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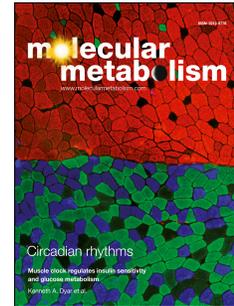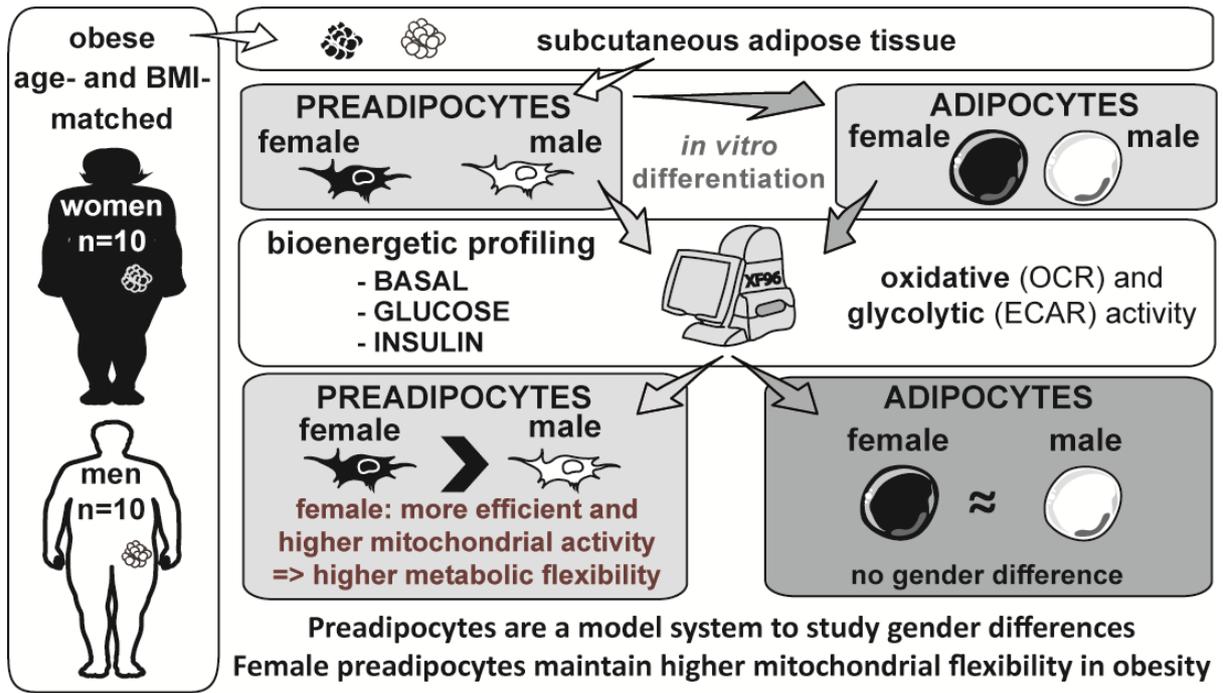
1 **Preadipocytes of obese humans display gender-specific bioenergetic responses to**

2 **glucose and insulin**

3

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7

8 **Running title:** Adipocytes' bioenergetics of obese men and women

9

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32

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35

36 **Abstract**

37

38 **Background/Objectives:** Although the prevalence of obesity and its associated

39 metabolic disorders is increasing in both sexes, the clinical phenotype differs between

40 men and women, highlighting the need for individual treatment options.

41 Mitochondrial dysfunction in various tissues, including white adipose tissue (WAT),

42 has been accepted as a key factor for obesity-associated comorbidities such as

43 diabetes. Given higher expression of mitochondria-related genes in the WAT of

44 women, we hypothesized that gender differences in the bioenergetic profile of white

45 (pre-) adipocytes from obese (age- and BMI-matched) donors must exist.

46 **Subjects/Methods:** Using Seahorse technology, we measured oxygen consumption

47 rates (OCR) and extracellular acidification rates (ECAR) of (pre-)adipocytes from

48 male (n=10) and female (n=10) deeply-phenotyped obese donors under hypo-, normo-

49 and hyperglycemic (0, 5 and 25 mM glucose) and insulin-stimulated conditions.

50 Additionally, expression levels (mRNA/protein) of mitochondria-related genes (e.g.

51 UQCRC2) and glycolytic enzymes (e.g. PKM2) were determined.

52 **Results:** Dissecting cellular OCR and ECAR into different functional modules

53 revealed that preadipocytes from female donors show significantly higher

54 mitochondrial to glycolytic activity (higher OCR/ECAR ratio, p=0.036), which is

55 supported by a higher ratio of UQCRC2 to PKM2 mRNA levels (p=0.021). However,

56 no major gender differences are detectable in *in vitro* differentiated adipocytes (e.g.

57 OCR/ECAR, p=0.248). Importantly, glucose and insulin suppress mitochondrial

58 activity (i.e. ATP-linked respiration) significantly only in preadipocytes of female

59 donors, reflecting their trends towards higher insulin sensitivity.

60 **Conclusions:** Collectively, we show that preadipocytes, but not *in vitro* differentiated

61 adipocytes, represent a model system to reveal gender differences with clinical

62 importance for metabolic disease status. In particular, females maintain enhanced

63 mitochondrial flexibility in preadipocytes, as demonstrated by pronounced responses

64 of ATP-linked respiration to glucose.

65

66   **1.   Introduction**

67

68   Obesity is characterized by reduced mitochondrial biogenesis and activity in several

69   tissues including the white adipose tissue (WAT) [1]. Decreased mitochondrial

70   function in white adipocytes leads to dysfunction of lipid storage and compromised

71   endocrine function of WAT [2,3]. These observations associate with obesity-induced

72   metabolic complications such as insulin resistance [4]. Several studies demonstrate

73   reduced mitochondrial content and activity of adipocytes from obese donors [5–9],

74   independent of fat cell size [8,9]. Furthermore, adipocytes from obese donors show

75   lower oxygen consumption after β-adrenergic stimulation as compared to lean

76   individuals [7]. Of note, gender-differences of lipid metabolism have been described

77   [10–13], showing that women exhibit higher lipolytic responses than men after

78   epinephrine infusion [14]. Based on these results, it has been suggested that

79   triglyceride synthesis rates in subcutaneous WAT (scWAT) of obese women are

80   higher than in obese men [15]. Notably, these gender differences are reflected at the

81   cellular level. Isolated adipocytes (subcutaneous and visceral) show gender-specific

82   differences in basal and norepinephrine-stimulated lipolysis [13,16]. Furthermore, the

83   lipolytic capacity of adipocytes appears to be differentially modulated by obesity [16]

84   and weight-reduction [11] in a gender-specific manner. Collectively, these gender-

85   specific differences in lipid metabolism of WAT may be caused by distinct cellular

86   metabolism. Molecular evidence supports the idea of gender differences in the

87   mitochondrial function of fat cells: Higher gene expression related to electron

88   transport chain (ETC) has been observed in the WAT of women, independently of fat

89   distribution and sex hormones [17]. However, a detailed characterization of the

90   cellular bioenergetics of preadipocytes and adipocytes, in particular distinguishing

4

91 obese men vs. women is still lacking. These studies would reveal whether gender

92 differences of cellular and mitochondrial bioenergetics exist.

93 The response of adipocytes bioenergetics to substrate supply (such as glucose) and

94 hormonal control (e.g. insulin) may also be gender specific as adipocytes from female

95 mice showed increased insulin sensitivity [10]. Furthermore, the maximally insulin-

96 stimulated glucose uptake is higher in adipocytes from obese women as compared to

97 obese men [18]. In human skeletal muscle, the modulation of bioenergetic parameters

98 by insulin was shown *in vitro* [19] and *in vivo* [20–22], and may relate to clinical

99 parameters such as HOMA-IR and insulin levels in a gender-specific manner [23].

100 Thus, we hypothesized the existence of gender-specific differences of the bioenergetic

101 machinery in adipocytes, and its differential modulation by insulin. Taking advantage

102 of new technologies simultaneously assessing in real-time time mitochondrial and

103 glycolytic activity in intact, undisturbed cell cultures, we determined the bioenergetic

104 profile of preadipocytes and adipocytes from female and male donors. To assess the

105 flexibility of cellular bioenergetics, we apply hypo-, normo- and hyperglycemic

106 conditions, which are modulators of human adipocytes bioenergetics [24]. The

107 responses to insulin are integrated in the experimental setup to determine acute

108 insulin-induced changes in human white adipocyte respiration and glycolytic activity.

109

110 **2.   Material and method**

111

112 **2.1. Subjects and cell culture**

113 Subcutaneous preadipocytes (stromal vascular fraction, SVF) isolated from 20

114 metabolically characterized obese patients (10 males, 10 females (6 pre- and 4 post-

115 menopausal), mean age: 41 (range: 26-62) years, mean BMI: 50 (range: 41-70)

116 kg/m²), who underwent bariatric surgery at the University Hospital Tübingen between

117 2006 and 2010, have been tested for absence of HIV, HBV, HCV, and mycoplasma.

118 Patients had not been on special diet prior to surgery. Details on donors'

119 characteristics (Table S1) and medication (Table S2) can be found in the Appendix A.

120 After expanding the SVF for two generations, cells were frozen in liquid nitrogen

121 until further expansion and experiments. Cells (no visible contamination with

122 epithelial or immune cells, third generation) were seeded and grown until confluency.

123 Then, cells were either subjected to analysis as preadipocytes (d0) or *in vitro*

124 adipogenic differentiation was induced as described [25]. 10 days after induction of

125 differentiation, cells accumulated visible lipid droplets and were analyzed as

126 adipocytes (d10). 24h before RNA/protein isolation and bioenergetic profiling, cells

127 were cultured in DMEM/F12 containing 0.5% FCS.

128

129 **2.2. Determination of adipocyte number and DNA quantification**

130 The number of adipocytes was estimated in the monolayers by direct counting using a

131 net micrometer (Leica, Wetzlar, Germany). Cells were counted as adipocytes, when

132 >5 lipid droplets were visible in the cell. DNA content as a surrogate for cell number

133 per XF96-well was determined using picogreen Quanti-iT assay (ThermoFisher).

134

6

135 **2.3. Gene expression analysis**

136 RNA was harvested and isolated from preadipocytes and adipocytes using the RNeasy

137 lipid tissue kit or miRNeasy Kit (Qiagen, Hilden, Germany). After reverse

138 transcription (SuperScript, Invitrogen or Transcriptor cDNA Synthesis Kit, Roche),

139 expression of genes together with the housekeeping gene RPS13 was analyzed with

140 Viia realtime PCR or Roche Lightcycler.

141

142 **2.4. Immunological detection of OXPHOS complexes and glycolytic enzymes**

143 Preadipocytes and adipocytes were lysed (30 min at 4°C), cleared by centrifugation

144 and protein concentrations were determined using BCA protein assay (Pierce). 15 or

145 30 µg protein lysate were separated on a 4-12% BisTris gel (Invitrogen) and blotted

146 onto a Nitrocellulose Membrane using iBlot (Invitrogen). Membrane was blocked for

147 1h in Odyssey Blocking Buffer (LiCor, Lincoln, NE USA) followed by incubation

148 with primary antibodies. Subsequently, IRDye® or AlexaFluor® secondary

149 antibodies (LiCor or Abcam, Cambridge, England) were used and signals were

150 detected using the Odyssey Sa or classic (LiCor). Following antibodies were use:

151 MitoProfile® Total OXPHOS Human WB Antibody Cocktail (#ab110411, abcam),

152 PFKP (D4B2, Cell Signaling), PKM2 (D78A4, Cell Signaling) and β-tubulin (Santa

153 Cruz, Heidelberg, Germany or Abcam).

154

155 **2.5. Energetic pathway studies**

156 Preadipocytes (d0) and adipocytes (d10) were washed with XF assay medium

157 containing 0 mM glucose (pH adjusted to 7.5) and incubated with indicated glucose

158 concentrations (0, 5 and 25 mM) for 1 h in a 37°C air incubator. The XF96 plate

159 (Seahorse Bioscience, Agilent Technologies) was then transferred to a temperature

160 controlled (37°C) Seahorse (extracellular flux) analyzer (Agilent Technologies) and

161 subjected to an equilibration period. One assay cycle comprised a 1-min mix, 2-min

162 wait, and 3-min measure period. Oxygen consumption rates (OCR) were analyzed as

163 follows: after 4 basal assay cycles, medium (0, 5 or 25 mM) or medium (5mM) with

164 insulin (1μM) was added by automatic pneumatic injection. After insulin stimulus,

165 OCR and ECAR were recorded for 6 assay cycles (approximately 40 min), before

166 oligomycin (1 µg/ml) injections were made to inhibit the ATP synthase for

167 determination of OCR related to ATP synthesis. After 3 further assay cycles, carbonyl

168 cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP; 0.5 µM) was injected to

169 stimulate maximal respiration by protonophoric action. After another 3 assay cycles,

170 rotenone (R, 4 µM) plus antimycin A (AA, 2 µM) was added followed by 4 assay

171 cycles to determine the non-mitochondrial OCR. The lowest OCR measurement after

172 addition of R/AA was subtracted from all other rates. To determine extracellular

173 acidification rates (ECARs) deriving from glycolysis, the last injection also contained

174 2-deoxy-glucose (2DG, 100 mM). Lowest ECAR after last injection was subtracted

175 from all ECAR values to obtain ECAR due to glycolysis. Coupling efficiency (CE)

176 was calculated as the oligomycin-sensitive fraction of mitochondrial respiratory

177 activity. Cellular respiratory control ratio (cRCR) is the ratio of maximal respiration

178 to proton leak respiration. ATP production from OXPHOS and glycolysis was

179 calculated as published previously [24]. OCR to ECAR ratio (OCR/ECAR) is the

180 ATP-linked OCR divided by glycolytic ECAR. After the measurement, cells were

181 lysed and total dsDNA amount per well was determined using Quant-iT PicoGreen

182 dsDNA Assay Kit (Thermo Fisher). All rates were normalized to 50 ng dsDNA

183 (~mean DNA content/well of all donors) which is approximately 7600 cells assuming

184 0.0065 ng dsDNA per mammalian cell [26].

185

2.6. Statistics

187 For statistical comparison, which served to support the group differences shown in the

188 figures, unpaired t-test (two-tailed) to compare male vs female group was performed

189 or Mann-Whitney test, if data failed to meet assumption of normal distribution (Fig.

190 1,2,4). To compare glucose effects and gender (Fig. 3), two-way ANOVA (post-hoc:

191 Bonferroni) were performed. To test effects of insulin, one-sample t-test to the value 1

192 was performed (Fig. 4). $p < 0.05$ was considered statistically significant. Statistical

193 tests were performed using Graph Pad Prism (GraphPad Software, Inc, La Jolla, CA,

194 USA) and Sigma Plot 12.0 (Systat Software, Inc., San Jose, California, USA).

195   **3.   Results**

196

197   **3.1. Electron Transport Chain (ETC) mRNA and protein expression reveal no**

198   **major gender differences in preadipocytes and adipocytes**

199   To study gender differences in the bioenergetics of fat cells from obese donors, we

200   analyzed gene expression of subcutaneous preadipocytes (d0) and adipocytes (d10) of

201   10 obese women and 10 obese men matched for BMI and age (Table S1). We focused

202   on genes encoding ETC components (NDUFB8, UQCRC2, SDHB) and UCP1, and

203   genes involved in glucose uptake and glycolysis (GLUT1, GLUT4, PKM2, and

204   PFKP). No significant differences were detected in ETC component and glycolysis-

205   related mRNA levels between preadipocytes of female vs male donors. In female

206   preadipocytes, UQCRC2 expression trended towards higher expression (p=0.076) and

207   PKM2 towards lower expression (p=0.088) as compared to male preadipocytes (Fig.

208   1A). The mRNA levels of ETC components and glycolysis related genes were not

209   significantly different between adipocytes of female donors as compared with male

210   donors (Fig. 1B). GLUT4 expression was below detection limit (Ct>35) in

211   preadipocytes (Fig. 1A), but robustly detectable in adipocytes (Fig. 1B), confirming

212   previous reports [27]. UCP1 mRNA levels were undetectable in preadipocytes and

213   adipocytes.

214   The protein abundance of ETC components (NUDFB8, SDHB, UQCRC2, MTCO2,

215   ATP5A) together with two rate-limiting enzymes of glycolysis (PFKP, PKM2) was

216   not significantly different between gender in preadipocytes (Fig. 1C). Adipocytes of

217   women displayed higher protein levels of SDHB and PFKP, and lower levels of

218   PKM2 (Fig. 1D). Together, the gene expression and protein data of cultured

10

219 adipocytes suggest no major differences of mitochondrial genes/proteins, contrasting

220 published results on WAT [17].

221

222 **3.2. Mitochondrial efficiency is higher in preadipocytes from obese women**

223 **under normoglycemic conditions**

224 To test if there are functional differences in cellular energy metabolism of

225 preadipocytes from obese women compared to men, we analyzed bioenergetic

226 function of preadipocytes under normoglycemic conditions. Total cell number per

227 well was identical, as estimated by DNA content of preadipocytes (Fig. S1A, B). The

228 traces for cellular oxygen consumptions rates (OCR) and acidification rates (ECAR)

229 of preadipocytes (Fig. S1C, D) were analyzed for differences between women and

230 men, as described in Methods and previous publications [24,28]. No gender

231 differences were found for non-mitochondrial respiration (Fig. S1E), maximal

232 substrate oxidation capacity (Fig. S1F), and ATP-linked respiration (Fig. 2A). ECAR

233 values, which report glycolytic activity, were similar between obese women and men

234 (Fig. S1I, J). Next, we calculated coupling efficiency (CE), cellular respiratory control

235 ratio (cRCR) and OCR/ECAR ratio, to internally normalize the traces, enabling us to

236 determine functional changes such as efficiency of energy turnover and switch of

237 metabolic routes with confidence. CE was significantly higher in female vs. male

238 preadipocytes (Fig. 2B). Higher mitochondrial efficiency in energy turnover was

239 further supported by significantly higher cRCR (p=0.012) (Fig. 2C). Estimating the

240 total ATP production rates from the Seahorse data with known stoichiometries [24]

241 revealed no differences between preadipocytes from men (50.4 pmol ATP/min ±7.9)

242 and women (56.7 pmol ATP/min ±9.3). However, we observed that the mitochondrial

243 contribution to ATP production was higher in preadipocytes of female donors, which

11

244 was demonstrated in a significantly higher OCR/ECAR ratio (Fig. 2D), and

245 complementary, the proportion of glycolytic ATP production in female was

246 significantly lower (Fig. 2E). Notably, the higher ratio of oxidative to glycolytic

247 activity in preadipocytes from women (Fig. 2D,E) was also reflected in a higher ratio

248 of UQCRC2 to PKM2 mRNA levels (Fig. 2F).

249

250 **3.3. Adipocytes of obese female and male donors display no differences in**

251 **cellular energy metabolism**

252 Adipogenic differentiation was induced in the preadipocytes of the identical donors

253 and the differentiated adipocytes subjected to bioenergetic analyses under

254 normoglycemic conditions. No differences in the rate of adipogenic differentiation

255 were detectable between adipocytes from men and women (DNA content and

256 adipocyte number per well (Fig. S2A-C)). As previously performed for preadipocytes,

257 the identical bioenergetic parameters were determined for adipocytes. However, we

258 found no significant gender differences of the bioenergetic parameters under basal,

259 normoglycemic conditions (Fig. S2D-K and Fig. 2G-K). Furthermore, differences in

260 the ratio of UQCRC2 to PKM2 mRNA levels disappeared in differentiated adipocytes

261 (Fig. 2L).

262

263 **3.4. Mitochondrial coupling efficiency (CE) and ATP-linked respiration**

264 **respond to glucose in preadipocytes of obese women**

265 Next, we studied the gender differences of preadipocyte energy metabolism in

266 response to hyperglycemic (25 mM glucose) and hypoglycemic (0 mM glucose)

267 conditions. We previously established the work-flow for in-depth bioenergetics in

268 SGBS adipocytes, which represent a subcutaneous human fat cell model [24]. In this

12

269   study, we apply this work-flow to compare the bioenergetic profile of preadipocytes

270   from men and women under hypo-, normo- and hyperglycemic conditions (c.f. Fig.

271   2). Preadipocytes from women show a wider scope to adapt mitochondrial energy

272   metabolism to glucose. This was evident in the respiration rates linked to ATP

273   synthesis, where ATP-linked respiration was significantly higher at hypoglycemic

274   conditions (p=0.034) (Fig. 3A). Preadipocytes from both genders increased glycolytic

275   rates with increasing glucose concentration (Fig. 3B), which was reflected in

276   significant changes of the OCR/ECAR ratio for preadipocytes from both genders (0

277   vs 25mM men: 3.4 vs 0.5; p=0.008; women 5.9 vs 0.6, p<0.001). In total, high

278   glucose levels provoked a higher contribution of glycolytic ATP to total ATP

279   production (Fig. 3C). Notably, preadipocytes from women significantly decreased CE

280   under hyperglycemic conditions as compared to hypoglycemic condition (Fig. 3D).

281   Cellular respiratory control ratio (cRCR) was not significantly changed with

282   increasing glucose concentrations, but preadipocytes from women showed higher

283   cRCRs under all three conditions as compared to men (0 mM: p=0.006; 5mM:

284   p=0.003; 25mM: p=0.045) (Fig. 3E).

285   Next, differentiated adipocytes were challenged with hypo-, normo- and

286   hyperglycemic conditions. ATP-linked respiration decreased with increasing glucose

287   concentration (Fig. 3F; 0mM vs 25mM, p= 0.039), which was paralleled by trends of

288   ECAR in the opposite direction (Fig. 3G; 0mM vs 25mM, p= 0.057). Both

289   parameters, however, show no gender differences. The relative contribution of

290   glycolysis to ATP production (% ATP from glycolysis, Fig. 3H) and CE (Fig. 3I)

291   were significantly different between hypo- and hyperglycemic condition in adipocytes

292   of women (p=0.004 and p=0.027), but not of men. In contrast, differences of cRCR in

293 response to hypo- and hyperglycemic conditions were found for both genders (Fig.

294 3J).

295 Taken together, when challenged with hypo- or hyperglycemic conditions,

296 preadipocytes only from women significantly decreased coupling efficiency and

297 reduced ATP-linked respiration, demonstrating gender differences in the adaption of

298 oxidative metabolism to glucose availability. These gender differences were

299 pronounced in preadipocytes and not detectable in ex vivo differentiated adipocytes.

300

301 **3.5. Distinct bioenergetics responses to insulin are specific for preadipocytes of**

302 **obese women**

303 Gender-dependent differences have been implicated in an important feature of

304 adipocytes, i.e. insulin-stimulated glucose uptake [10,18]. Thus, we integrated insulin

305 stimulation during the bioenergetic analysis to investigate gender differences.

306 Insulin significantly suppressed ATP-linked respiration in preadipocytes of females

307 (Fig. 4A). Insulin treatment increased ECAR ~1.7-fold in preadipocytes, suggesting

308 insulin-induced glycolysis as expected. Interestingly, insulin-dependent glycolytic

309 rates responded irrespective of gender (Fig. 4B). Insulin action in preadipocytes from

310 women was reflected in decreased CE (Fig. 4C) and cRCR (Fig. 4D). Notably, insulin

311 decreased the OCR/ECAR ratio in preadipocytes, thereby indicating the switch from

312 oxidative towards glycolytic metabolism. The OCR/ECAR was significant different

313 between gender, and thus demonstrates increased flexibility of overall metabolism in

314 female preadipocytes (~ -50% for women vs ~-30% for men) (Fig. 4E).

315 In differentiated adipocytes, we detected insulin-induced suppression of ATP-linked

316 respiration for both genders (Fig. 4F). In parallel, the ECAR increased almost 2-fold

317 after insulin treatment independent of gender (Fig. 4G). CE (Fig. 4H), cRCR (Fig. 4I)

318 and OCR/ECAR (Fig. 4J) significantly decreased after insulin treatment without

319 gender-specific effects.

320 Taken together, the responses of mitochondrial bioenergetics to glucose and insulin,

321 in particular ATP-linked respiration and CE, are more pronounced in preadipocytes

322 from obese women, and thus more flexible, allowing higher metabolic plasticity.

323 Importantly, these gender-dependent differences are no longer detectable in

324 differentiated adipocytes, suggesting that cell intrinsic differences between women

325 and men vanish during adipogenic differentiation, at least in our experimental *in vitro*

326 setting.

327

328

329   **4.   Discussion**

330

331   In the present study we report on gender differences in the cellular energy metabolism

332   of preadipocytes. In particular the differences in insulin-dependent glucose handling

333   may have profound implications for gender-specific treatment of metabolic diseases.

334   Pronounced gender differences of ATP-linked respiration were found in

335   preadipocytes from obese donors in response to glucose and insulin. The results from

336   female donors suggest that preadipocytes display greater plasticity of oxidative

337   metabolism that may be related to differences in anabolic or catabolic processes.

338   Gender differences have also been found in other progenitor cells, e.g. muscle-derived

339   stem cell (MDSC) and embryonic cells (neurons) from mice and rats, which display

340   sex differences in the susceptibility to stressor-induced cell death [29–31].

341   Preadipocytes appear to be a more robust test system to interrogate gender-related

342   hypotheses in relation to metabolism and metabolic diseases, contrasting (in vitro)

343   differentiated adipocytes which showed no, or only minor, bioenergetic gender

344   differences. We cannot exclude that the absence of gender differences may relate to

345   commonly applied experimental conditions, using a hormonal cocktail to induce

346   adipogenic differentiation that putatively overrides existing genetic and epigenetic

347   differences. Thus, the absence of differences in the capacity of *in vitro* differentiation

348   (Fig. S2A-C) could be due to experimental conditions. However, the differences seen

349   in preadipocytes could potentially impact differentiation *in vivo*, contributing to

350   gender differences in subcutaneous WAT cellularity/hyperplasia [32]. Vice versa, our

351   observations in preadipocytes *in vitro* may be primed (e.g. epigenetic changes) by

352   gender-specific differences of the *in vivo* WAT environment, including sex hormones

353   and nutrients (e.g. glucose, Table S1). For example, whether the menstrual cycle has

16

354 an impact in this study has not been assessed. Although we cannot formally exclude

355 these confounding factors, it should be noted that the preadipocytes were cultivated

356 for at least three generations in the medium with identical hormone and nutrient

357 concentrations.

358

359 Obesity significantly disturbs WAT cellular metabolism [5]. Importantly, this

360 bioenergetic fingerprint is preserved in *in vitro* differentiated adipocytes, contrasting

361 vanished gender differences. In previous studies, we comprehensively characterized

362 the bioenergetics of human SGBS adipocytes, which represents a "lean", insulin-

363 sensitive preadipocyte cell strain [24,33]. Compared to SGBS cells, the ATP-linked

364 respiration of *in vitro* differentiated adipocytes from obese donors was about 60%

365 lower, suggesting impact of the obesity state on ATP turnover. The depression of

366 ATP metabolism in obesity is further supported by data of Yeo and colleagues, who

367 directly compared SGBS to *in vitro* differentiated subcutaneous adipocytes from

368 obese donors, the latter showing lower mitochondrial activity and reduced lipid

369 accumulation and insulin-stimulated glucose uptake [34]. Furthermore, isolated

370 mitochondria from human primary ("floating") adipocytes revealed strong BMI-

371 dependent decreases of mitochondrial activity (measured as ATP-linked respiration)

372 [8,9]. In line with our observations, a study on basal heat production of primary

373 ("floating") adipocytes from lean vs obese women and men revealed no gender

374 difference but decreased heat output by obesity of ~50% [35]. Concerning

375 mitochondrial differences between pre- and mature adipocytes, we calculated for

376 SGBS adipocytes vs preadipocytes from our previously published data ~4.4- fold

377 higher oxygen consumption [24], which is in a similar range as data from von

378 Heimburg and colleagues (who detected ~4.8-fold higher respiration in adipocytes vs

379 preadipocytes from lean donors) [36]. In the present study focusing on obese donors,

380 cellular respiration in preadipocytes vs adipocytes increased only ~3-fold (18 pmol

381 O_2/min/50 ng dsDNA vs 53 pmol O_2/min/50 ng dsDNA), further supporting the idea

382 that obesity disturbs cellular metabolism. Our data suggest that obesity-induced (epi-

383)genetic, molecular and metabolic perturbations remain in *ex vivo* differentiated

384 adipocytes, despite the lack of potential *in vivo* gender differences. This also confirms

385 previous studies of *in vitro* differentiated adipocytes, showing molecular differences

386 for metabolic healthy vs unhealthy obese donors [37], together justifying the value of

387 *in vitro* studies in adipocytes for metabolic disease.

388

389 To the best of our knowledge, we report for the first time on significant gender

390 differences of oxidative to glycolytic activity ratios (OCR/ECAR) in preadipocytes

391 from obese donors. This functional difference was also reflected on the gene

392 expression level showing higher levels of UQCRC2 to PKM2 in women (p=0.021,

393 Fig. 2F). However, other molecular bioenergetic markers do not reveal gender

394 difference, contrasting analyses on whole WAT [17], indicating that there is either no

395 robust link of mRNA/protein levels with function, in particular during acute exposure

396 to hormones and nutrients, or that the gender-specific microenvironment created by

397 hormones (e.g. adiponectin [38]) or inflammation (e.g. TNFa [39]) is strongly

398 affecting the expression of bioenergetic markers.

399 The functional differences in preadipocyte energy metabolism may at least partially

400 provide the basis for well-described systemic gender differences in substrate

401 metabolism [40–42]. Females display higher net lipid oxidation than males in resting

402 conditions [42]. In particular, when energy demand increases (e.g. during physical

403 activity), women show a higher contribution of fat oxidation to total energy

404 expenditure [40,41]. For other tissues such as muscle, higher oxidative to glycolytic

405 activity has been suggested for women [43,44]. In obese and diabetic individuals,

406 increased glycolytic to oxidative muscle metabolism has been reported [45,46],

407 indicating the link between insulin sensitivity and the balance of oxidative to

408 glycolytic pathways. The over-proportioned reliance on glycolytic pathways, possibly

409 due to compromised oxidative pathways, may be a hallmark of insulin resistance [45].

410 Our functional studies on adipose cells are comprehensive but would not allow for

411 gender-stratified correlation analyses testing the link between function and clinical

412 parameters at this stage, as the number of 20 donors is too low. Nevertheless, our data

413 indicate that improvement of mitochondrial function and the higher oxidative to

414 glycolytic ratio of preadipocytes from obese women could be beneficial. Improved

415 glucose homeostasis and insulin sensitivity is more frequently observed in obese

416 women as compared to age- and BMI-matched men [47–52]. Our cohort showed

417 trends towards higher insulin sensitivity in women (Appendix A, Table S1).

418

419 The acute insulin stimulus significantly lowered cRCR and coupling efficiency (Fig.

420 4) in human adipocytes from women, contrasting insulin-effects in human myotubes

421 where insulin acutely increases cRCR and coupling efficiency by decreasing proton

422 leak respiration [19]. In adipocytes, neither basal nor FCCP-stimulated respiration

423 rates were robustly altered by insulin (Fig S3 A-D); thus, the changes in cRCR and

424 CE are mainly due to changes in proton leak respiration (Fig. S3E,F). Increased

425 proton leak respiration is either caused by changes in the conductance of protons or

426 other ions at the mitochondrial inner membrane. However, similar to human

427 myotubes [19] we found a robust increase of ECAR reporting glycolytic activity in

428 response to insulin, that should also enhance glucose uptake. This is in line with

429 reports on increased glycolytic activities in murine adipocytes [53,54]. Therefore,

430 extracellular flux analyses are instrumental to monitor insulin sensitivity, and offers

431 simultaneous real-time measurements of both, glycolysis/glucose uptake and

432 mitochondrial oxidation.

433

5.   Conclusions

435 In summary, preadipocytes retain gender differences *in vitro*, and cells from obese

436 women possess a higher metabolic flexibility involving oxidative metabolism.

437 Metabolic flexibility may assist to sustain metabolic health better as age- and BMI-

438 matched men. Therapies targeting obesity, adipose tissue and dysfunctional

439 mitochondrial properties must consider gender differences.

440

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Acknowledgments

453

454 **Conflict of interest**

455 The authors have nothing to disclose.

456

457

458 **APPENDIX A. SUPPLEMENTARY DATA**

459 Supplementary data related to this article can be found online

460

461

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Figure legends

Figure 1: **The expression of genes/proteins involved in oxidative and glucose metabolism of male and female preadipocytes and adipocytes**

(A, B) Total mRNA of preadipocytes (d0) and adipocytes (d10) were prepared and analyzed by qPCR. The relative mRNA expression of genes involved in oxidative metabolism (NDUFB8, SDHB, and UQCRC2) and glucose metabolism (GLUT1, GLUT4, PFKP, and PKM2) was normalized by ΔCt to housekeeper RPS13. Data are fold change to men and are the mean + SEM of 10 male and 10 female donors for which bioenergetic pathway analyses were performed. **(C, D)** Protein lysates of preadipocytes (d0) and adipocytes (d10) were prepared and analyzed by western blot. Representative western blots for preadipocytes and adipocytes of 5 female and 5 male donors using total OXPHOS human antibody cocktail, PFKP, PKM2 and β-tubulin antibody. Quantification of signals for OPXHOS antibodies (NDUFB8, SDHB, UQCRC2, MTCO2 and ATP5A) and glycolytic enzymes (PFKP and PKM2) presented as ratio to β-tubulin and as fold change to the mean of male signal/β-tubulin per membrane. Data are the mean + SEM of 10 male and 10 female donors for which bioenergetic pathway analyses were performed. * < 0.05, ** $p < 0.01$

Figure 2: **The cellular metabolism of preadipocytes and adipocytes from obese women and men under normoglycemic conditions**

(A-E,G-K) Oxygen consumption (OCR) and extracellular acidification (ECAR) after interference of energetic pathways with specific inhibitors were analyzed under normoglycemic (5 mM) conditions using a XF96 extracellular flux analyzer as described previously [24] and in Materials and Methods. All data were normalized to

693   50 ng dsDNA per well. OCR and ECAR traces vs time are shown in SI (Fig. S1 and

694   S2). Mitochondrial respiration (Fig. S1G, S2F) was dissected into **(A,G)** ATP-linked

695   respiration and proton leak respiration (Fig. S1H, S2H). Coupling efficiency (CE,

696   **(B,H)**) and cell respiratory control ratio (cRCR, **(C,I)**) were calculated as described in

697   Methods. The OCR/ECAR ratio **(D,J)** and the percentage of ATP produced by

698   glycolysis **(E,K)** of male and female preadipocytes and adipocytes**. (F,L)** The mRNA

699   levels of UQCRC2 and PKM2 mRNA levels were analyzed by qPCR, normalized by

700   ΔCt to RSP13 (c.f. Fig 1) and are presented as ratio of UQCRC2 to PKM2 mRNA

701   levels for preadipocytes and adipoctyes. All data are the mean of 10 men and 10

702   women + SEM. * < 0.05, ** $p<0.01$

703

704   **Figure 3**: **The cellular metabolism of preadipocytes and adipocytes from obese**

705   **women and men under hypo- and hyperglycemic conditions**

706   **(A-J)** Oxygen consumption (OCR) and extracellular acidification (ECAR) after

707   interference of energetic pathways with specific inhibitors were analyzed under hypo-

708   (0 mM) and hyperglycemic (25 mM) conditions together with normoglycemic

709   conditions (c.f. Fig. 2) using a XF96 extracellular flux analyzer as described

710   previously [24] and in Materials and Methods. OCR and ECAR traces vs time are

711   shown in SI (Fig. S1 and S2). The effect of glucose on ATP-linked respiration **(A, F)**,

712   glycolysis **(B, G)**, the percentage of ATP produced by glycolysis **(C, H)**, coupling

713   efficiency **(D, I)** and cRCR **(E, J)** are shown for preadipocytes and adipocytes**.** All

714   data were normalized to 50 ng dsDNA per well and are the mean of 10 men and 10

715   women + SEM. Glucose: * < 0.05, ** $p<0.01$, *** $p<0.001$. Gender: [a] $p<0.05$ vs

716   women

717

718 **Figure 4**: **Acute effects of insulin on the cellular metabolism of preadipocytes and**

719 **adipocytes from obese men and women**

720 OCR and ECAR were recorded and detailed dissection/analysis was performed as

721 described in Method section. All data are presented as fold change to medium control

722 (normoglycemic condition, c.f. Fig 2) of each donor. Insulin-induced changes in ATP-

723 linked respiration **(A, F),** glycolysis **(B, G),** coupling efficiency (CE, **(C, H)**), cell

724 respiratory control ratio (cRCR, **(D, I**)) and OCR/ECAR **(E, J)** are presented for

725 preadipocytes and adipocytes. All data were normalized to 50 ng dsDNA per well and

726 are the mean of 10 men and 10 women + SEM. Gender: * $p < 0.05$; insulin: # $p < 0.05$,

727 ## $p < 0.01$, ### $p < 0.001$ vs basal.

Figure 1

Figure 2

■ female □ male
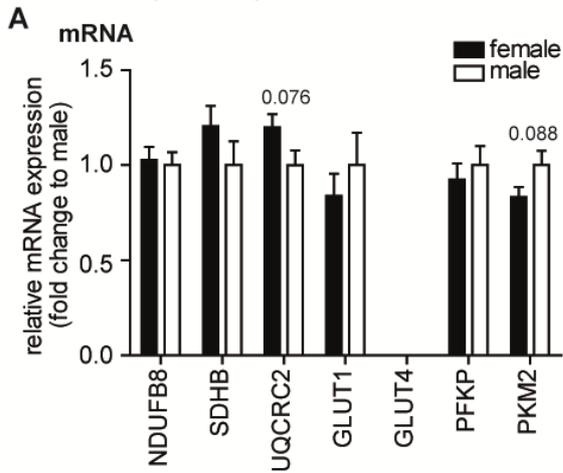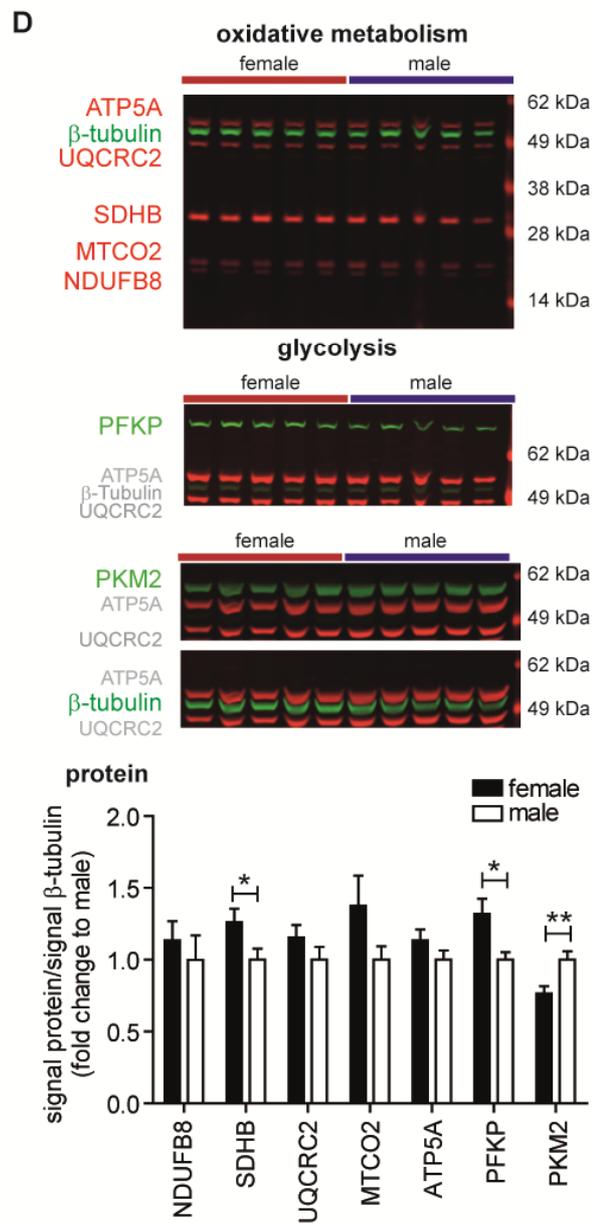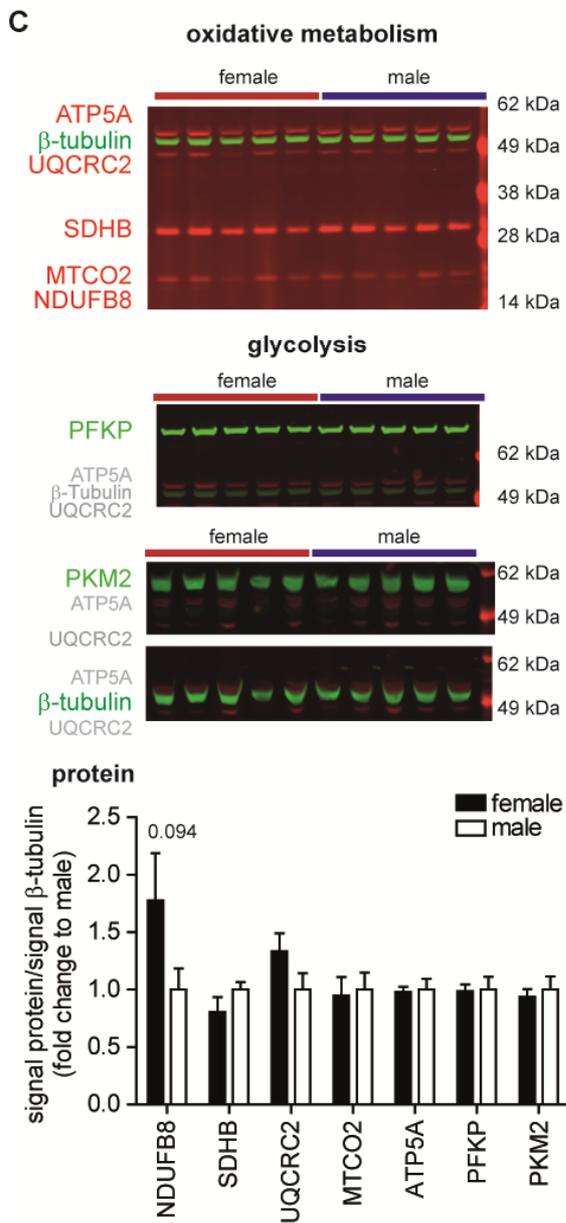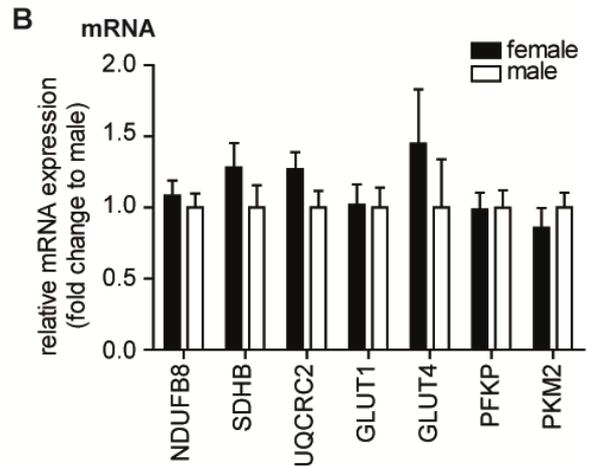
PREADIPOCYTES **ADIPOCYTES**
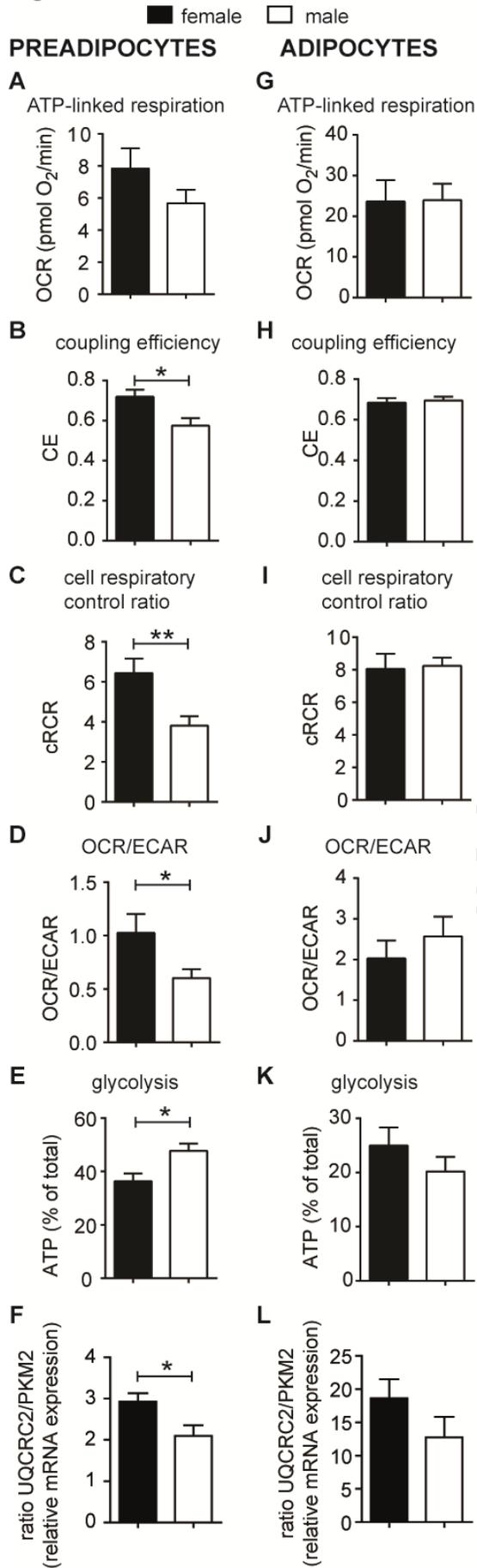
Figure 3
Effects of Glucose

☐ 0 mM   ☐ 5 mM   ■ 25 mM

PREADIPOCYTES

A ATP-linked respiration
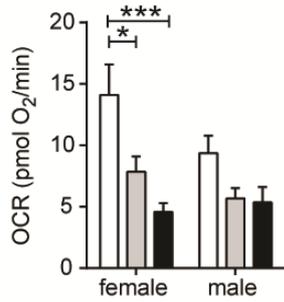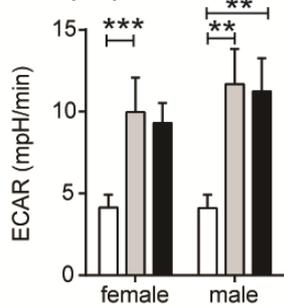
B Glycolysis

C % ATP glycolysis
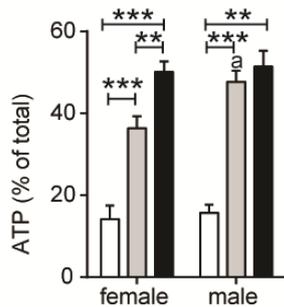
D coupling efficiency
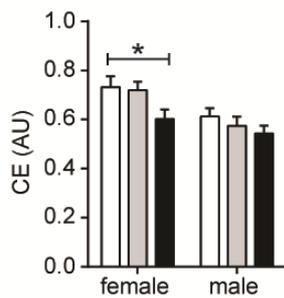
E cell respiratory control ratio
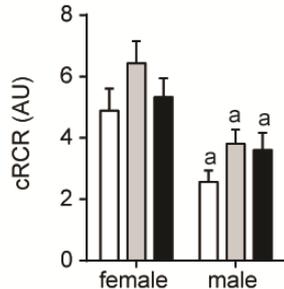
ADIPOCYTES

F ATP-linked respiration
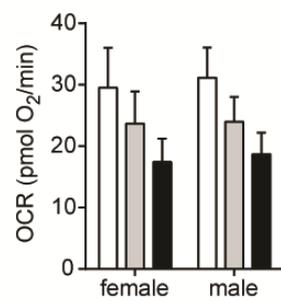
G Glycolysis
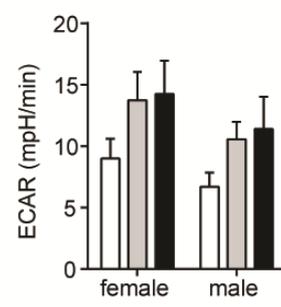
H % ATP glycolysis
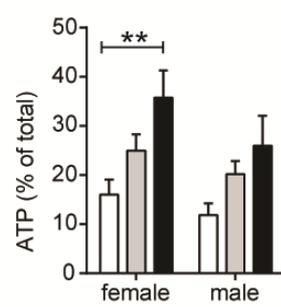
I coupling efficiency
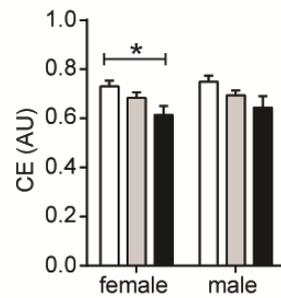
J cell respiratory control ratio
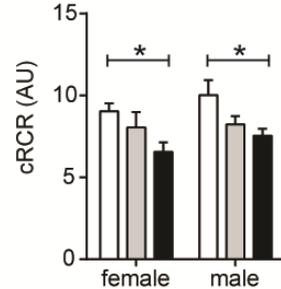
Figure 4
Effects of Insulin

■ female □ male

PREADIPOCYTES   **ADIPOCYTES**

A ATP-linked respiration

F ATP-linked respiration
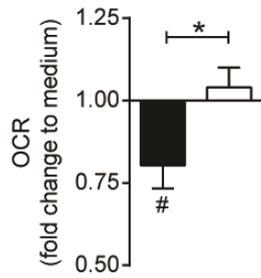
B Glycolysis
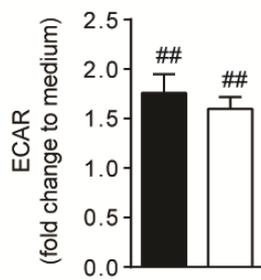
G Glycolysis

C coupling efficiency

H coupling efficiency
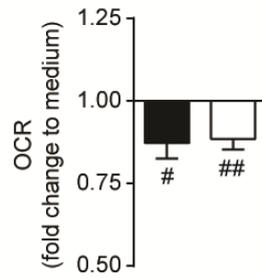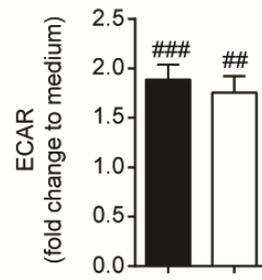
D cell respiratory control ratio
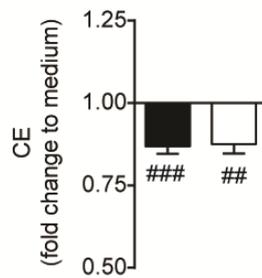
I cell respiratory control ratio
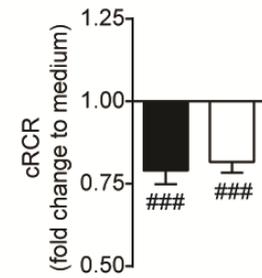
E OCR/ECAR

J OCR/ECAR
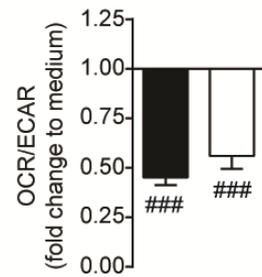
Highlights

- Preadipocytes may represent a model system to study gender differences

- Female vs male preadipocytes show higher mitochondrial to glycolytic activity

- ATP-linked respiration of female preadipocytes is suppressed by glucose and insulin

- Female vs. male preadipocytes have higher metabolic flexibility via mitochondria

- Gender differences are not detectable in *in vitro* differentiated adipocytes