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Title:

The role of adipose tissue and subsequent liver tissue hypoxia in obesity and early stage metabolic dysfunction associated steatotic liver disease

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Competing interest statement

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23 **Abstract**

24 *Background*: Obesity is linked to several health complication, including Metabolic Dysfunction
25 Associated Steatotic Liver Disease (MASLD). Adipose tissue hypoxia has been suggested as an
26 important player in the pathophysiological mechanism leading to chronic inflammation in obesity, and
27 in the progression of MASLD. The study aims to investigate the effect of progressive obesity on adipose
28 and liver tissue hypoxia.

29 *Methods*: Male 8-week-old C57BL/6J mice were fed a high-fat high-fructose diet (HFHFD) or control
30 diet (CD) for 4, 8, 12, 16 and 20 weeks. Serum ALT, AST and lipid levels were determined, and glucose
31 and insulin tolerance testing was performed. Liver, gonadal and subcutaneous adipose tissue was
32 assessed histologically. In vivo tissue pO_2 measurements were performed in gonadal adipose tissue
33 and liver under anaesthesia. A PCR array for hypoxia responsive genes was performed in liver and
34 adipose tissue. The main findings in the liver were validated in another diet-induced MASLD mice
35 model, the choline-deficient L-amino acid defined high-fat diet (CDAHFD).

36 *Results:* HFHFD feeding induced a progressive obesity, dyslipidaemia, insulin resistance and MASLD. In
37 vivo pO_2 was decreased in gonadal adipose tissue after 8 weeks of HFHFD compared to CD, and
38 decreased further until 20 weeks. Liver pO_2 was only significantly decreased after 16 and 20 weeks of
39 HFHFD. Gene expression and histology confirmed the presence of hypoxia in liver and adipose tissue.
40 Hypoxia could not be confirmed in mice fed a CDAHFD.

41 *Conclusion:* Diet-induced obesity in mice is associated with hypoxia in liver and adipose tissue. Adipose
42 tissue hypoxia develops early in obesity, while liver hypoxia occurs later in the obesity development
43 but still within the early stages of MASLD. Liver hypoxia could not be directly confirmed in a non-obese
44 liver-only MASLD mice model, indicating that obesity-related processes such as adipose tissue hypoxia
45 are important in the pathophysiology of obesity and MASLD.

46

47 **Introduction**

48 Obesity represents an enormous health burden, affecting over 650 million adults in 2016 according to
49 the WHO(1). Obesity is related to an increased risk of developing several health problems. The main
50 comorbidities of obesity are included in the metabolic syndrome and non-alcoholic fatty liver disease
51 (NAFLD) is considered as the hepatic manifestation of the metabolic syndrome. It was therefore
52 recently renamed and re-defined as Metabolic Dysfunction Associated Steatotic Liver Disease
53 (MASLD)(2). MASLD consists of a spectrum of disease, ranging from isolated steatosis to non-alcoholic
54 or metabolic dysfunction associated steatohepatitis (MASH). MASH is characterized by the concurrent
55 presence of steatosis, ballooning and lobular inflammation, and predisposes to complications such as
56 cirrhosis, hepatocellular carcinoma and cardiovascular disease(3, 4). Although often considered as a
57 benign condition, isolated steatosis can predispose the liver to further injury, and is a precursor and
58 independent risk factor for type 2 diabetes and cardiovascular disease(5).

59 Obesity is characterized by a state of chronic inflammation and increasing evidence points to a critical
60 role of hypoxia in mediating the proinflammatory responses in adipose tissue (AT) in obesity(6)
61 resulting in systemic inflammation. With the expansion of the AT, there is an increased angiogenesis
62 to ensure sufficient supply of oxygen and nutrients. As adipocyte hypertrophy continues, local tissue
63 hypoxia develops leading to the activation of hypoxia-inducible transcription factors, in particular
64 hypoxia-inducible factor (HIF)-1(7). This sets a cascade in motion that contributes to further adipocyte
65 and metabolic dysfunction. Studies in mice with diet-induced obesity show that AT hypoxia can occur
66 as soon as 1-3 days after starting the high-fat diet (HFD)(8). However, little is known about how AT
67 hypoxia develops during the progression towards severe obesity.

68 In MASLD progression, hypoxia has also been implicated, as increased intrahepatic resistance results
69 in a chronic flow impairment in the microcirculation of steatotic livers(9, 10). This inadequate perfusion
70 potentially leads to hepatic hypoxia most distally along the sinusoids(11). Indeed, hypoxia is suggested
71 to be present in the centrilobular region of steatotic livers in mice on a HFD(12, 13). Furthermore, AT

72 hypoxia has also been implicated in MASLD, as adipocyte HIF-modulation affects liver lipid
73 accumulation(5). Under normal physiological circumstances, the AT and liver strive to maintain
74 metabolic homeostasis through the secretion of adipokines and growth factors(14). However, chronic
75 nutrient excess, causing obesity, leads to a disruption of the adipose tissue-liver axis and further
76 contributes to the metabolic derangement. It is unclear if and to what extent the development of
77 hypoxia in the liver and AT contributes to an imbalance in the adipose-liver axis.

78 Therefore, the aim of this study was to investigate the effect of progressive severe obesity on the role
79 of hypoxia in the AT and liver tissue in a mice model for obesity.

80

81

82 **Methodology**

83 *Mice and experimental design model 1: high-fat high-fructose diet*

84 C57BL/6J 8-week-old male mice (Janviers Labs, Le GenestSaint-Isle, France) were kept on a 12:12-hour
85 light/dark cycle with controlled temperature (21-24°C) and humidity (45-60%) and housed in enriched
86 cages with a stainless-steel grid, filter top, and free access to tap water. All experiments were
87 performed during the light cycle.

88 Mice had free access to standard chow in the control group (Ssniff Specialdiäten GmbH, Soest,
89 Germany), with 9kJ% from fat and 67 kJ% from carbohydrates, or a high-fat high-fructose diet (HFHFD)
90 in the experimental group (D16042610; Research Diets, New Brunswick, NJ), containing 55 kcal% fat
91 and 25kcal% carbohydrates of which 14 kcal% are from fructose. Mice were fed either the standard
92 chow or HFHFD and sacrificed after 4, 8, 12, 16 or 20 weeks after the start of the experiment. A more
93 detailed description of the 2 diets can be found in the supplemental materials. Mice were selected at
94 random for each experimental group and the researchers were blinded for all tissue analysis
95 performed after sacrifice.

96 This study was approved by the Ethical Committee for Animal Testing of the University of Antwerp
97 (2016-16). Based on a difference in gene expression of *HIF-1α* between animals on an HFD and animals
98 on a standardised diet found in a pilot study, a sample size of 10 animals per group per time point was
99 calculated. The animals were included trough 2 separate replicate experiments. Animals were
100 excluded from the analysis when a significant drop in body weight was noticed (for example due to
101 fighting among the animals housed together).

102 *Glucose and Insulin Tolerance Testing*

103 Glucose and insulin tolerance testing were performed by injecting mice intraperitoneally with a 20%
104 glucose solution (Baxter, Deerfield, IL) at 2 mg glucose per gram of body weight or a 0.1U/mL insulin
105 solution (Novo Nordisk, Bagsvaerd, Denmark) at 0.75 mU per gram of body weight, after being fasted

106 for 6 hours. Glucose levels were determined with a portable glucometer (GlucoMen LX; A. Menarini
107 Diagnostics, Florence, Italy) after 15, 30, 60, 90, and 120 minutes. The area under the curve (AUC) was
108 calculated with the trapezoidal rule for both glucose and insulin.

109 *Tissue pO_2 measurements*

110 *In vivo* pO_2 measurements were made in the gonadal AT and liver tissue of anesthetized mice with an
111 oxygen Clark-type microsensor (25 µm tip diameter, Unisense, Aarhus, Denmark). The procedure was
112 performed in the mice after laparotomy under general anaesthesia with isoflurane (3%, 0.2 l/min, in
113 combination with room air).

114 The oxygen microsensor was connected to a high-sensitivity picoampere amplifier and was calibrated
115 in water saturated with 21% O_2 and an oxygen-free solution (0.1M sodium ascorbate in 0.1M NaOH).
116 In gonadal AT, the microsensor was inserted with the help of a micromanipulator 2mm into the tissue
117 until a stable signal was obtained. For liver tissue, the microsensor was inserted 3mm into the tissue
118 of the left lateral lobe and retracted 0.5mm. The mean adipose and liver pO_2 was measured for 90
119 seconds after positioning. This was repeated three times per animal per tissue, and the average of
120 these measurements was calculated.

121 *Biochemistry*

122 A blood sample was collected through cardiac puncture. Whole blood samples were centrifuged for 10
123 minutes at 10 000g and serum was collected. ALT (Limit of detection (LoD) ≤7U/L), AST (LoD ≤8U/L),
124 triglycerides (LoD ≤8mg/dl), HDL (LoD ≤4 mg/dl), LDL (LoD ≤4 mg/dl) and total cholesterol (LoD ≤10
125 mg/dl were determined by means of an automated system (Atellica, Siemens Healthineers, Erlanger,
126 Germany).

127 *Histology*

128 Samples of gonadal AT, subcutaneous AT and liver were fixed in 4% formaldehyde and embedded in
129 paraffin. Subsequently, 5 µm sections were stained with haematoxylin and eosin (H&E) according to

130 standard protocols. Liver sections were also stained with picrosirius red. The histological characteristics
131 of MASH in the liver were scored on H&E-stained slides using the NASH Clinical Research Network
132 Scoring System, including the NAFLD Activity Score (NAS)(15). The adipocyte diameter of AT was
133 determined by means of the ImageJ plugin Adiposoft(16). Liver fibrosis was quantified with ImageJ on
134 picrosirius red stained slides.

135 Hypoxia was immunohistochemically stained in AT with pimonidazole, which is reductively activated
136 in hypoxic cells and forms stable adducts with thiol groups of proteins, peptides and amino acids(17).
137 Pimonidazole was injected intraperitoneally at 100 mg/kg bodyweight 1 hour before tissue collection.
138 The adducts were then stained on paraffin embedded tissue samples according to the manufacturer's
139 protocol (Hypoxyprobe, NPI, USA).

140 *PCR Array*

141 A Hypoxia signal Pathway RT² Profiler PCR Array was used to screen a panel of 84 genes involved in
142 hypoxia-related processes and 5 housekeeping genes. Samples of mice after 4, 12 and 20 weeks of diet
143 were used. Four animals per group (control vs HFHFD) were randomly selected per time point.

144 Total RNA was isolated from gonadal adipose tissue, subcutaneous adipose tissue and liver using the
145 RNeasy Plus Universal Tissue Mini Kit (Qiagen) following the manufacturer's instructions. The
146 concentration and quality of the RNA was evaluated using the NanoDrop® ND1000 UV-Vis
147 Spectrophotometer (Thermo Fisher Scientific). Next, 0.5 µg RNA was converted to cDNA by reverse
148 transcription using the RT2 First Strand Kit (Qiagen). Gene expression was then determined with the
149 RT² Profiler PCR Array using RT² SYBR Green ROX qPCR Mastermix (Qiagen) on an ABI 7500 Fast Real-
150 Time PCR system (Thermo Fisher Scientific) under the following conditions: an initial activation step of
151 the DNA Taq Polymerase for 10 minutes at 95°, followed by 40 cycles of denaturation at 95°C for 15
152 sec and annealing/extension for 1 min at 60°C.

153

154 *Mice and experimental design model 2: choline-deficient L-amino acid defined high-fat diet*

155 *In vivo* hypoxia results were validated in a second dietary MASLD model. C57BL/6J 8-week-old male
156 mice were fed a choline-deficient L-amino acid defined high-fat diet (CDAHFD, A06071302, Research
157 Diets, New Brunswick, NJ, USA) as reported previously(18) and compared to lean control animals on a
158 standardized diet. Animals were sacrificed after 1, 2, 3, 4 and 6 weeks of diet. As this is a non-obese
159 liver-only MASH mice model, only measurements in liver tissue were repeated for this model.

160 This study was approved by the Ethical Committee for Animal Testing of the University of Antwerp
161 (2019-42).

162 *Statistical and data analysis*

163 Statistical analyses were performed in SPSS 27.0 (SPSS, Chigaco, Illinois, USA), Graphpad Prism 9
164 (Boston, Massachusetts, USA) and R version 4.0.1 and higher running Rstudio (2022.07.0). A p-value
165 ≤0.05 was considered as statistically significant. Differences between groups were determined via
166 Mann-Whitney U test adjusted for multiple testing using the two-stage step-up method of Benjamini,
167 Krieger and Yekutieli(19). A mixed effects analysis was used to determine group and time effects on
168 the pO_2 measurements. The associations between hypoxia and metabolic parameters was assessed
169 using a spearman correlation analysis. A detailed description of the analysis of the PCR array data can
170 be found in the supplemental materials.

171

172 **Results**

173 *Body weight and metabolic profile after HFHFD*

174 The body weight of the mice increased progressively over 20 weeks of HFHFD (Figure 1 and Table 1).
175 This was associated with an increase in total cholesterol and LDL levels (Table 1). Glucose and insulin
176 tolerance testing showed decreased glucose tolerance after 4 weeks of HFHFD and a decreased insulin
177 sensitivity after 16 weeks of HFHFD (Figure S1).

178 After 20 weeks, liver weight was significantly increased in the animals receiving HFHFD (Table 1).
179 Histological assessment of the liver after HFHFD showed progressive stages of MASLD, with an
180 increasing NAS the longer the diet was administered (Figure 2). This was not observed in animals on
181 standard chow, displaying normal liver histology. No evidence of fibrosis was found in the animals
182 (Table S1).

183 Histological assessment of the gonadal and subcutaneous AT after HFHFD showed development of
184 adipocyte hypertrophy at all time points (Figure S2). Over time, in gonadal AT, the adipocyte diameter
185 increased from 59.29 (42.54 – 65.34) µm to 71.42 (67.28-75.53) µm after 20 weeks of HFHFD ($p<0.002$).
186 On standard chow the adipocyte diameter also increased in the gonadal AT after 20 weeks (41.25
187 (38.10-45.26) µm compared to 46.38 (43.86 – 49.04) µm, $p=0.004$), but less compared to a HFHFD. In
188 the subcutaneous AT an increase in adipocyte diameter was also observed for the animals on HFHFD,
189 with an increase from 43.53 (31.03 – 54.31) µm at 4 weeks to 68.63 (53.04 – 71.08) µm after 20 weeks
190 of HFHFD ($p=0.001$). In the animals on standard chow no increase in adipocyte diameter in
191 subcutaneous AT was found after 20 weeks (32.90 (27.66-35.27) µm at 4 weeks vs. 32.03 (29.46 –
192 44.10) µm after 20 weeks; $p=0.7$).

193 Overall, the area of pimonidazole in the gonadal AT was increased in animals on a HFHFD compared to
194 animals on a standard diet ($p=0.029$; 0.39(0.058 – 2.60)% vs. 0.22 (0.014 – 1.42)%). However, no
195 significant difference could be found on the different time points separately. No significant difference

196 in %area pimonidazole was found in subcutaneous AT overall or at any timepoint ($p>0.05$). Figure S3
197 summarizes these data.

198 *Body weight and liver characteristics after CDAHFD*

199 The characterisation of the mice fed a CDAHFD have been previously reported(18). Briefly, the body
200 weight decreased compared to a standard diet (Figure S4 and Table S2), while the liver weight
201 increased, albeit not significant, over time. In contrast, the liver/body weight ratio was significantly
202 increased after 2, 3, 4 and 6 weeks of CDAHFD. This corresponded with a significant increase in ALT.
203 (Table S2). Histological assessment of the liver of CDAHFD mice already showed an increased NAS after
204 1 week (median NAS of 4), with increasing severity over time (median NAS of 8 at 6 weeks).

205 *In vivo pO_2 measurements*

206 Increasing obesity was associated with a decreasing pO_2 in the gonadal AT after 8 weeks of HFHFD
207 which remained until 20 weeks of HFHFD (Figure 3A). In the liver, a decrease in pO_2 was only observed
208 after 16 and 20 weeks of HFHFD (Figure 3B).

209 A mixed effects analysis was used to investigate the effect of time on the pO_2 measurements (Table 2).
210 For both gonadal AT and liver a significant effect of group (control *vs*. HFHFD) was found on pO_2
211 measurements (both $p<0.001$). Time did not contribute separately to the pO_2 in gonadal AT or liver
212 tissue ($p=0.06$ and $p=0.08$), however a significant time-group interaction was found for gonadal AT pO_2
213 ($p=0.007$). The AT pO_2 also correlated significantly with the stained AT area of pimonidazole on
214 histology after correction for adipocyte diameter ($r=-0.27$, $p= 0.02$).

215 In animals fed CDAHFD no significant differences in liver pO_2 were found compared to control animals
216 (Figure 5C), however, there was a trend for a decreased pO_2 after 6 weeks of CDAHFD diet. No effect
217 for group or time were found on liver pO_2 in this experiment.

218 Correlations between pO_2 measurements and several metabolic parameters were found in the HFHFD
219 fed mice model, which are summarized in Table 3.

220 *Hypoxia signalling pathway PCR Array*

221 The expression of hypoxia-related genes was determined after 4, 12 and 20 weeks of HFHFD on liver,
222 and gonadal and subcutaneous AT using a PCR array. A clear tissue dependent effect was seen in the
223 expression data (Figure S5), therefore all tissues were analysed separately.

224 In the gonadal AT, 7 genes were upregulated after 12 weeks of HFHFD in obese animals compared to
225 control animals (Table S3). Two genes were related to angiogenesis (*Lox, Plau*), 2 genes were HIF1-
226 interactors (*NfkB1, Trp53*), two were other hypoxia-responsive genes (*Lgals3, Ctsa*) and one was
227 involved in metabolism-related processes (*Gusb*). After 20 weeks, 28 genes were differentially
228 expressed in the gonadal AT of obese animals compared to control animals (Table S4). Four genes were
229 involved in the regulation of apoptosis (*Bnip3, Adm, Nos3* (all downregulated*) and Mif* (upregulated)),
230 7 genes in metabolism-related processes (upregulated: *Pkm2, Ero1l, Slc2a1, Pdk1 and Gusb;*
231 downregulated: *Pfkfb3 and Gys1*), 6 genes were related to angiogenesis (upregulated: *Plau, Hmox1;*
232 downregulated: *Vegf, Angptl4, Edn1, Serpine1*), 2 were HIF1-interactors (*Egln1* (downregulated)*, Trp53*
233 (upregulated)), 7 were other hypoxia-responsive genes (upregulated: *Map3k1, Car9, Lgals3, Ctsa,*
234 *Dnajc5;* downregulated: *Eif4ebp1, Gbe1, ,*), 1 gene was involved in the regulation of Cell Proliferation
235 (*Txnip,* downregulated) and 1 gene expressed in transporters, channels and receptors (*Tfrc,*
236 upregulated).

237 In the subcutaneous AT, 5 genes were downregulated after 12 weeks of HFHFD in obese animals
238 compared to control animals (Table S3). This was one HIF1-interactor (*Egln2*), one gene related to DNA
239 damage and repair (*Ruvbl2*), one gene involved in the regulation of cell proliferation (*Odc1*) and one
240 gene expressed in transporters, channels and receptors (*Tfrc*). One gene could not be related to any
241 relevant pathways (*Hsp90ab1*). After 20 weeks, 7 genes were differentially expressed in the
242 subcutaneous AT (Table S4). Five genes were involved in metabolism-related processes (*Eno1, Pfkl,*
243 *Pdk1, Pgam1* (all upregulated) *and Slc2a3* (downregulated)), one gene was a HIF1-interactor (*Egln2,*
244 downregulated) and one gene was related to DNA damage and repair (*Ruvbl2,* downregulated).

245 In the liver, 8 genes were differentially expressed after 12 weeks of HFHFD in control animals compared
246 to obese animals (Table S3). Of these genes, 3 were related to angiogenesis (*F3* (upregulated), *Hmox1*
247 and *Mmp9* (both downregulated)), 2 to the regulation of Cell Proliferation (*Met* and *Nampt,* both
248 upregulated), one gene was involved in Metabolism-related processes (*Gbe1,* upregulated) and 2
249 genes were hypoxia-responsive genes (*Trp53* and *Map3k1,* both downregulated). After 20 weeks of
250 HFHFD, 11 genes were differentially expressed in the liver of control animals compared to animals fed
251 a HFHFD (Table S4), of which 10 genes were down regulated and only 1 gene was upregulated in obese
252 animals compared to the controls. Six of these genes were HIF1 co-transcription factors or HIF1-
253 interactors (*Apex1, Nfkb1, Map3k1, P4ha1, Trp53, Arnt, Hnf4a*), 2 genes were involved in angiogenesis
254 or coagulation (*Anxa2* (upregulated), *Amp9*), 1 in the regulation of cell proliferation (*Pim1*) and 1 in
255 metabolism-related processes (*Ero1l*).

256 The influence of covariates on the gene expression pattern in the different tissues was determined
257 through a principal component analysis (PCA). In gonadal AT, three principal components (PC) could
258 be identified. The loading of the different PC can be found in Figure S6. PC2 could distinguish based on
259 group (control vs HFHFD) and correlated strongly with both liver and AT pO_2 (Figure 4A). Also in
260 subcutaneous AT three PC could be identified (Figure S6), where PC3 strongly correlated with glycemia
261 (Figure 4B). In liver tissue, four PC could be identified (Figure S6). PC1 in the liver was able to distinguish
262 based on group (control vs HFHFD) and correlated with fasting glycemia (Figure 4C).

263 The association between the *in vivo* oxygen measurements and 11 core hypoxia genes(20) in liver and
264 gonadal AT is shown in Figure 5. The hypoxia signature was optimized for each tissue (Figure 6). Both
265 in liver and gonadal AT 5 core hypoxia genes were included, resulting in a strong correlation with the
266 *in vivo* pO_2 measurements (figure 6). Table 4 shows the core hypoxia genes included for each tissue.

267 **Discussion**

268 This study shows the development of visceral AT and liver hypoxia during progressive obesity, with AT
269 hypoxia preceding liver hypoxia, and the latter developing at the stage of isolated steatosis already.
270 We indeed found adipocyte hypoxia in early obesity after 8 weeks of HFHFD. Liver pO_2 only decreased
271 in a later stage of obesity development, *i.e.* after 16 weeks of HFHFD. Nevertheless, the hypoxia
272 development seen in the liver was during the early MASLD stadium when steatohepatitis and fibrosis
273 had not yet developed, supporting our hypothesis that early vascular changes in steatosis can impair
274 intrahepatic blood flow and induce local tissue hypoxia.

275 As previously demonstrated, 20 weeks of HFHFD resulted in obesity, MASL as well as several metabolic
276 alterations such as dyslipidaemia and insulin resistance(21). The HFHFD mouse model therefore
277 mimics the human pathology relatively closely in terms of obesity-associated metabolic derangements
278 and early MASLD lesions. We showed that these alterations are accompanied by significant adipose
279 and liver tissue hypoxia, and by an altered gene expression in several hypoxia-related genes in both
280 liver and AT.

281 AT hypoxia was found after 8 weeks of HFHFD. Normally, AT expands trough hyperplasia and
282 hypertrophy. However, in obesity, the rapid adipocyte expansion occurs almost solely through
283 hypertrophy, which is not matched by blood vessel formation, resulting in AT hypoxia(22). Indeed, the
284 adipocyte diameter already increased in the gonadal AT after 4 weeks of HFHFD. Several studies have
285 previously indicated AT hypoxia in animal models trough histology(23-25). Additionally, Rausch *et al.*
286 and Ye *et al.*, measured partial oxygen pressure via a fibre-optic sensor with both studies indicating a
287 decreased pO_2 in *ob/ob* mice with obesity after 28 weeks and 7 weeks of HFD respectively(24, 25).
288 However, previous studies only measured hypoxia at a single time point and lack information on the
289 evolution of hypoxia during the development of obesity. To the best of our knowledge, we are the first
290 to report the longitudinal evolution of directly and indirectly measured pO_2 levels and hypoxia in AT

291 during the progression of obesity, where we show decreasing pO_2 levels in gonadal AT of HFHFD fed
292 mice.

293 Hepatic tissue hypoxia appeared after 16 weeks of HFHFD, which was accompanied by steatosis but
294 with very few other lesions, at this stage thus insufficient to diagnose steatohepatitis (with a median
295 NAS score of 1.5 which was attributed to isolated steatosis with limited ballooning but no
296 inflammation). We have previously showed that early MASLD is associated with portal hypertension
297 as a result of an increased intrahepatic vascular resistance that impairs the intrahepatic blood flow(9,
298 26). These pathophysiological changes in liver vasculature could possibly underlie the hepatic tissue
299 hypoxia seen in our experiment. We hypothesize that this local tissue hypoxia could then trigger
300 several pathways of cell damage and inflammation that will ultimately lead to the progression of
301 isolated steatosis to MASH, fibrosis and its further hepatic and extrahepatic complications(11). Other
302 studies indirectly support a potential role of hypoxia in MASLD and its progression to MASH. For
303 example, steatotic donor livers appear to be more vulnerable to ischaemia-reperfusion injury
304 compared to normal donor livers(27-29), and furthermore a higher complication rate and mortality of
305 primary surgery of steatotic livers has been observed(27). Also, the relationship between obstructive
306 sleep apnoea and MASLD is well established(30-32), which could be partly due to the presence of
307 (intermittent) hypoxia. Previous research in experimental models of diet-induced obesity showed that
308 exposure to intermittent hypoxia exacerbates insulin intolerance and hepatic steatosis(33), and in
309 models of liver cirrhosis intermittent hypoxia impaired liver microvasculature function and increased
310 oxidative stress(34).

311 To investigate if the effects seen in the liver could be repeated in another model, some experiments
312 were repeated in a CDAHFD mouse model which is a liver-specific model with rapid progression
313 towards steatohepatitis with fibrosis but without developing obesity(35). In this model of more severe
314 MASH compared to HFHFD, no significant decrease in liver pO_2 could be found after 6 weeks CDAHFD
315 feeding despite liver histology already showing an increased average NAS score of 5 after 1 week of

316 CDAHFD diet (corresponding with steatohepatitis) which further increased to an average score of 8
317 after 6 weeks. With a longer duration of the diet, liver tissue pO_2 tended to decrease but without
318 reaching significance. It is important to emphasise that the CDAHFD model, in which steatosis develops
319 due to impaired hepatic VLDL and triglyceride secretive capacity, histologically and functionally
320 resembles sever human MASH at the level of the liver but does not resemble the metabolic context of
321 obesity and peripheral insulin resistance in which MAFLD usually develops(35). The lack of significant
322 hypoxia, demonstrated by direct measurement, in this model with primary hepatocellular damage
323 potentially indicates that the metabolic alteration as seen in a more physiological model as the HFHFD
324 mouse model could be essential for the development of (direct) measurable liver hypoxia in MASLD.
325 Previous research has shown the presence of panlobular hypoxia in the liver of CDAHFD mice model
326 trough immunohistochemistry (18). However, the differences in liver hypoxia between the HFHFD and
327 CDAHFD model indicate that the metabolic changes seen during obesity are important in several
328 pathophysiological mechanism involved. Indeed, basal lipolysis, a key feature in AT dysfunction, is
329 stimulated by AT hypoxia(5), which was further indicated in the study by Pasarica *et al.* where lipolysis
330 was inversely correlated with oxygen tension in the white AT of patients(36). The associated release
331 of fatty acids into the bloodstream can lead to re-esterification and deposition of fatty acids in insulin-
332 sensitive organs such as the liver, leading to both insulin resistance and MASLD. Furthermore,
333 adipocyte HIF modulation can affect liver lipid accumulation. Studies have shown that a knockout of
334 adipocyte HIF-1α leads to improved insulin sensitivity, and that HIF-1α inhibition is able to prevent
335 MASLD and hepatocyte inflammation(8, 37, 38). The adipose tissue-liver axis is hence potentially of
336 great pathophysiological relevance in human MASLD(39, 40).

337 By using a PCR hypoxia-signalling pathway array, the expression of multiple genes could be
338 simultaneously screened while using real-time PCR for the accurate quantification of changes in mRNA
339 level. The development of obesity involved changes in many hypoxia-related genes, including changes
340 in angiogenesis and metabolism-related processes. However, liver and AT showed a clear distinct
341 expression pattern. Furthermore, progressive obesity resulted in changes in the expression pattern in

342 the different tissues. For instance, we found that *Slc2a1*, a classic hypoxia-sensitive gene, was
343 upregulated in gonadal AT after 20 weeks of diet, while this was not found for the liver or subcutaneous
344 AT. This again indicates the importance of longitudinal changes in the hypoxia response during obesity,
345 which is relevant to study disease progression and also implies the data generated on a single time
346 point in disease evolution should be interpreted with caution. Future studies should also study the
347 effect of weight loss on hypoxia in these tissues, as it is not yet known if the longitudinal changes found
348 in our experiment are reversible.

349 We utilized several different methodologies to determine hypoxia. Tissue hypoxia is a challenging
350 marker to determine, and is often limited to only immunohistochemistry. Indirect hypoxia markers can
351 be influenced by various processes and may not (solely) reflect hypoxia. Especially in the liver, hypoxia
352 is difficult to measure because of the unique vascularization of the liver that results in an oxygen
353 gradient across the liver lobule with lower oxygen tensions in the perivenous regions(18, 41). A direct
354 measurement of tissue hypoxia is the best standard to objectify hypoxia and to link observed changes
355 to hypoxia, but indirect hypoxia markers support the data obtained by direct measurements, which is
356 important as the direct measurement can be technically challenging. An important strength of this
357 study is hence the determination of *in vivo* hypoxia by means of a microsensor combined with histology
358 and gene expression patterns. A good correlation between the *in vivo* pO_2 measurements and the
359 expression of core hypoxia genes(20) was found in both liver and AT. Also, a significant correlation
360 between the pimonidazole stain and *in vivo* oxygen measurements in the gonadal AT was found, all in
361 line with other tests related to hypoxia. Consequently, we established a core hypoxia signature model
362 for both liver (*Ldha, Slc2a1, Eno1, Aldoa* and *Mif*) and AT (*Eno1, Mif, Slc2a1, Tpi1* and *Pgam1*) that best
363 corresponded with the direct hypoxia measurements.

364 In conclusion, the development of diet-induced obesity was associated with a gradual decrease in pO_2
365 in the liver and AT of mice. While AT hypoxia develops in the early stages of obesity, liver hypoxia
366 occurs later in progressive obesity development but before the occurrence of MASH. Liver hypoxia

367 could not be directly confirmed in a non-obese liver-only MASH mice model, indicating that obesity-
368 related processes such as AT hypoxia are important in the pathophysiology of obesity and its hepatic
369 manifestation MASLD.

370

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376

377 **Credit author statement**

378 **AVE**: conceptualization, methodology, formal analysis, investigation, writing original draft, project
379 administration, funding acquisition; **WK**: methodology, writing – review & editing; **CP**: investigation,
380 writing – review & editing; **SM**: investigation, writing – review & editing; **SVL**: formal analysis, writing
381 – review & editing; **KVDM**: writing – review & editing; **KVH**: writing – review & editing; **JDM**:
382 methodology, writing – review & editing; **BDW**: methodology, writing – review & editing, supervision;
383 **SF**: conceptualization, methodology, writing – review & editing, supervision; **SV**: conceptualization,
384 writing – review & editing, supervision, funding acquisition

385

386 **Competing Interests**

387 We have no conflict of interest to declare. Funding was received by an Unrestricted Research Grant of
388 the American Thoracic Society Foundation, but this organization had no conflict of interest with the
389 result, nor a role in the study design.

390

391 **Data Availability Statement**

392 The raw data supporting the conclusions of this article will be made available from the corresponding

393 authors, without undue reservation on reviewers' request.

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509 **Figure legends**

510 **Fig 1:** Effect of HFHFD on total body weight presented as median and IQR. (n per time point and
511 group can be found in table 1)

512 **Fig 2:** Histology of the liver. (A) NAS score represented as median with individual data points;
513 Representative 10x images of (B) H&E stained liver tissue and (C) picrosirius red stained liver tissue
514 after 20 weeks of diet (left control, right HFHFD).

515 **Fig 3:** In vivo pO_2 in (A) gonadal AT and (B) liver tissue of mice fed either a control diet or HFHFD; and
516 (C) liver pO_2 in mice fed either a control diet or CDAHFD. Graph represents median and individual
517 data points.

518 **Fig 4:** Covariate analysis for the different principal components in (A) gonadal adipose tissue, (B)
519 subcutaneous adipose tissue and (C) liver tissue, represented as a heat map.

520 **Fig 5:** Correlation analysis between in vivo pO_2 measurements and core hypoxia genes in (A) liver
521 tissue and (B) gonadal adipose tissue.

522 **Fig 6:** AIC curve for a hypoxia gene signature model and the corresponding correlation plots between
523 the best model and pO_2 measurements in (A) liver and (B) gonadal adipose tissue

524

525 **Table Legends**

526 **Table 1:** Effect of HFHFD on weight and biochemistry.

527 **Table 2:** Mixed effects analysis to determine group and time effect on pO_2 measurements in liver and

528 adipose tissue.

529 **Table 3:** Spearman correlation analysis between in vivo pO_2 and metabolic parameters.

530 **Table 4:** Core hypoxia genes included in the hypoxia signature model in liver and adipose tissue.

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