S3A-C). OBE-NASH had higher fasting glycemia than both other groups and higher glycosylated hemoglobin A1c and serum alanine aminotransferase activity than OBE-CON (Table 1).

Compared to OBE-NASH, fasting AT IR was 34% lower in OBE-CON and tended to be 30% lower in OBE-NAFL (p = 0.073) (Fig. 1A). Fasting hepatic IR was similar between groups (Fig. 1B).

During hyperinsulinemic-euglycemic clamps, reflecting postprandial metabolic conditions, AT insulin sensitivity (IS) tended to be lower in OBE-NASH than in OBE-CON (p = 0.080) (Fig. 1C), while hepatic IS was similar between groups (Fig. S3D). Whole-body IS tended to be lower in OBE-NASH than in OBE-CON (p = 0.058) (Fig. S3E), mainly due to decreased insulin-stimulated glucose oxidation rates (Fig. 1D), but not non-oxidative glucose disposal (Fig. S3F). Insulin-suppressed lipid oxidation rates were 51% and 59% lower in OBE-CON than in OBE-NAFL and OBE-NASH, respectively (Fig. 1E), resulting in decreased metabolic flexibility in OBE-NASH compared to OBE-CON (Fig. 1F).

Individuals with NASH exhibit lower mitochondrial respiration, as well as complex IV expression in VAT, but not SAT

Using a substrate-uncoupler-inhibitor protocol (Fig. S4), VAT oxidative phosphorylation (OXPHOS) capacity ($_P$) with electron input through electron transferring flavoprotein (ETF) ($[ETF]_P$), was 32% and 33% lower in OBE-NAFL and OBE-NASH, respectively, than in OBE-CON (Fig. 2A), with similar differences for ETF and complex (C)I combined ($[ETF+CI]_P$). Maximal ADP-stimulated mitochondrial respiration with convergent electron input through ETF, CI and CII combined, $[ETF+CI+II]_P$, was 30% and 33% lower in OBE-NAFL and OBE-NASH than in OBE-CON (Fig. 2A). Instead, in SAT, mitochondrial respiration was similar in all groups (Fig. 2B). Although mitochondrial respiration can decline with aging,[16] adjustment for age did not affect results of mitochondrial respiration in both AT compartments (data not shown). Both CSA and mitochondrial DNA (mtDNA) copy number were similar between groups in both compartments (Fig. 2C-D). Consequently, mitochondrial respiration normalized by mtDNA remained lower in VAT of OBE-NAFL and OBE-NASH than in OBE-CON and similar in SAT of all groups (Fig. S5A-B).

To further characterize differences in mitochondrial respiration, we assessed mitochondrial coupling efficiency by calculating respiratory control ratio (RCR) and leak control ratio (LCR), which were similar between groups in both SAT and VAT (Fig. S5C-F).

On the other hand, the protein expression of OXPHOS CIV was lower in VAT of OBE-NASH than OBE-CON, but this difference was not seen in SAT (Fig. 2E-F). Also, the sum of all bands related to OXPHOS CI-CV tended to be lower in OBE-NASH than in OBE-CON (p = 0.062) only in VAT (OBE-CON median: 8.5 [first quartile: 7.0, third quartile: 9.7] arbitrary units [AU], OBE-NAFL 9.4 [6.2, 12.2] AU, OBE-NASH 6.8 [5.7, 8.8] AU), but not SAT (OBE-CON

Table 1. Characteristics of the participants.

Variable	OBE-CON	OBE-NAFL	OBE-NASH
Age (years)	35 (31–45)	40 (35–44)	45 (32–51)
Female/male (n)	20/2	18/4	18/4
Body weight (kg)	155 (130–165)	150 (139–162)	148 (136–173)
BMI (kg/m^2)	53 (46–59)	51 (47–53)	51 (47–55)
Waist circumference (cm)	132 (124–140)	138 (128–143)	137 (128–144)
Hip circumference (cm)	148 (142–162)	153 (140–160)	145 (141–158)
HbA1c (%)	5.4 (5.2–5.6)	5.5 (5.3–5.9)	6.0 (5.2–6.7)[a]
Fasting glucose (mg/dl)	80 (76–93)	88 (83–93)	98 (85–124)[a,b]
Fasting insulin (mU/L)	17 (11–33)	21 (16–30)	26 (21–33)
Fasting triglycerides (mg/dl)	99 (81–124)	125 (90–158)	152 (107–178)
Fasting FFA (μmol/L)	536 (418–658)	635 (490–794)	705 (585–792)
hsCRP (mg/dl)	0.84 (0.60–2.67)	0.84 (0.48–1.32)	0.74 (0.44–0.94)
ALT (U/L)	25 (17–32)	30 (20–41)	41 (30–55)[a]

ALT, alanine aminotransferase; FFA, free fatty acids; HbA1c, hemoglobin A1c; hsCRP, high-sensitivity C-reactive protein; OBE-CON, obese humans without non-alcoholic fatty liver; OBE-NAFL, obese humans with NAFL; OBE-NASH, obese humans with NASH.
Data are median (IQR) and absolute numbers, as applicable. [a] p <0.05 *vs.* OBE-CON, [b] p <0.05 *vs.* OBE-NAFL, one-way ANOVA corrected for multiple comparisons with Tukey-Kramer multiple comparisons test.

7.3 [4.9, 10.5] AU, OBE-NAFL 6.1 [4.3, 7.8] AU, OBE-NASH 6.6 [4.8, 7.8] AU).

It is conceivable that adipocyte size and number drives the observed decreased mitochondrial respiration in VAT.[17,18] However, analysis of AT morphology in a subgroup of the study population revealed that both adipocyte number and mean adipocyte size were similar between all groups and in both compartments (Fig. S6A-D).

Levels of one biomarker of mitophagy are higher only in SAT of individuals with NAFL compared to those with NASH

Maintaining mitochondrial respiration requires a balance between biogenesis, reserve capacity, mitophagy and the mitophagy-induced recruitment of the autophagic machinery.[19] In both AT compartments, protein biomarkers of macroautophagy, including the autophagy-related gene 5, p62 and the microtubule-associated protein 1 light chain 3 A/B II/I ratio, were similar between groups (Fig. S7A-B).

Despite no differences in VAT (Fig. 3A), OBE-NAFL exhibited higher levels of the phosphorylated Parkin (pParkin) than OBE-NASH in SAT (Fig. 3B). Protein markers of mitochondrial fusion (optic atrophy 1, mitofusin 1 and 2) and fission (dynamin-related protein 1 and serine 616 phosphorylated DRP1 and their ratio) were similar between groups in both compartments (Fig. S7C-F).

The same applied for the AT mRNA expression of transcription factors regulating mitochondrial biogenesis, including nuclear respiratory factor (*NRF*)*1*, mitochondrial transcription factor A (*TFAM*) and PPARGC1A (peroxisomal proliferator activated receptor γ-coactivator 1A) (Fig. 3C-D).

Selective upregulation of inflammation in VAT of NAFL and of ER stress in SAT of NASH

Next, we examined the role of AT oxidative stress and inflammation on NAFLD.[3] There were neither differences in lipid peroxidation, as assessed by thiobarbituric acid reactive substances (TBARS) (Fig. S8A-B) nor measures of anti-oxidant defense, as assessed by the protein expression of superoxide dismutase 1 and catalase, between groups, in either VAT or SAT (Fig. S8C-F). Despite similar interleukin 6 (*IL6)* expression (Fig. 4A-B), the mRNA expression of tumor necrosis factor A (*TNFA)* was higher only in VAT of OBE-NAFL compared to OBE-CON (Fig. 4A-B).

In VAT, expression of activating transcription factor 4 (ATF4) and the transcription factor C/EBP homologous protein (CHOP) were similar between the groups (Fig. 4C). On the other hand, in SAT, ATF4 was higher in OBE-NASH than in OBE-CON and CHOP tended to be higher in OBE-NASH than in OBE-NAFL (p = 0.054) (Fig. 4D). Indeed, ATF4 regulates the expression of CHOP, which upregulates pro-apoptotic protein expression.[20] Other biomarkers of ER stress, including protein kinase-like ER kinase, inositol-requiring enzyme 1α, eukaryotic translation initiation factor 2α and binding immunoglobulin protein 1 were similar across all groups and in both compartments (data not shown).

Finally, mRNA expression of the macrophage marker, *CD68,* was higher in OBE-NASH than in OBE-CON, but only in VAT, not in SAT (Fig. 4E-F). Other macrophage markers including *CD163* and monocyte chemoattractant protein 1 were comparable across all groups in SAT and VAT (Fig. 4E-F). Also, the immunohistochemical analysis of macrophage-specific antigens revealed similar numbers of CD68+, CD163+ and CD11c+-expressing cells in both SAT and VAT in a subgroup (n = 4 per group) (Fig. S9A-F).

Mitochondrial respiration is higher in VAT of obese persons without NAFLD than in SAT of obese people with or without NAFLD

Comparison of the features of SAT and VAT within and between groups showed higher $[ETF]_P$ in the VAT of OBE-CON than in SAT of all three studied groups (Fig. S10A), with similar differences for $[ETF+CI]_P$ and for maximal ADP-stimulated mitochondrial respiration ($[ETF+CI+II]_P$) (Fig. S10A). Of note, VAT of OBE-NAFL and OBE-NASH displayed similar mitochondrial respiration as SAT of all groups (Fig. S10A).

Accordingly, protein expression of OXPHOS CIV and CV was higher in VAT than in SAT of OBE-CON and OBE-NAFL (Fig. S10B). Protein expression of OXPHOS CIII-CV was also higher in VAT of OBE-CON compared to SAT of both OBE-NAFL and OBE-NASH.

Of note, OBE-CON and OBE-NAFL had lower pParkin/Parkin ratio in VAT than in SAT (Fig. S10C). OBE-CON also featured reduced mRNA expression of *NRF1,* but not *TFAM*, in VAT compared to SAT (Fig. S10D).

Finally, the mRNA expression of *TNFA*, was higher in SAT than in VAT of OBE-CON (Fig. S10E), despite the similar *IL6* (p = 0.885, data not shown). Similarly, TBARS were increased in SAT of OBE-

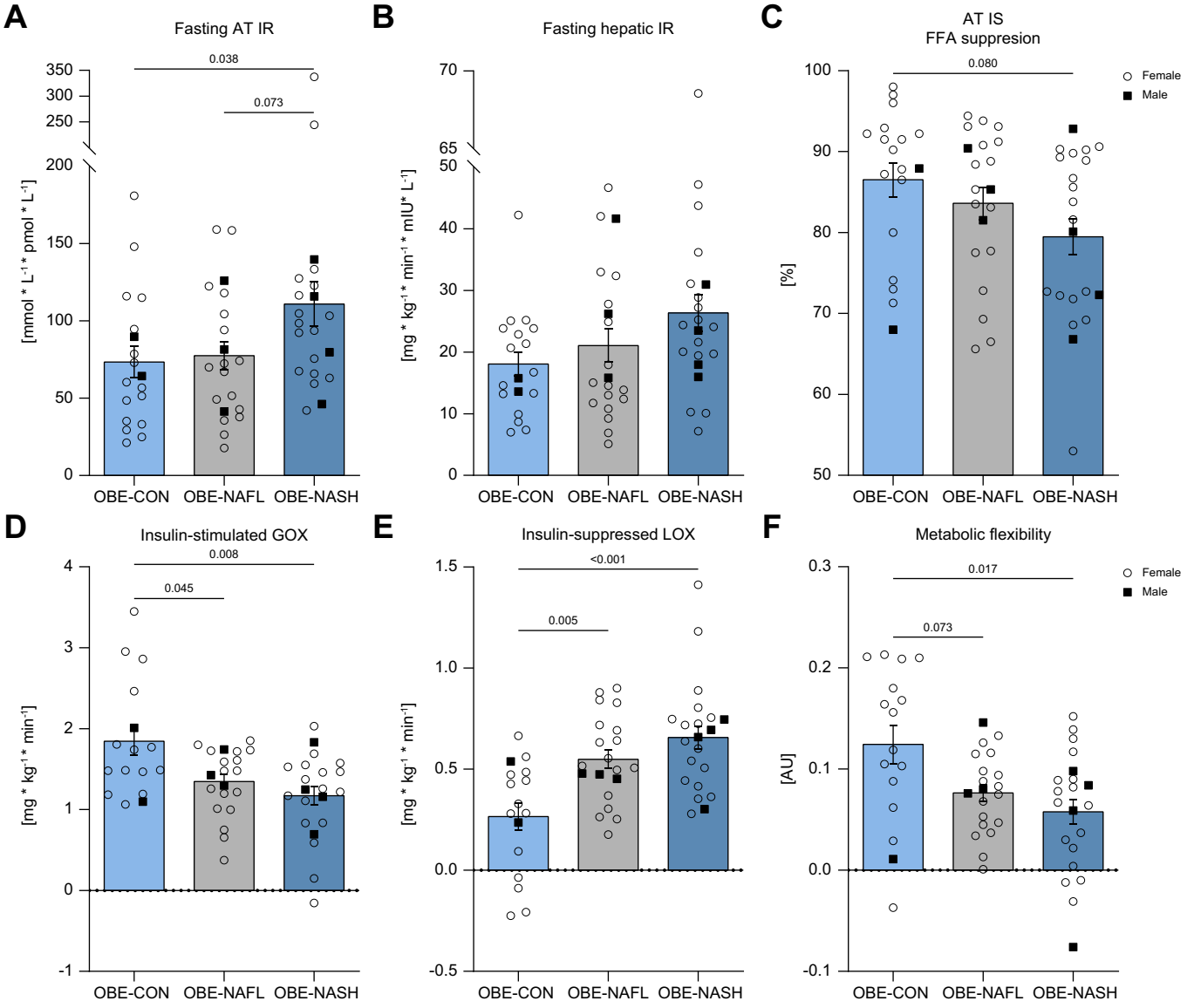

Fig. 1. Tissue-specific IR and substrate oxidation in OBE-CON, OBE-NAFL or OBE-NASH. (A) AT IR and (B) hepatic IR during fasting as well as (C) AT IS, (D) insulin-stimulated GOX and (E) insulin-suppressed LOX rates during clamp, (F) metabolic flexibility during fasting and clamp conditions. Means ± SEM, levels of significance of each significant and borderline significant difference are marked in the figure (one-way ANOVA corrected for multiple comparisons with Tukey-Kramer multiple comparisons test). AT, adipose tissue; AU, arbitrary units; FFA, free fatty acid; GOX, glucose oxidation; IR, insulin resistance; IS, insulin sensitivity; LOX, lipid oxidation; OBE-CON, obese humans without non-alcoholic fatty liver; OBE-NAFL, obese humans with NAFL; OBE-NASH, obese humans with NASH.

CON and OBE-NAFL compared to VAT of all three groups (Fig. S10E). On the other hand, VAT showed increases in ATF4 when compared to SAT of OBE-CON (Fig. S10F), and in CHOP when compared to SAT of all respective groups (Fig. S10F).

Obese individuals with NAFLD show no differences in systemic oxidative stress, or inflammation when compared to obese humans without NAFLD

Plasma TBARS, reflecting systemic oxidative stress, were not different between the groups (Fig. 5A), as were plasma TNFA and plasma IL6 levels (Fig. 5B-C). Only plasma fibroblast growth factor 21 (FGF21) was higher in OBE-NAFL than in OBE-CON (Fig. 5D). Finally, high-molecular weight adiponectin and leptin were similar across all groups (Fig. 5E-F).

Mitochondrial respiration in VAT associates positively with AT IS, but negatively with inflammation of VAT

In VAT, maximal ADP-stimulated mitochondrial respiration ($[ETF+CI+II]_P$) associated positively with AT IS (β = 0.985, p = 0.041) and tended to associate negatively with the degree of hepatic steatosis (β = −0.004, p = 0.099) across all groups combined (Table S3). The sum of OXPHOS CI-CV in VAT associated negatively with the degree of hepatic steatosis upon adjustment for T2D (β = −0.006, p = 0.048) (Table S3). Maximal ADP-stimulated respiration further associated negatively with *TNFA* expression in VAT (β = −0.085, p = 0.040) and with plasma FGF21 (β = −0.196, p = 0.041) (Table S3). Plasma FGF21 also associated positively with the degree of liver steatosis across all groups, only upon adjustment for T2D (β=0.009, p = 0.022) (Table S3).

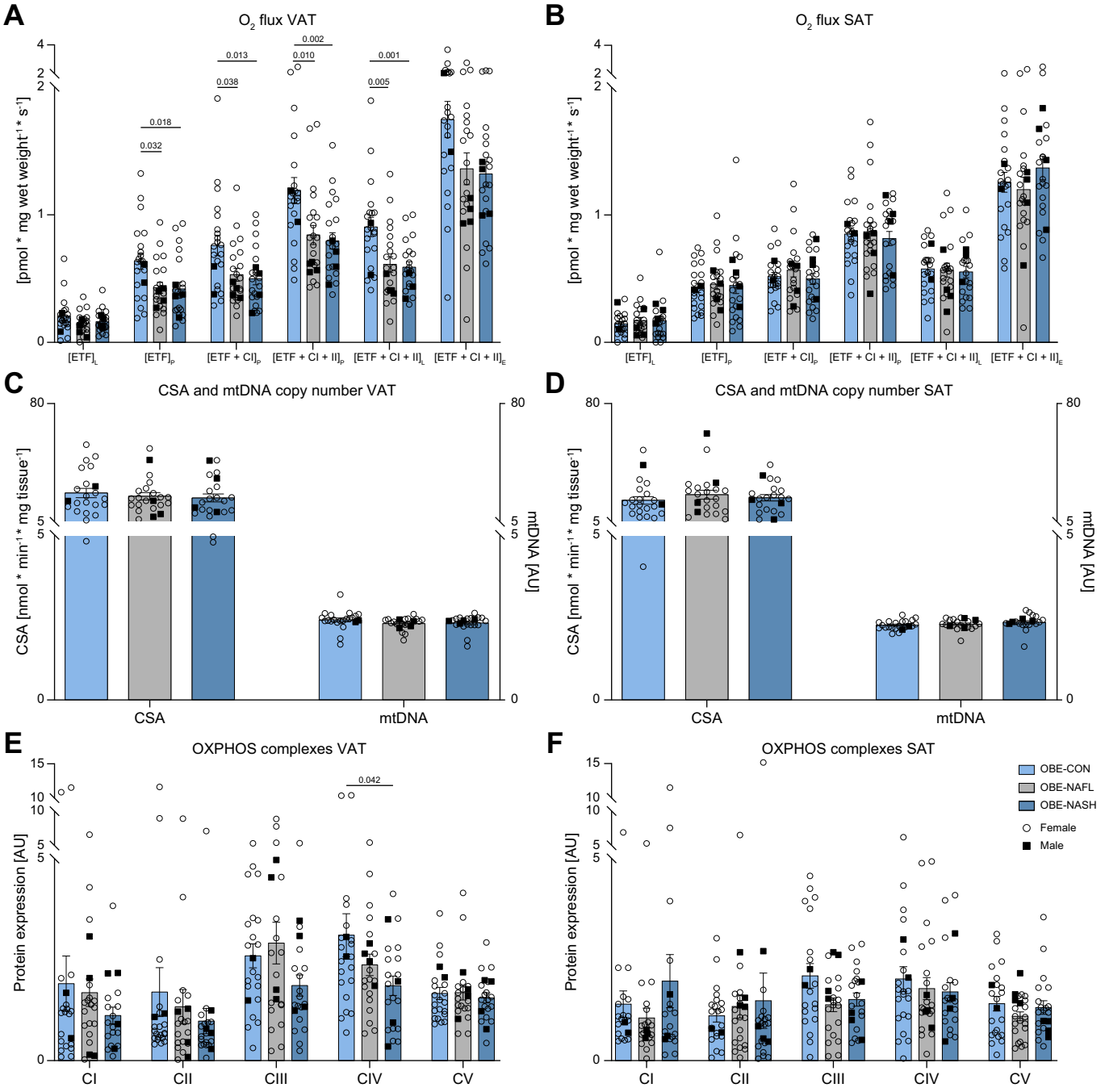

Fig. 2. Mitochondrial respiration and content in OBE-CON, OBE-NAFL or OBE-NASH. Mitochondrial respiration in (A) VAT and (B) SAT, with electron flow through ETF. ADP-stimulated respiration ($[ETF]_P$;$[ETF+CI]_P$;$[ETF+CI+II]_P$), leak respiration ($_L$) ($[ETF+CI+II]_L$) and maximal uncoupled respiration ($[ETF+CI+II]_E$), (C) CSA and mtDNA copy number in VAT and (D) SAT, (E) protein expression of OXPHOS CI-CV in VAT and (F) SAT. Means ± SEM, levels of significance of each significant difference are marked in the figure (one-way ANOVA corrected for multiple comparisons with Tukey-Kramer multiple comparisons test). AU, arbitrary units; C, complex; CSA, citrate synthase activity; $_E$, electron transport system capacity; ETF, electron transferring flavoprotein; mtDNA, mitochondrial DNA; OBE-CON, obese humans without non-alcoholic fatty liver; OBE-NAFL, obese humans with NAFL; OBE-NASH, obese humans with NASH; OXPHOS, oxidative phosphorylation; $_P$, oxidative phosphorylation capacity; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.

Only in OBE-NASH, maximal ADP-stimulated respiration of SAT associated with the degree of hepatic steatosis (β = −0.010, p = 0.002, p for the interaction = 0.005) (Table S3).

Discussion

This study showed that obese humans with NAFL and NASH exhibit marked reductions of mitochondrial respiration in VAT when compared to similarly obese humans without NAFLD. Mitochondrial respiration in VAT associated positively with insulin-stimulated IS of whole-body AT, but negatively with local inflammation. Nevertheless, compared to those without NAFL, SAT of obese humans with NAFLD showed no further alterations of mitochondrial function, but only differences in biomarkers of mitophagy and ER stress.

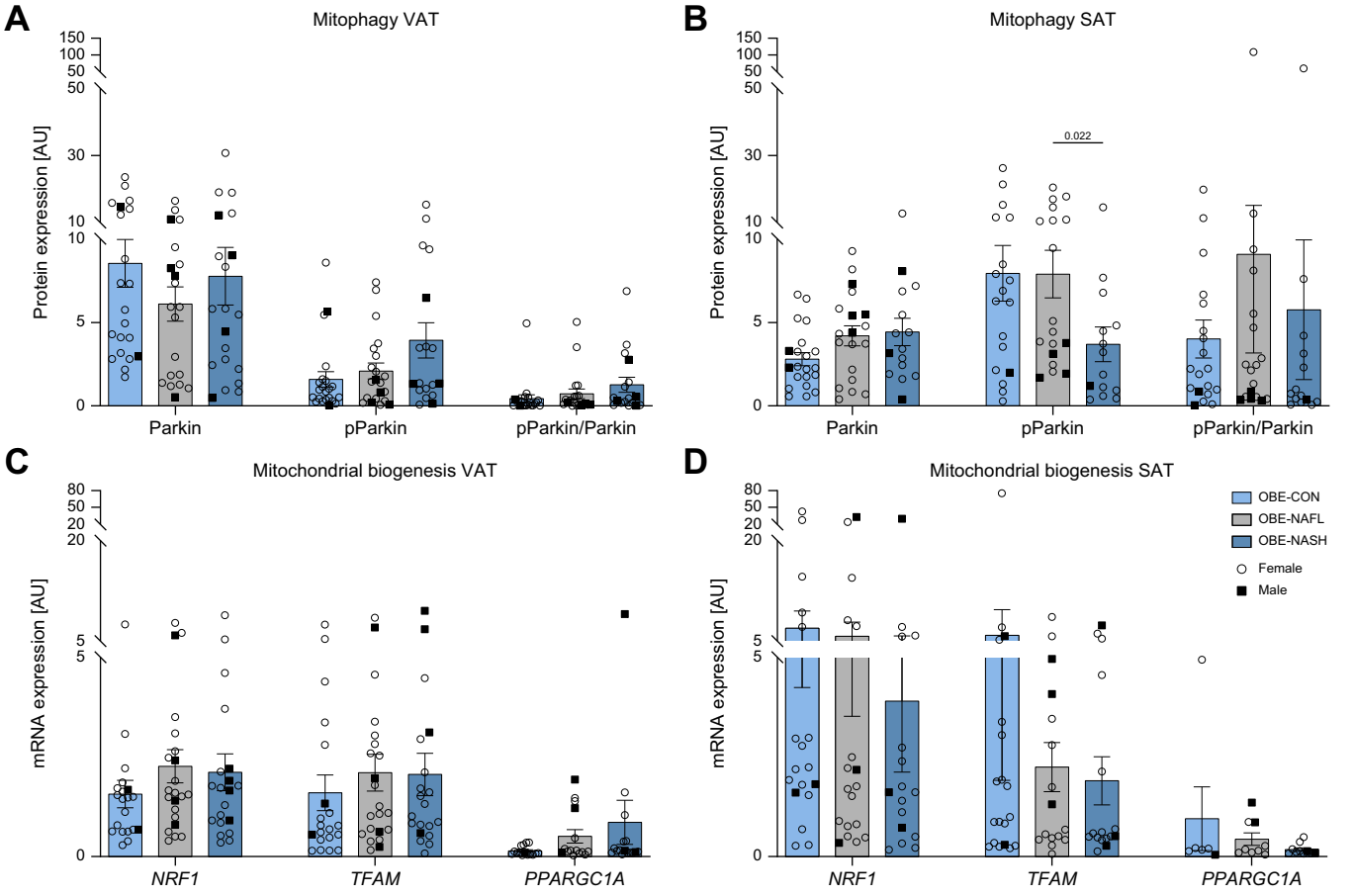

Fig. 3. Mitophagy and mitochondrial biogenesis in OBE-CON, OBE-NAFL or OBE-NASH. (A) Protein content of mitophagy markers (Parkin; phosphorylated Parkin (pParkin); pParkin/Parkin ratio) in VAT and (B) SAT, (C) transcript levels of regulators of mitochondrial biogenesis in VAT and (D) in SAT. Means ± SEM, levels of significance of each significant difference are marked in the figure (one-way ANOVA corrected for multiple comparisons with Tukey-Kramer multiple comparisons test). AU, arbitrary units; *NRF1*, nuclear respiratory factor 1; OBE-CON, obese humans without non-alcoholic fatty liver; OBE-NAFL, obese humans with NAFL; OBE-NASH, obese humans with NASH; *PPARGC1A*, peroxisomal proliferator activated receptor γ coactivator 1A; SAT, subcutaneous adipose tissue; *TFAM*, mitochondrial transcriptional factor A; VAT, visceral adipose tissue.

The present data cannot confirm the previously reported uniform impairment of mitochondrial respiration in both fat depots of obese humans. For instance, in both VAT and SAT of obese humans, proteomic analyses revealed decreased abundance of enzymes involved in fatty acid oxidation.[21] In contrast, the present findings rather support the hypothesis of a specific downregulation of energy metabolism in VAT, at least in persons with NAFLD and when compared to a carefully matched control group with comparable whole-body and abdominal obesity. Importantly, comparison of both fat depots revealed that only VAT of obese humans without NAFLD exhibits elevated mitochondrial respiration compared to SAT of all studied groups. Interestingly, mitochondrial respiration in VAT of obese people with NAFLD was comparable to that measured in SAT of obese non-steatotic people. This is in line with studies showing that VAT is generally characterized by higher oxidative metabolism in obese humans[22] and that only mitochondrial respiration in SAT, but not VAT, associates negatively with BMI in overweight people.[23] Our findings differ from previous evidence showing lower mitochondrial respiration in VAT of obese humans, whose liver histology was, however, not reported.[24]

Of note, the present study cannot assess whether mitochondrial respiration of both AT compartments is different between lean non-steatotic and obese humans without NAFLD, as reported for human livers.[13] But other studies have already suggested a reduction in O_2 consumption rates in both SAT and VAT when comparing overweight/obese humans with non-obese individuals.[25]

The markedly lower mitochondrial respiration in VAT of both OBE-NAFL and OBE-NASH could stem from impaired respiratory control. However, the observed comparable RCR and LCR across all groups and both fat depots suggests an intact coupling in both compartments. These findings are consistent with previous studies showing that adaptation of mitochondrial respiration coexists with unchanged RCR in SAT of murine obesity.[26] The rather high values of $[ETF+CI+II]_L$ compared to $[ETF+CI+II]_P$ and the relatively low RCR are in line with findings in the deep compartment of SAT,[27] and slightly higher when compared to abdominal SAT.[28] Use of different methods for tissue permeabilization, such as digitonin in the chamber[27] (like in the present study) or saponin prior to addition of AT in the chamber,[28] may explain the differences between studies.

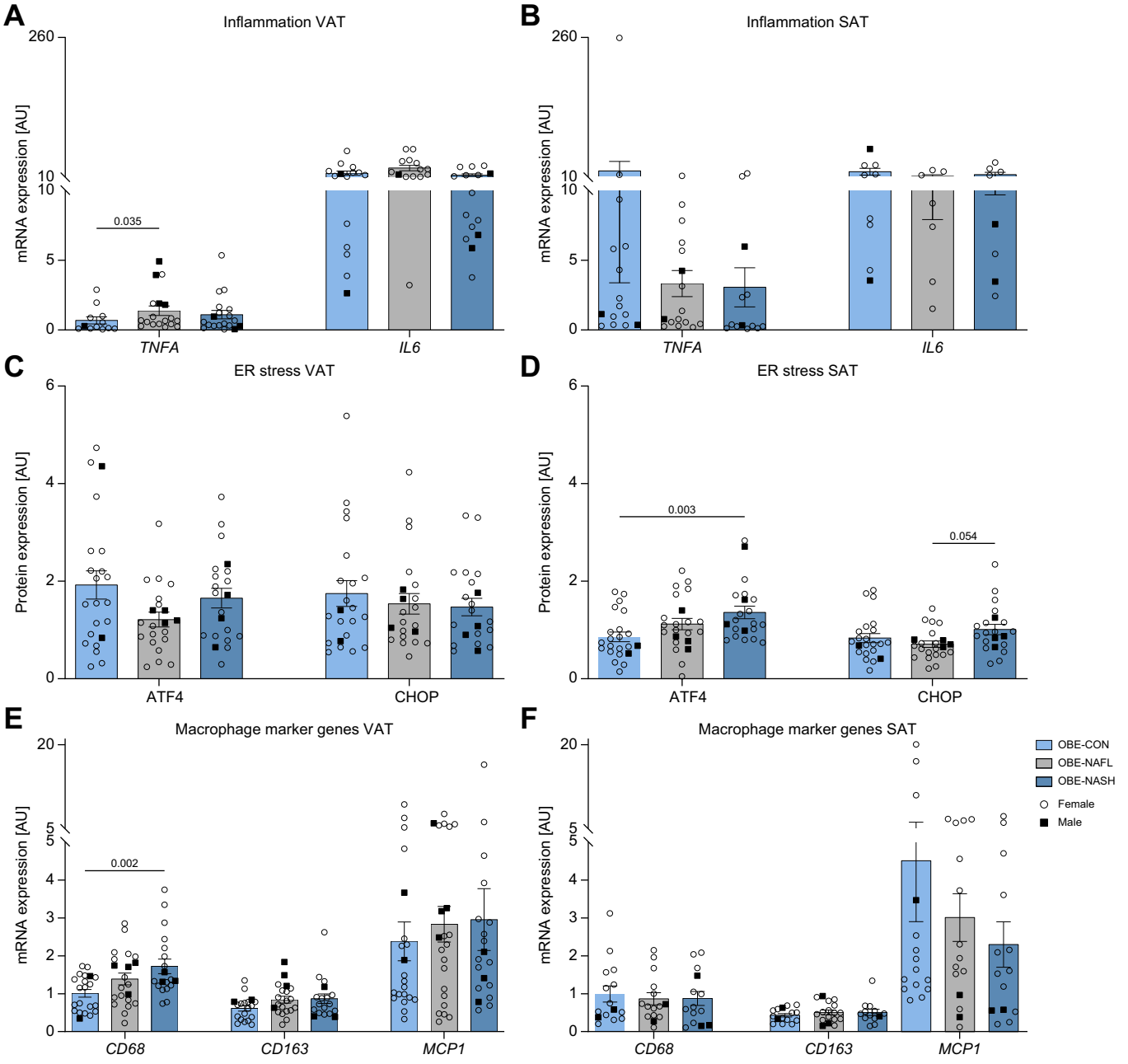

Fig. 4. Inflammation, ER stress and macrophage markers in OBE-CON, OBE-NAFL or OBE-NASH. (A) Transcript levels of *TNFA* and *IL6* in VAT and (B) SAT, (C) protein expression of ATF4 and CHOP in VAT and (D) in SAT, (E) transcript levels of *CD68*, *CD163* and *MCP1* in VAT and (F) in SAT. Means ± SEM, levels of significance of each significant and borderline significant difference are marked in the figure (one-way ANOVA corrected for multiple comparisons with Tukey-Kramer multiple comparisons test). ATF4, activating transcription factor 4; AU, arbitrary units; CHOP, C/EBP homologous protein; ER, endoplasmic reticulum; *IL6*, interleukin-6; *MCP1*, monocyte chemoattractant protein; OBE-CON, obese humans without non-alcoholic fatty liver; OBE-NAFL, obese humans with NAFL; OBE-NASH, obese humans with NASH; SAT, subcutaneous adipose tissue; *TNFA*, tumor necrosis factor A; VAT, visceral adipose tissue.

Lower mitochondrial mass may represent another possible reason for the reduced mitochondrial respiration in OBE-NAFL and NASH. In the absence of a single validated gold-standard, the present study employed 3 independent parameters, CSA, mtDNA and protein levels of OXPHOS CI-CV, as a proxy for mitochondrial content, as previously suggested.[24,25,29,30] While reduced CSA was reported in SAT and omental AT of obese humans,[25] the present study revealed similar CSA between groups in both compartments. No differences were detectable for mtDNA, in contrast to previous studies reporting lower mtDNA in VAT than in SAT of less obese humans.[24] Despite the slightly decreased expression of OXPHOS CIV in VAT of OBE-NASH than in OBE-CON, the lack of changes in RCR, LCR and other biomarkers of mitochondrial mass suggests an adaptive response to the altered metabolic conditions in NAFL and NASH. As the respirometry analysis in this study allows us to completely assess the OXPHOS system,[19] the results point to a true lower intrinsic mitochondrial functionality within VAT of humans with NAFLD.

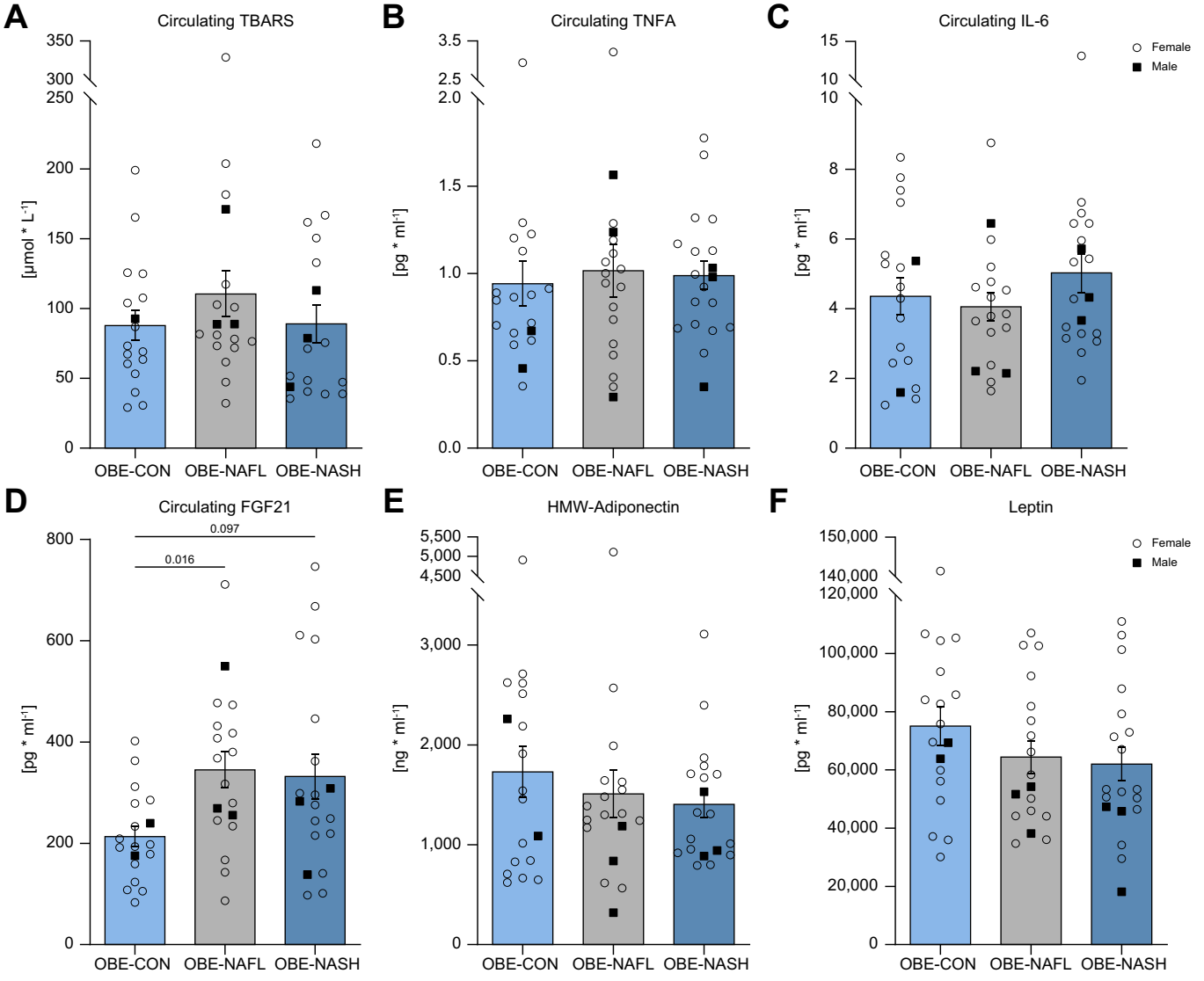

Fig. 5. Systemic oxidative stress and inflammation in OBE-CON, OBE-NAFL or OBE-NASH. Plasma concentrations of (A) TBARS, (B) TNFA, (C) IL6, (D) FGF21, (E) HMW-adiponectin and (F) leptin. Means ± SEM, levels of significance of each significant and borderline significant difference are marked in the figure (one-way ANOVA corrected for multiple comparisons with Tukey-Kramer multiple comparisons test). FGF21, fibroblast growth factor 21; HMW, high-molecular weight; IL6, interleukin-6; OBE-CON, obese humans without non-alcoholic fatty liver; OBE-NAFL, obese humans with NAFL; OBE-NASH, obese humans with NASH; TBARS, thiobarbituric acid reactive substances; TNFA, tumor necrosis factor A.

Likewise, comparison of both fat compartments revealed lower CIV-V expression in SAT of all groups than in VAT of OBE-CON, thus supporting differential adaptation of VAT in NAFLD.

Mitochondrial dynamics cannot account for the observed differences, as biomarkers of these pathways were similar in VAT between groups. Only in SAT, lower expression of pParkin in OBE-NASH may suggest altered mitophagy. Of note, VAT of OBE-CON and OBE-NAFL featured lower pParkin and pParkin/Parkin ratios, but increased expression of ER stress markers than SAT of the respective groups. The downregulated mitophagy in these groups could contribute to the gradual increase in ER stress markers, due to the previously described negative feedback mechanisms between autophagy and ER stress.[31]

Another novel finding of this study is the association between maximal ADP-stimulated mitochondrial respiration ($[ETF+CI+II]_P$) in VAT with AT IS, which remains a matter of debate. While some studies found a positive correlation between mitochondrial oxidative capacity in SAT and AT glucose metabolism,[29] others dissociated AT respiration from tissue-specific IR.[18] Differences among these results and our study may rely on the lack of data from human VAT.[18] Although the current study detected elevated AT IR only in OBE-NASH, data from another cohort of BARIA_DDZ indicate that obese humans with NAFL also have higher AT IR.[32] These findings support a role for AT IR in the previously reported association between increased AT lipolysis and NAFLD in humans.[33]

Recent evidence indicates that SAT shows adequate expandability in obese people with and without NAFLD[34] and that SAT inflammation and increased fatty acid release in relation to fat-free mass are unrelated to IR.[35] Given the critical role of AT mitochondria in fatty acid oxidation[36] and FFA release into the circulation, VAT can contribute up to 50% of the FFA delivered to

the liver,[37] as well as contributing to IR by interfering with insulin-stimulated glucose transport.[3] Of note, $[ETF]_P$, which was reduced only in VAT of obese individuals with NAFLD, reflects oxygen consumption linked to fatty acid oxidation. Taken together, these data support that downregulation of mitochondrial respiration in VAT contributes to impaired insulin-mediated suppression of AT lipolysis, leading to increased FFA release to the portal vein and NAFLD progression in obese people.

Despite no changes in circulating pro-inflammatory biomarkers, OBE-NAFL showed higher *TNFA* expression in VAT, which correlated negatively with maximal ADP-stimulated mitochondrial respiration ($[ETF+CI+II]_P$) across all groups. This extends and supports previous findings on the TNFA-mediated downregulation of OXPHOS genes in VAT of obese females.[38] We also found greater levels of *TNFA* in SAT of OBE-CON and of TBARS in SAT of OBE-CON and OBE-NAFL than in VAT of the corresponding groups. Nevertheless, VAT *TNFA* levels were not increased in OBE-NASH, in contrast to previous evidence showing higher human VAT *TNFA* mRNA expression in participants with NASH, but with higher BMI, compared to those with NAFL.[39] Our findings suggest a link between VAT-derived *TNFA* and early hepatic damage, in line with evidence pointing to hepatocytes and Kupffer cells as the primary sources of TNFA production in the context of NASH.[40] Both expression and systemic IL6 concentrations were similar between groups, in line with equally high IL6 levels in obese insulin-sensitive and insulin-resistant humans.[35] Only in VAT, mRNA expression of the macrophage marker *CD68* was increased in OBE-NASH, supporting previous evidence for a linear correlation between CD68+ VAT macrophages and hepatic inflammation in obese humans.[41]

Finally, FGF21 also correlated negatively with maximal ADP-stimulated mitochondrial respiration ($[ETF+CI+II]_P$) in VAT across all groups. This finding lends further support to the suggested link between FGF21 and impaired muscle mitochondrial respiration in humans,[42] but does not allow for conclusions on causality given the complex function of FGF21 in the metabolism of various tissues.[43]

The present study benefits from the assessment of various independent features of mitochondrial function in a cohort tightly matched for measures of obesity. Our participants underwent comprehensive phenotyping of energy metabolism and detailed biopsy assessment of liver histology. Although liver biopsies were assessed by a validated score,[15] our data need to be carefully interpreted in the context of the limitations of the evaluation tool. As the primary aim was to examine AT mitochondrial function in obese NAFLD, the lack of a lean control group does not represent a study limitation, but means our findings are not generalizable to individuals with lean NAFLD. Also, the cross-sectional design does not allow for conclusions on causality. This study found no trend towards differences in mitochondrial respiration and content between males and females. However, the higher proportion of female volunteers, also known from other bariatric surgery cohorts,[44] cannot exclude that such sex differences might occur, as reported in AT of high-fat fed and obese mice.[45] Further, the experimental setup, *i.e.* assessing AT respiration during the presurgical period, comprising diet, fasting and weight loss, does not necessarily reflect physiological day-to-day conditions in people with or without NAFLD.[46] Finally, expression of mitochondrial respiration normalized to mitochondrial content remains a matter of debate.[19] Nevertheless, the present study revealed similar results when expressing respiration rates either per protein or additionally per mtDNA, confirming their robustness.

In conclusion, maximal ADP-stimulated mitochondrial respiration ($[ETF+CI+II]_P$) in VAT is reduced in obese individuals with NAFL or NASH compared to those without NAFLD and correlates positively with AT IS, but negatively with local VAT inflammation. These data indicate an important role of compartment-specific AT energy metabolism for IR and hepatic lipid accumulation in the context of obesity. It is tempting to speculate that improvement of mitochondrial respiration in VAT could serve as a future therapeutic target to prevent the manifestation and progression of NAFLD.

Abbreviations

AT, adipose tissue; ATF4, activating transcription factor 4; AU, arbitrary units; C, complex; CHOP, C/EBP homologous protein; CSA, citrate synthase activity; $_E$, electron transport system capacity; ER, endoplasmic reticulum; ETF, electron transferring flavoprotein; FFA, free fatty acids; FGF21, fibroblast growth factor 21; GOX, glucose oxidation; hsCRP, high-sensitivity C-reactive protein; IL, interleukin; IR, insulin resistance; IS, insulin sensitivity; $_L$, leak respiration; LCR, leak control ratio; LOX, lipid oxidation; mtDNA, mitochondrial DNA; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NOXGD, non-oxidative glucose disposal; NRF, nuclear respiratory factor; OBE-CON, obese humans without non-alcoholic fatty liver; OBE-NAFL, obese humans with NAFL; OBE-NASH, obese humans with NASH; OXPHOS, oxidative phosphorylation; $_P$, OXPHOS capacity; POX, protein oxidation; pParkin, phosphorylated Parkin; RCR, respiratory control ratio; SAT, subcutaneous adipose tissue; T2D, type 2 diabetes; TBARS, thiobarbituric acid reactive substances; TFAM, mitochondrial transcription factor A; TNFA, tumor necrosis factor A; VAT, visceral adipose tissue.

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Conflict of interest

M.R. received lecture fees or served on advisory boards for Allergan, Astra-Zeneca, Bristol-Myers-Squibb, Eli Lilly, Fishawack Group, Gilead Sciences, Intercept Pharma, Inventiva, Novartis, Novo Nordisk, Pfizer, Prosciento, Sanofi US and Target RWE and performed investigator-initiated research with support from Boehringer-Ingelheim, Nutricia/Danone and Sanofi-Aventis to the German Diabetes Center (DDZ). C.H. received grant support from Sanofi-Aventis. No conflicts of interest, financial or otherwise, are declared by the other authors.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

M.R., S.K. and K.P. designed the study and analyzed the results. M.R. and K.P. drafted the manuscript. K.P., S.K., L.M., D.P., C.H., J.P., B.D., M.H., C.G, N.S., A.Y., S.G., G.H., I.E., M.S. contributed to acquisition of the data. K.S. provided statistical analysis of the data. K.P., S.K., L.M., K.S., D.P., C.H., J.P., B.D., M.H., C.G., N.S., A.Y., S.G., G.H., I.E., M.S. and M.R. revised the manuscript and approved the final version of the manuscript.

Data availability statement

Data are available upon reasonable request. The data sets generated during and/or analyzed during the current study are not publicly available, since they are subject to national data protection laws and restrictions imposed by the ethics committee to ensure data privacy of the study participants. However, they can be applied for through an individual project agreement with the principal investigator of the German Diabetes Study.

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Supplementary data

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Author names in bold designate shared co-first authorship

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