Effect of sex on glucose handling by adipocytes isolated from rat subcutaneous, mesenteric and perigonadal adipose tissue

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Background. Adult rat epididymal adipocytes are able to convert large amounts of glucose to lactate and glycerol. However, fatty acid efflux is much lower than that expected from glycerol levels if they were the product of lipolysis. Use of glucose for lipogenesis is limited, in contrast with the active glycolysis-derived lactate (and other 3-carbon substrates). In this study, we analyzed whether white adipose tissue (WAT) site and sex affect these processes.

Methods. Mature adipocytes from perigonadal, mesenteric and subcutaneous WAT of female and male rats were isolated, and incubated with 7 or 14 mM glucose during 1 or 2 days. Glucose consumption, metabolite efflux and gene expression of glycolytic and lipogenesis-related genes were measured.

Results. The effects of medium initial glucose concentration were minimal on most parameters studied. Sex-induced differences that were more extensive; however, the most marked, distinct, effects between WAT sites, were dependent on the time of incubation. In general, the production of lactate was maintained during the incubation, but glycerol release rates increased with time, shifting from a largely glycolytic origin to its triacylglycerol (TAG) lipolytic release. Glycerol incorporation was concurrent with increased TAG turnover: lipolytic glycerol was selectively secreted, while most fatty acids were recycled again into TAG. Fatty acid efflux increased with incubation, but was, nevertheless, minimal compared with that of glycerol. Production of lactate and glycerol from glucose were maximal in mesenteric WAT.

Discussion. Female rats showed a higher adipocyte metabolic activity than males. In mesenteric WAT, gene expression (and substrate efflux) data suggested that adipocyte oxidation of pyruvate to acetyl-CoA was higher in females than in males, with enhanced return of oxaloacetate to the cytoplasm for its final conversion to lactate. WAT site differences showed marked tissue specialization-related differences. Use of glucose for lipogenesis was seriously hampered over time, when TAG turnover-related lipolysis was activated. We postulate that these mechanisms may help decrease glycaemia and fat storage, producing, instead, a higher availability of less-regulated 3-carbon substrates, used for energy elsewhere.

5 **Effect of sex on glucose handling by adipocytes isolated from rat**

6 **subcutaneous, mesenteric and perigonadal adipose tissue**

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24 **Abstract**

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39 incorporation was concurrent with increased TAG turnover: lipolytic glycerol was selectively

40 secreted, while most fatty acids were recycled again into TAG. Fatty acid efflux increased with

41 incubation, but was, nevertheless, minimal compared with that of glycerol. Production of lactate

42 and glycerol from glucose were maximal in mesenteric WAT.

43 **Discussion**. Female rats showed a higher adipocyte metabolic activity than males. In mesenteric

44 WAT, gene expression (and substrate efflux) data suggested that adipocyte oxidation of pyruvate

45 to acetyl-CoA was higher in females than in males, with enhanced return of oxaloacetate to the

46 cytoplasm for its final conversion to lactate. WAT site differences showed marked tissue

47 specialization-related differences. Use of glucose for lipogenesis was seriously hampered over

48 time, when TAG turnover-related lipolysis was activated. We postulate that these mechanisms

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50 regulated 3-carbon substrates, used for energy elsewhere.

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52

53 **Introduction**

54   Adipose tissue is a large disperse organ, closely related to most organs, and distributed in
55   anatomically differentiated masses, traditionally considered storage depots of fat reserves. There
56   are marked differences between these sites (Jamdar 1978; Prunet-Marcassus et al. 2006), up to
57   the point that their main common characteristic is the massive accumulation of fat (essentially
58   triacylglycerols, TAG), stored in vacuoles, taking most of the cell space (Rotondo et al. 2016).
59   The marked oxidative metabolism of brown adipose tissue, with multiple (and smaller) fat
60   vacuoles and a large number of mitochondria, contrasts with the predominantly glycolytic
61   activity of the huge single-vacuole adipocytes of white adipose tissue (WAT) (Ho-Palma et al.
62   2016). Between these extremes, we find a number of intermediate adipocyte phenotypes, such as
63   beige (Wu et al. 2012) or pre-adipocytes (Torio-Padron et al. 2010). The array of cell types,
64   which retain a number of similar characteristics, result in an ample variability of functions of
65   BAT, WAT; but also beige and *brite* or brown-in-white adipose tissue (Giralt & Villarroya
66   2013). Nevertheless, for most of the earlier research, the prevailing idea of WAT function was
67   just that of a fat (energy) storage organ. The dangers derived from WAT (and/or individual
68   adipocytes) hypertrophic growth are closely related to the development of metabolic syndrome
69   (Kaplan 1989), a chronic state of inflammation (Cancello & Clément 2006). In the last twenty
70   years, many other functions of WAT have been discovered, including the synthesis of hormones
71   and growth factors, which interaction with other organs and cell types potentially affecting
72   overall health. WAT regulatory effects are often exerted by small masses of adipose tissue, such
73   as perivascular (Guzik et al. 2007), pericardial (Greif et al. 2013) or intramuscular (Smith et al.
74   1998).

75   The active presence of a large number of other cell types interspersed between
76   adipocytes, such as stem (Zuk et al. 2002), immune system (Qiu et al. 2016), stromal vascular
77   (Ribeiro Silva et al. 2017) and even hematopoietic (Luche et al. 2015) is seldom taken into
78   account from the point of view of substrate handling. Nevertheless, their role is critical for
79   regulation and for other WAT functions. The diversity of cell proportions, functions and site
80   distribution contribute to extend, diversify and specialize the roles of WAT in a number of roles,
81   from the control of energy partition to defense and the control of the function of other organs
82   (Scherer 2006; Alemany 2011). This is largely achieved through hormonal (Kershaw & Flier
83   2004) and cytokine (Wisse 2004) signaling.

84   In mammals, body fat distribution is sex-related, and is more marked in humans (Kuk et
85   al. 2005) than in rodents. Essentially, the differences in WAT distribution are a consequence of
86   differences in overall metabolic function (Ailhaud et al. 1991; Fried et al. 2015); i.e. we have
87   observed, recently, a marked effect of sex on rat WAT amino acid metabolism (Arriarán et al.
88   2015a; Arriarán et al. 2015b). We also found that WAT, and isolated adipocytes, are able to
89   convert large amounts of glucose into lactate and glycerol (Arriarán et al. 2015c; Ho-Palma et al.
90   2016), despite its small percentage of active cytoplasm with respect to tissue weight (Rotondo et
91   al. 2016). This anaerobic conversion of glucose to 3-carbon substrates (3C), was not related to
92   hypoxia, since it took place in the presence of abundant oxygen. In addition, epididymal
93   adipocytes from adult rats converted most of ^{14}C-labelled glucose to labelled 3C Ho-Palma et al.
94   2018 in press).

95   The analysis of how a disperse organ (Vitali et al. 2012) in which the different sites play
96   different functions, could maintain its distinctive organization and uniform control was one of
97   the critical objectives of the present study. The objective was to find out whether the site
98   differences were a question of adipocyte metabolic specialization within a frame of metabolic
99   capabilities and parameters that define WAT as a specific (albeit disperse) organ. Consequently,
100   we selected three quite different WAT sites: perigonadal (PG), mesenteric (MES) and
101   subcutaneous (SC) WAT. PG, i.e. epididymal or periovaric, contains large cells, and a high
102   percentage of fat per g of tissue (Romero et al. 2014); it is considered essentially an example of
103   storage WAT (Ruan et al. 2003). In rats, MES WAT is less consistent, lax, and structurally
104   complex in its connections, in part bridging the gap between intestine (and dietary nutrients) and
105   the liver (Novelle et al. 2017). In humans, MES accounts for most of "visceral" WAT. SC WAT
106   is even more diverse, with extreme differences between locations. Subcutaneous WAT is one of
107   the most studied adipose tissues in humans (Gensanne et al. 2009) because of its accessibility.
108   We used the inguinal fat pads, which provide a clearly distinguishable (and uniform) site with
109   sufficient material for analysis.

110   In brief, our main objective in carrying this study was to test whether widely different
111   (location, structure, fat content, blood flow, relationship with other organs, and median cell size)
112   WAT sites shared a basic web of metabolic pathways, and, alternatively, showed distinct ways to
113   handle energy substrates. The study was focused on the known relationships of glucose with
114   lipogenesis, glycerogenesis, and especially, glycolysis to lactate (Sabater et al. 2014; Rotondo et

115   al. 2017). We included sex as another potentially important variable, since most of our previous

116   work had been done using males, and the sexual differentiation of adipose tissue metabolism

117   plays a critical role in obesity and other metabolic syndrome pathologies (Medrikova et al.

118   2012).

119

120   **Materials and Methods**

121   *Animals and sampling*

122   The experimental design and the rat handling procedures were applied following the

123   animal treatment guidelines established by the corresponding European, Spanish and Catalan

124   Authorities. The Committee on Animal Experimentation of the University of Barcelona

125   specifically authorized the procedures used in this study [Approval number: 9443].

126   Wistar rats (Janvier, Le Genest-Saint Isle, France), 14-week old (8 male and 8 female),

127   were used after at least 7 days of adaptation to the new environment. The animals were kept in

128   two-rat cages under standard conditions: i.e. 21.5-22.5ºC, and 50-60% relative humidity; lights

129   were on from 08:00 to 20:00. The rats had free access to water and standard rat chow (#2014,

130   Teklad Diets, Madison WI USA) available in excess at any time.

131   When the females were in the proestrus phase, the rats were weighed, and killed at the

132   beginning of a light cycle. After complete anesthesia using isoflurane, the animals were

133   exsanguinated with syringes by puncturing the aorta. They were rapidly dissected, excising

134   samples of mesenteric (MES) WAT, cleaned of attachments and pancreatic tissue, epididymal

135   /periovaric (i.e. PG) WAT, and both inguinal pads of SC WAT.

136   *Experimental groups*

137   Tissue samples of a pair of same-sex rats with similar weight were coarsely minced and

138   pooled prior to the separation of adipocytes. In all, four 2-rat samples were used for adipocyte

139   extraction of each WAT site and sex. The cells were distributed in groups, incubated

140   independently in at least four wells each (one for each pool of 2 rats of the same sex): The

141   variables to study were: a) Sex: female and male. b) Site: SC, MES and PG. c) Initial glucose

142   concentration: 7 mM and 14 mM. And. d) time of incubation: 24 h or 48 h. In total, there were

143   48 groups of four different rat pools. In all cases, at the end of the incubation, the cells were

144   harvested and used for gene expression analysis, while the media were used for analysis of

145   glucose and metabolites as described below.

Preparation and incubation of adipocytes

147   Adipocytes were isolated by incubation with collagenase (type I #LS004196, Worthington

148   Biomedicals, Lakewood, NJ USA), as described in a previous paper (Rotondo et al. 2016),

149   essentially following the Rodbell procedure (Rodbell 1964). Adipocyte numbers were counted

150   from the final washed suspensions. Their (spherical when free) diameters were measured using

151   serial microphotographs (Figure 1) and the ImageJ software (http://imagej.nih.gov/ij/) (Baviskar

152   2011). The recovery of adipocytes with respect to the mass of WAT used was estimated in a

153   number of randomly selected samples, as previously described (Rotondo et al. 2016).

154   Incubations were carried out using 12-well plates (#734-2324VWR International BVBA/Sprl.,

155   Leuven Belgium). The incubation medium consisted of 1.7 ml of DMEM (#11966-DMEM-no

156   glucose; Gibco, Thermo-Fisher Scientific, Waltham MA USA), supplemented with, 30 mL/L

157   fetal bovine serum (FBS, Gibco). The medium contained added glucose at a final nominal

158   concentration of 7 mM or 14 mM. The medium also contained 25 mM hepes (Sigma-Aldrich, St

159   Louis MO USA), 2mM glutamine (Lonza Biowhittaker, Radnor, PA USA), 1 mM pyruvate

160   (Gibco), 30 mg/mL delipidated bovine serum albumin (Millipore Calbiochem, MA USA), 100

161   nM adenosine, 100 mU/mL penicillin and 100 mg/L streptomycin (all from Sigma-Aldrich).

162   Each well contained 400 µL of the cell suspension. Since 0.1 mL of medium was used for

163   initial measurements (metabolite zero values, cell counting), the final incubation volume was 2.0

164   mL. The plates were kept at 37°C in an incubation chamber, ventilated with air supplemented

165   with 5% CO_2. The cells were incubated for 24 h or 48 h, without any further intervention, as

166   previously described (Ho-Palma et al. 2016; Rotondo et al. 2016).

167   The incubation of adipocytes was stopped by pipetting out the whole contents of the well,

168   allowing the adipocytes to float and form a defined layer, which was taken out. The infranatant

169   medium was centrifuged (to recover any remaining adipocytes), mixed, aliquoted and frozen.

170   The media were used for the estimation of glucose, lactate, glycerol and NEFA. Glucose

171   was measured using a glucose oxidase-peroxidase kit (#11504, Biosystems, Barcelona Spain)

172   containing 750 nkat/mL mutarrotase (porcine kidney, 136A5000, Calzyme, St Louis, MO USA)

173   (Oliva et al. 2015). Lactate was measured with kit 1001330 (Spinreact, Sant Esteve d'en Bas,

174 Spain); glycerol was estimated with kit #F6428 (Sigma-Aldrich). NEFA were measured using kit

175 NEFA-HR (Wako Life Sciences, Mountain View, CA USA).

176 *Gene expression analyses*

177   Total cell RNA was extracted from the packed washed adipocytes using the Tripure

178 reagent (Roche Applied Science, Indianapolis IN USA), and were quantified using a ND-1000

179 spectrophotometer (Nanodrop Technologies, Wilmington DE USA). RNA samples were reverse

180 transcribed using the MMLV reverse transcriptase (Promega, Madison, WI USA) system and

181 oligo-dT primers. Real-time PCR (RT-PCR) amplification was carried out using 10 μL

182 amplification mixtures containing the Power SYBR Green PCR Master Mix (Applied

183 Biosystems, Foster City, CA USA), 4 ng of reverse-transcribed RNA and 150 nM primers.

184 Reactions and measurement of evolved fluorescence were analyzed in an ABI PRISM 7900 HT

185 detection system (Applied Biosystems),

186   A semi-quantitative approach for the estimation of the concentration of specific gene

187 mRNAs per unit of tissue weight was used (Romero et al. 2007). *Arbp* was the charge control

188 gene (Bamias et al. 2013). The results were expressed as the number of transcript copies per cell

189 in order to obtain comparable data between the groups. The genes analyzed and a list of primers

190 used are presented in Table 1.

191 *Statistics*

192   The experimental design combined two different animal origins of WAT (female, male),

193 from three different anatomical locations (site: subcutaneous, mesenteric and perigonadal). In

194 addition, the length of incubation (24h or 48 h) and the initial level of glucose (7 mM or 14 mM).

195 Since the effects of the parameters studied could influence the results in widely different ways,

196 we tried to apply a escalating statistical comparison set up that will allow us to go from a general

197 view to the specifics of which factors were more (or less) responsible for the differences. We

198 considered that the intermixing of data could only be discerned using ANOVA analyses, since

199 our experimental design had low N values, because of the need for integral comparison of data

200 and cooperative analysis in the individual wells,. Thus, we scaled the statistical analyses between

201 groups using four-, three- or two-way ANOVAs on the same sets of data. Analyses were carried

202 out with the Stratgraphics program (Statpoint Technologies, Warrington, VA USA).

203

204   **Results**

205   *Cells, glucose uptake and metabolite efflux*

206   Mean adipocyte volumes for the suspensions of cells used are shown in Figure 1. There

207   were significant differences for site (but not for sex) in mean cell size; the largest cells were

208   those of PG WAT. We expressed the metabolite efflux (or uptake in the case of glucose) as rates

209   akin to the unit of enzyme activity, katal, and taking the cell as unit for comparison.

210   Consequently, the data (shown in Figure 2) were expressed as attomoles per second and cell.

211   Data were analyzed using ANOVAs, four way, as indicated in the legend of the Figure, three

212   way, independently for each site, and two-way for sex in each site (both series shown in the

213   Figure itself).

214   Four-way ANOVA showed the general trend: there were differences between sites

215   affecting all parameters; sex also showed significant differences for everything except lactate

216   efflux. On the other side, doubling glucose concentration only affected glucose uptake, whereas

217   the time of incubation affected all parameters except glucose uptake. The latter parameter was

218   closely linked to the incubation time, with limited effects of sex (except in PG WAT).

219   Lactate efflux was remarkably uniform and not related to glucose concentration or the

220   time of incubation (except in PG WAT), but there was an overall significant effect of sex on SC

221   WAT (not observed in the other sites). Glycerol and NEFA efflux showed a similar pattern,

222   despite glycerol efflux being, in all cases, much larger than that of NEFA. There was a

223   significant effect of sex in all WAT sites (except for NEFA in PG WAT), and a practically nil

224   effect of glucose concentration. However, the duration of incubation markedly raised the efflux

225   of both glycerol and NEFA in all sites, irrespective of sex, the sole exception being adipocytes of

226   MES WAT from females, which were unaffected by glucose and/or incubation time. Glycerol

227   efflux (and NEFA to a lower extent) by MES cells was higher than those of SC or PG cells.

228   *Analysis of gene expression*

229   We used the same statistical comparative approach described above for metabolites to

230   analyze the changes in gene expression at 24 and 48 h of incubation. Here we present the data as

231   number of copies of the gene mRNA transcripts per cell. Figures 3 to 6 show the gene

232   expressions of main enzymes and transporters affecting the metabolism of glucose, lipogenesis

233   and 3C fragment handling in the adipocyte. The statistical analysis of the differences between

234   groups, related to WAT sites, sexes and the medium glucose concentration were shown in the

235   graphs and Figure legends. The presentation –and initial analysis– of the gene expression data

236   was done along three main lines: a) Glucose and glycerol, b) Fatty acid metabolism, c) Pyruvate,

237   lactate and oxaloacetate. Figure 7 shows a schematic view of the genes studied. superimposed to

238   the main metabolic pathways of carbohydrate-lipid relationships in the adipocyte.

239   *a) Glucose and glycerol*

240   In MES, the expression of *Glut1* was higher in males than in females; it was practically

241   unaltered by glucose and incubation time. In SC WAT, the *Glut1* expression was lower than in

242   the other sites. Males showed a marked interaction between glucose level and incubation time.

243   The expression of *Hk1* repeated the trend of *Glut1,* but in MES WAT, the effects of sex were

244   more marked, with male-origin adipocytes decreasing their gene expression with incubation

245   time. Sex differences were more marked in *Pfkl*, repeating the pattern of higher male expression

246   in MES and lower in PG WAT; no effect of glucose levels were observed, either, but incubation

247   time increased the expression of this gene, especially in PG WAT. The expressions of *Phgdh*

248   (not a pathway control enzyme) were rather uniform, and lower than the other glycolytic

249   enzymes analyzed. There was a trend to increase expression with incubation time, and to

250   maintain the male-female predominance described for the other enzymes, but variability was

251   high and the statistical significance of the differences was low. In sum, no marked changes were

252   found to be influenced by the conditions of the study, suggesting a fluid and uniform operation

253   of glycolysis down to pyruvate with practically no effects of external glucose concentration,

254   combined with increased expressions with incubation time.

255   Glycerol efflux was paralleled by a marked trend to increase the already high expression of

256   *Gpd1* (i.e. compared with *Phgdh*) with incubation time, but –again– it was not affected by the

257   concentration of glucose. The maximal increase in expression, not linked to sex, was observed in

258   PG WAT. The phosphatase pathway (*Pgp*) showed no changes at all, except for higher values in

259   females of its (also high) expression in PG WAT. This was not the case for the enzyme

260   catalyzing the reverse reaction, glycerokinase, which gene (*Gk*) maintained the differences

261   between sexes, but showed a higher increase of its expression with incubation time. The overall

262   number of copies per cell of *Gk* was, however, about one order of magnitude lower than that of

263   *Pgp*. The patterns for Aquaporin 7 gene (*Aqp7*) expression closely resembled those of *Pgp*,

264   including the range of copies per cell.

265   The assumed incorporation of newly formed glycerol-3P into acyl-glycerols increased with
266   incubation time, judging from the expression of *Gpam*, following a pattern comparable to that of
267   *Gpd1*, deeply affected by time but not by medium glucose levels. Sex differences were
268   maintained: higher values for females in SC, and, especially, PG WAT. and higher levels of
269   expression for males in MES.

270   *b) Fatty acid metabolism*

271   Lipogenesis did not seem to represent a quantitatively important process under the
272   conditions tested because of the limited possibility of producing acetyl-CoA from the main
273   medium substrate, glucose (down to pyruvate). Pyruvate dehydrogenase activity may be
274   hampered, both because of the relatively scarce number of mitochondria and because of the
275   proportionally high expression of the main controller of the enzyme, pyruvate dehydrogenase
276   kinase 4. The expression of its gene (*Pdk4*) showed a marked effect of incubation time,
277   increasing (in all three sites) differently according to sex. Males and females' *Pdk4* expression in
278   SC and PG WAT increased about one order of magnitude in 24 h. In MES WAT, males followed
279   the same pattern, but no significant increase with incubation time was observed in females
280   (despite its spectacular rise in PG WAT). Under these conditions, pyruvate dehydrogenase could
281   not operate fully at 48 h, but these effects should be less marked in the MES WAT of females.

282   The transfer of acetyl-CoA to the cytoplasm via citrate: ATP lyase was not affected by the
283   treatment received by adipocytes, as shown by the little change found in the expression of its
284   gene *Acly*. Carboxylation of acetyl-CoA to malonyl-CoA was probably unchanged (or decreased
285   in male adipocytes of MES WAT) from the expression of *Acaca*. A similar pattern, with higher
286   number of copies per cell, was observed in *Fas*, fatty acid synthase. As for the availability of
287   NADPH in the cytoplasm, the indicator gene of the pentose phosphate pathway *G6pdx* showed
288   high numbers of copies and a marked sexual differentiation in MES and PG WAT, but no
289   changes induced by glucose or incubation time. These data suggest an absence of change in the
290   main NADPH provider, in parallel to the glycolytic and pyruvate-handling pathways already
291   analyzed. The other main NADPH generator, malic enzyme (*Me1*), also showed little change in
292   its expression, with a tendency to lower the number of copies in MES-WAT with incubation
293   time.

294    Probably, the uptake of medium fatty acids (at least from cell remnants), was activated,

295 since the expression of one of the main transporters *Cd36* increased with time. Sex affected only

296 PG WAT, and no effects of glucose levels were observed at all. Mitochondrial utilization of

297 acyl-CoA was assumed to be low (if any), first because of the nature of the tissue and its energy

298 needs (expected low oxidative metabolism); second because of the ample availability of glucose;

299 and third because the expression of *Cpt1b* was very low in all three sites, thus making difficult

300 the entry of acyl-CoA into the mitochondria. The already described differences related to sex

301 were maintained, and there was a marked increase with time in the expression of this gene in

302 both sexes.

303    The main lipases of adipose tissue: the extracellular lipoprotein lipase (*Lpl*), and the

304 internal adipose TAG lipase (*Atgl*) and hormone-sensitive lipase (*Hsl*) showed essentially the

305 same trend. All had uniform increases in gene expression with incubation time, lack of effects of

306 glucose and sex-related differences of limited extent. *Lpl* showed the highest number of copies

307 per cell found in this study. In the case of MES WAT, females' increase of expression with time

308 was less marked than that of males, a reminiscence of the discordance described for *Pdk4*. The

309 high coordinated increase in lipase activity was not correlated to the limited release of NEFA

310 into the medium, and these levels were far from being correlated with any of the expressions of

311 the lipases studied.

312   *c) Pyruvate, lactate and oxaloacetate*

313    In addition to the different sex-related response of *Pdk4* expression of adipocytes with

314 incubation, further limiting the synthesis of acetyl-CoA in most of the conditions analyzed, the

315 obviously major outlet for excess pyruvate generation in the cytoplasm was its conversion to

316 lactate, completing the classic glycolytic pathway. The number of copies of *Ldha* was high, with

317 practically no effect of sex, but showing a trend to increase its expression with time of incubation

318 in SC and PG WAT. Initial glucose affected more intensely the MES values, decreasing *Ldha*

319 expression at lower-, and increasing at higher medium glucose, with a clear differentiation by sex

320 (higher values for males). *Ldhb* showed less change and lower number of copies per cell. The

321 ratio of expression of both lactate dehydrogenase isoform genes (*Ldha / Ldhb*) was rather

322 constant, with a mean value of 6.2 for both sexes. The expression of the monocarboxylate

323 transporter gene (*Mct1*) presented a pattern closely similar to that of *Ldha*, hinting at a

324 coordinated regulation. As described above, *Me1* showed only limited changes in expression,

325   which agrees with the lower needs for NADPH (mainly used for lipogenesis) observed under the

326   conditions of incubation. Last, but not least, the key marker enzyme gene *Pck1* (P-enol-pyruvate

327   carboxy-kinase), a critical enzyme bridging the pyruvate kinase gap to favor the arrival of

328   oxaloacetate to the triose-P pool under conditions of scarcity of glucose, showed a marked sex-

329   related difference in its response to incubation. Irrespective of glucose concentration, both sexes

330   in PG and only males in SC and MES WAT increased dramatically its expression with time of

331   incubation, suggesting the need for the conversion of excess cytoplasmic oxaloacetate into P-

332   enol pyruvate. This change was not observed in female adipocytes of SC and MES WAT, in a

333   pattern closely resembling that of MES *Pdk4*.

334

335   **Discussion**

336   The main outcome of this study is a reinforcement of the widely accepted idea that WAT

337   sites are remarkably uniform (Arriarán et al. 2015b) from a qualitative point of view, but also

338   that they are adjusted to play different metabolic functions depending on their location (Prunet-

339   Marcassus et al. 2006). Thus, the differences between sites were mainly quantitative. This idea is

340   not new, but the comparative analyses of the capacity of metabolic pathways are too scarce and

341   incomplete to provide sufficient support. Our contribution may help reinforce the assumption

342   that WAT plays a role in glucose handling, sufficient to participate in the maintenance of

343   glycaemia (Arriarán et al. 2015b; Ho-Palma et al. 2016). We also present additional evidence

344   that key metabolic functions of WAT are deeply affected by sex, as previously indicated

345   (Amengual-Cladera et al. 2012; White & Tchoukalova 2014). This aspect is more clearly

346   observed in MES and SC WAT than in the fat pads (PG) attached to reproductive organs. Our

347   data also agree with the postulated role of glycolysis to lactate as main provider of energy (ATP)

348   to sustain most of the white adipocyte normal function (Crandall et al. 1983; Hagström et al.

349   1990; Sabater et al. 2014), thus reducing its need for oxidative metabolism, and, consequently, of

350   oxygen (Sjöström 1990). This aspect is also sustained, by the WAT relative scarcity of

351   mitochondria and, logically, of the oxidative metabolism they sustain (Deveaud et al. 2004;

352   Frayn et al. 2008). We assume that glucose is taken up as needed (mainly for energy); and,

353   consequently, lactate is released not as a response to glucose availability (and uptake) but

354   depending on cytoplasm energy requirements. The consequence is an extensive conversion of

355   glucose to 3C metabolites because of the energetic inefficiency of the (anaerobic) glycolytic use

356   of glucose. The 3C thus generated can be easily used for energy or as carbon source elsewhere

357   (Frayn & Coppack 1990; Sabater et al. 2014; Arriarán et al. 2015c; Rotondo et al. 2017b).

358   Our previous studies on adipocyte primary cultures were carried out using only male rats'

359   epididymal adipose tissue (Rotondo et al. 2017b), a classical depot WAT site; the comparison

360   done here with female rat periovaric WAT showed little differences between them, even with

361   respect to potential fatty acid synthesis. These results were not expected, given the higher

362   tendency to fat accumulation of adult males versus females, both in rats and humans (Schemmel

363   et al. 1969; Fried et al. 2015). However, as a rule, the number of copies per cell for most of the

364   genes studied was higher in females than in males, in contrast with the smaller (NS) mean size of

365   their adipocytes, hinting at a possibly higher overall metabolic activity of female WAT (Blaak

366   2001).

367   MES WAT showed a markedly higher efflux of glycerol, and NEFA (to a proportionally

368   lower extent in molar terms), compared with the other sites investigated. Previous analyses of

369   glucose carbon fate in epididymal WAT showed that a sizeable proportion of glucose ended

370   forming part of glyceride-glycerol (Cahill et al. 1959; Ho-Palma et al. 2016). Results were more

371   marked after incubation of adipocytes for more than one day in the presence of glucose (Rotondo

372   et al. 2017b).There was, also, a significant lipase-driven TAG turnover, in which most of fatty

373   acids were recycled to TAG (Hammond & Johnston 1987), but glycerol was excreted (Vaughan

374   1962; Jansson et al. 1992). The data of the present study agree with this interpretation. However,

375   since no labelled C has been used, we can only deduce (not prove) the origin of glyceride-

376   glycerol in the adipocytes. We know, however, that the rates of glycerol release to the medium

377   by MES were much higher than in PG WAT (both epididymal and periovaric). We can, assume,

378   thus, that glycerol production in MES WAT may be higher than that of the only tissue

379   quantitatively analyzed in detail with tracers, male PG WAT (Ho-Palma et al. 2016). Since MES

380   WAT plays an ancillary energy-handling role to the liver, its massive production of glycerol (to

381   our knowledge not previously described) may help facilitate the hepatic handling of fatty acids.

382   Intestinal absorption-derived fatty acids, not used in the synthesis of the TAG carried out by

383   lymph, as well as excess systemic blood NEFA arrived to the liver mixed in the portal blood with

384   the efflux of MES-WAT. This WAT site may also help to lower the portal intestine-released

385   glucose load carried to the liver, as shown by the higher glucose uptake and 3C substrate efflux

386   rates in MES when compared with SC and, especially, PG WAT. We can speculate that MES-

387   WAT activity may help buffer the impact of large digestive glucose loads, and thus facilitate its

388   hepatic handling.

389   The differences in response between female- and male-derived MES adipocytes facing

390   periodic exposure to excess energy (in this case, glucose) seem minimal, but may be far-

391   reaching. In addition to higher overall glycerol efflux, in female rats, pyruvate dehydrogenase

392   inhibition by its kinase 4 seems not to be altered by either glucose levels or time of incubation.

393   This occurs in contrast with the high increases elicited in males (Rotondo et al. 2017b) of the

394   number of copies of *Pdk4*, a powerful inhibitor of the dehydrogenase (Cadoudal et al. 2008),

395   which is mainly regulated via transcription (Jeong et al. 2012). This increase was also observed

396   in SC and PG WAT of both male and female rats, being, thus, a unique effect restricted to

397   (female) MES WAT. This assumed "lower potential inhibition" of pyruvate dehydrogenase hints

398   at a potentially higher flow of 3C (pyruvate) into mitochondrial acetyl-CoA, thus facilitating

399   either its oxidation or incorporation into the lipogenic pathway. This could not proceed so easily

400   in males (or in other WAT sites of females), which *Pdk4* expression increased with time and

401   exposure to glucose, preventing the decarboxylation of mitochondrial pyruvate to acetyl-CoA.

402   Since in female MES, excess mitochondrial pyruvate could not be processed to acetyl-CoA, it

403   must be returned to the cytosol. The limitation of mitochondrial acetyl-CoA means that it could

404   not be derived through the Krebs cycle, which in any case needs oxygen to oxidize it. Thus, the

405   most probable way of utilization of the 'surplus' mitochondrial pyruvate is via carboxylation

406   (Ballard & Hanson 1967) to oxaloacetate and its transfer to the cytoplasm via the

407   pyruvate/malate shuttle, partly using the machinery of fatty acid synthesis (Patel et al. 1971).

408   This implies a considerable activity of the malate-oxaloacetate metabolism in the mitochondria,

409   albeit not implicating citrate. After malate is transferred into the cytosol, it could be either used

410   by the malic enzyme to provide NADPH (improbable in this case as explained above) or

411   oxidized to oxaloacetate by the cytoplasmic malate dehydrogenase, providing NADH (Nye et al.

412   2008). Cytoplasmic oxaloacetate can be converted into P-enol-pyruvate by its carboxy-kinase

413   (Ballard et al. 1967). In males, the expression of its gene, *Pck1*, was raised with incubation time

414   in parallel to *Pdk4*, but it was stabilized in females following the same pattern than the kinase, an

415   effect extended to SC-WAT. The differences in the cytosol-mitochondria handling of pyruvate

416   suggest a deep sex-related divergence in the metabolic fate of pyruvate, which, ultimately, may

417 help explain the known different metabolic handling of lipids (and, probably glucose) by visceral

418 WAT depending on sex (Franckhauser et al. 2002).

419 Curiously, the alternatives for disposal of the oxaloacetate assumedly extracted from the

420 mitochondria by the malate shunt point to the regeneration of P-enol pyruvate. This high-energy

421 compound could not go further up the glycolytic pathway because of massive thrust of glycolysis

422 carbon flow towards pyruvate/lactate. The high lactate efflux is proof of the unequivocal

423 direction of glycolysis in the adipocytes, at least under the conditions and cell sizes used in this

424 study, leaving open only the conversion (again) of P-enol-pyruvate to pyruvate by pyruvate

425 kinase. However, the difference (in females) lies, precisely, in the assumed provision of

426 cytoplasmic NADH by malate dehydrogenase, which may provide reducing power for the final

427 conversion of pyruvate to lactate (Rotondo et al. 2017b) easing its disposal via excretion.

428 Since pyruvate is a good substrate for lipogenesis (Schmidt & Katz 1969) and WAT is the

429 largest depot for body lipid storage, one can expect lipogenesis to be fully activated when

430 glucose availability is high. The process requires, however, the massive production of acetyl-

431 CoA, its transfer to the cytoplasm via citrate and then a full activation of lipogenesis, including

432 necessarily higher expressions of *Acaca* and *Fas* and the activation of NADPH providers (such

433 as *G6pdx* or *Me1*). None of these signs was detected; no effects of glucose or duration of the

434 incubation affected the expressions of these genes. Probably, quantitative conversion of glucose

435 to fatty acids under high glucose conditions was not as a function as relevant for the adipocyte

436 (or WAT) as to dispose of excess glucose, in agreement with previous studies using labelled

437 glucose (Rotondo et al. 2017b).

438 In addition to the paradoxical apparent inactivity of lipogenesis, there was a high increment

439 with time (but not with higher glucose) of lipase gene *Lpl* and *Atgl* expressions (in *Hsl* we

440 observed a tendency to "restraint" resembling those of *Pdk4* and *Pck1*) (Sabater et al. 2014;

441 Rotondo et al. 2016; Rotondo et al. 2017b). However, the expected massive efflux of NEFA did

442 not occur. In any case, NEFA were released to the medium in much smaller proportions than the

443 canonical 3-to-1 molar ratio *vs*. glycerol expected from TAG hydrolysis, in disagreement with

444 the frank liberation of NEFA described for the initial phase of catecholamine-elicited WAT

445 lipolysis (Jocken et al. 2008). The results shown here were, however, fully compatible with an

446 activation of TAG turnover, a critical adipocyte regulatory system (Arner et al. 2011). In the end,

447 this process, selectively released glycerol as a 3C substrate ultimately derived from glucose, as

448   we have previously established (Arriarán et al. 2015c; Rotondo et al. 2017b). The highest

449   number of lipase gene mRNA copies per cell, and the steepest increase with time was observed

450   in PG WAT (Rotondo et al. 2016; Rotondo et al. 2017b), with values even higher for females as

451   found in the present study. Nevertheless, the glycerol release rates observed for this site were

452   smaller than in MES and SC WAT.

453   The higher female number of copies for *Gk* (glycerol kinase gene) may suggest the

454   existence of an additional restrain of the whole process, i.e. free glycerol being recycled to *sn-*

455   glycerol-3P (Lee et al. 2005). This process may potentially decrease the actual release of

456   glycerol, in parallel to lower NEFA liberation. It can unbalance, also, the equilibrium between

457   glycerol-3P synthesis and hydrolysis (Margolis & Vaughan 1962). However, we have not

458   sufficient data to prove or disprove the operation of this alternative pathway, which would help

459   define the postulated increase in WAT turnover as a 'futile cycle' in which energy was wasted

460   (for thermogenesis?).

461   WAT production of glycerol has been known for a long time (Vaughan 1962; van der

462   Merwe et al. 1998; Bolinder et al. 2000). Glycerol has been related to glucose in WAT as source

463   of carbon (Wood et al. 1960; Smith 1972), but its immediate origin has been linked to lipolysis

464   (Karpe et al. 2005; Langin 2006) within the context of the glucose-fatty acid cycle (Randle et al.

465   1963). However, glycerol and fatty acid effluxes seldom have been analyzed together (Jocken et

466   al. 2008). The quantitative approach used here shows that there is a direct relationship between

467   glycerol and NEFA release, but not at the ratios expected, and points to lipolysis as part of TAG

468   turnover. This turnover was accelerated with time, apparently to favor the release of more

469   glycerol. Nevertheless, the main factor affected by these changes, and that most modulated by

470   sex (at least in MES adipocytes) was assumed to be the equilibrium between NADPH and

471   NADH in the thin layer of adipocyte cytoplasm. Intercellular cooperation, perhaps close

472   neighboring cells, smaller than adipocytes, providers of mitochondria oxidative power, are

473   needed to fully understand how WAT works, and, also, to uncover the intricate effects of sex on

474   WAT operation (Randle et al. 1963).

475

476   **Conclusions**

477   The fairly uniform (or coordinated) pattern of gene expressions, with limited overall

478   changes, and the actually small amount of "live matter" in the adipocytes used in the incubations

479   hint at an active but stabilized energy sustaining machinery working along the 2-day incubation.

480   This resulted in a high proportion of glucose converted to 3C substrates, largely lactate. The

481   length of the incubation period modulated the development and timing of the alternative

482   mechanisms that allow the synthesis and release of free glycerol, especially addressed to the liver

483   (at least by the highest WAT glycerol producer, the MES site), and the considerable restraint

484   observed in the synthesis of fatty acids in the presence of high medium glucose concentrations.

485   These findings are not completely new, but its concatenation is.

486   In fact, the resilience of adipocytes to limit the production of additional fatty acids under

487   excess glucose availability, breaking up, instead, most of the glucose, then released as lactate and

488   glycerol, is remarkable. It also makes us wonder whether we can keep considering true the

489   current assumption that WAT 'has to take up and use for fat synthesis the surplus dietary

490   glucose', under conditions of excess glucose/energy available (a principle often used to help

491   explain obesity).

492

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Figure 1

WAT sites and adipocyte size

Panel [A] depicts microphotographs of representative isolated adipocyte preparations from subcutaneous, mesenteric and epididymal WAT. The cells were viewed in a Neubauer chamber. Main grid squares were 250 µm wide (and had a volume of 6.25 nL each). Panel [B] shows the mean adipocyte volume in WAT sites extracted from undisturbed female and male adult Wistar rats. The data are the mean ± sem of 4 groups of 2 rat-pools for sex and site. SC = subcutaneous WAT; MES = mesenteric WAT; PG perigonadal (epididymal – males, periovaric – females) WAT; blue bars, M = male rats; mauve (clear) bars, F = female rats. Statistical comparison between groups: two-way ANOVA; the p values for sex (green) and site (red) are included in the Figure. NS = not significant (p>0.05).

A   SUBCUTANEOUS   MESENTERIC   EPIDIDYMAL

SITE: p=0.0015
SEX: NS

B

ADIPOCYTE VOLUME (pL)

500
400
300
200
100
0

SC-M SC-F MES-M MES-F PG-M PG-F

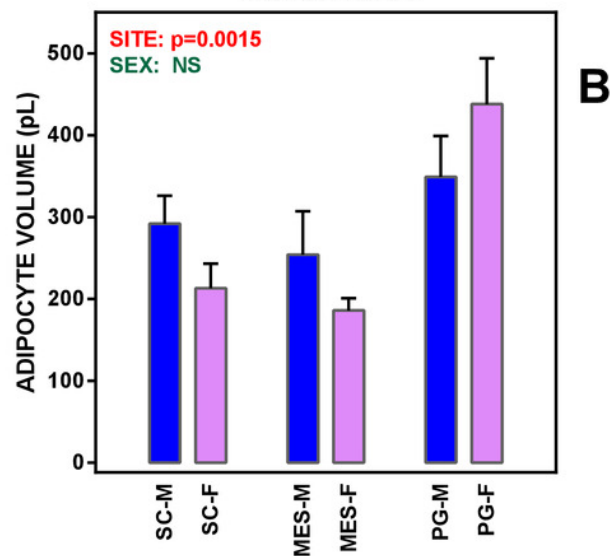
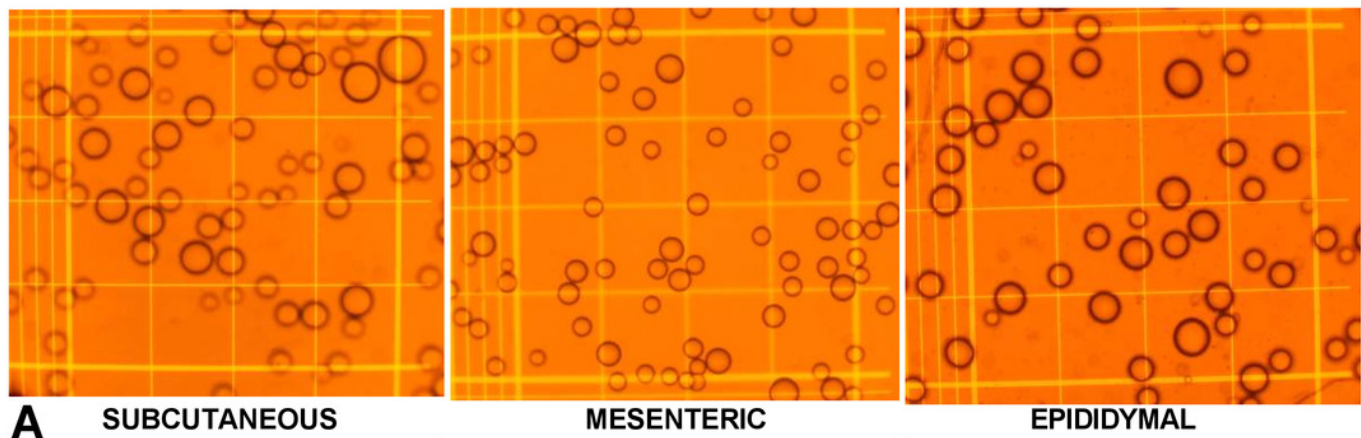



Figure 2

Glucose uptake rates and lactate, glycerol and NEFA efflux rates (all in attomoles per second and cell) of adipocytes obtained from three WAT sites of male and female adult Wistar rats

The data are the mean ± sem of 4 groups of 2 rat-pools for sex and site, incubated for 24 or 48 h. The abbreviations for site and sex are the same as in Figure 1. [A] Glucose uptake, [B lactate effl, [C] glycerol efflux, [D] NEFA efflux. The statistical significance of the differences between groups was investigated using 2-, 3- and 4-way ANOVA analyses and the variables: WAT Site, Sex (S), medium Glucose concentration (G) and Time of incubation (T). The Figure shows the 3- (S, G, T) and 2- way ANOVA (G,T) analyses for each site; the data are included in blue boxes (3-way) encompassing each site and showing the corresponding p values when statistically significant (p<0.05), or gray boxes (2-way) with significant p values for each sex within a given WAT site. The 4-way ANOVA values were, Site: p<0.001 for all parameters except p=0.0081 (lactate): Sex: p<0.0001 for glycerol and NEFA, and p=0.0046 for glucose; Glucose concentration: p<0.0001 for glucose; Time of incubation: p<0.0001 for NEFA, p=0.0001 for glycerol and p=0.0133 for lactate.

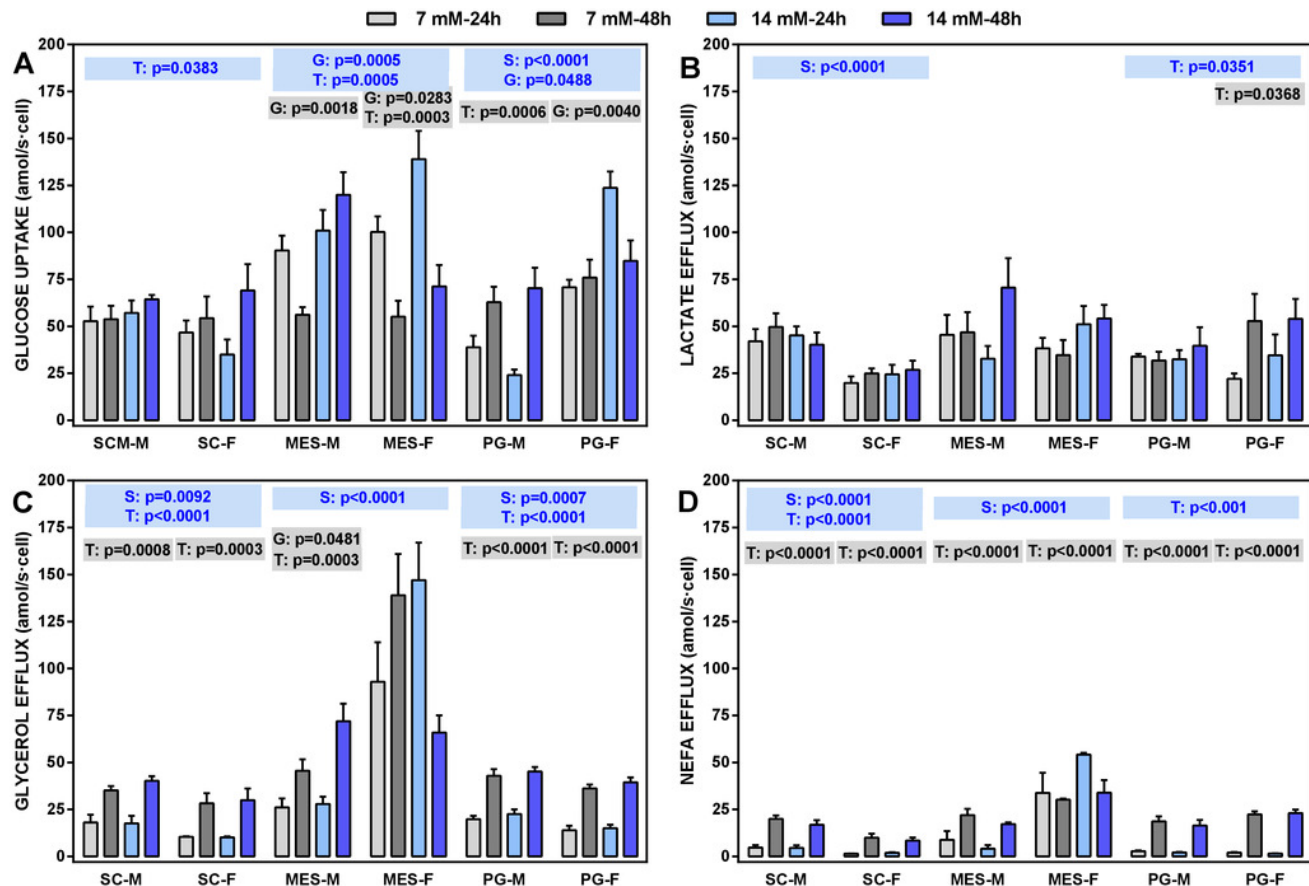



Figure 3

Expression of genes related with the metabolism of glucose, lipogenesis and 3C handling in adipocytes of male and female adult rats incubated in the presence of 7 mM or 14 mM glucose (I)

The data are the mean ± sem of 4 (or 3) groups of 2 rat-pools for sex and site, incubated for 24 or 48 h, and represent the number of mRNA copies per cell of each gene. The data are presented on a log scale. Blue circles indicate males: deep blue 14 mM glucose, light blue 7 mM glucose; purple circles represent females: violet-purple 14 mM glucose, mauve 7 mM glucose. SC = subcutaneous, MES = mesenteric and PG = perigonadal WAT. [A] *Glut1*, [B] *Hk1*, [C] *Pfkl*. [D] *Phgdh*, [E] *G6pdx*, [F] *Me1*. Statistical analysis of the differences between groups: The figures contain the p values for a 3-way ANOVA analysis (sex [S in green], glucose [G in blue] and time of incubation [T in purple]) applied to each site. Results of two-way ANOVA for these paramers are also represented: the letters and colors are the same, adding m for male and f for female subgroups; for 2-way ANOVAs, the p values are represented by up to three stars, corresponding to three levels of statistical significance of the differences (p<0.05, p<0.01 and p<0.001). Only significant differences have been represented. The results for 4-way ANOVAs were significant (p<0.0001) for time in all gene expressions. The effect of sex was significant only for *Pfkl* (p=0.0006) and *Me1* (p=0.0007), that of glucose concentration was significant for *Hk1* (p=0.0371), *Phgdh* (p=0.0164) and *Me1* (p=0.206). The effect of time of incubation was significant for *Glut1* (p=0.0023) and *Pfkl*, *Phgdh*, and *G6pdx* (p<0.0001). Non-statistically significant data (p>0.05) were not represented.

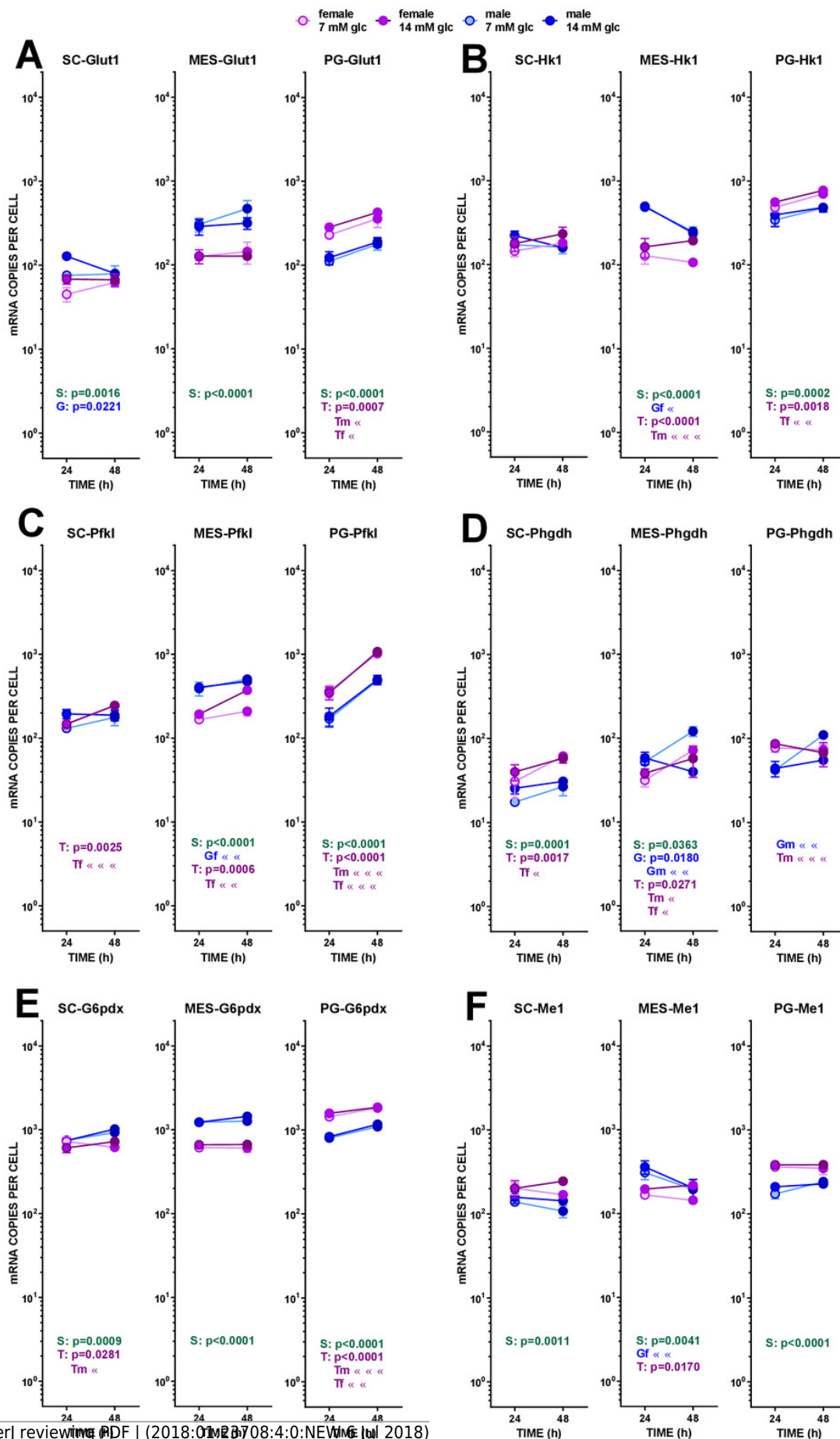



Figure 4

Expression of genes related with the metabolism of glucose, lipogenesis and 3C handling in adipocytes of male and female adult rats incubated in the presence of 7 mM or 14 mM glucose (II)

The data are the mean ± sem of 4 (or 3) groups of 2 rat-pools for sex and site, incubated for 24 or 48 h, and represent the number of mRNA copies per cell of each gene. The distribution figure set-up and other conventions are those described in Figure 4. [1] *Ldha*, [B] *Ldhb*, [C] *Pdk4*, [D] *Pck1*, [E] *Mct1*, [F] *Cpt1b*. The results for 4-way ANOVAs were significant (p<0.0001) for time in all gene expressions. The effect of sex was significant only for *Ldhb* (p=0.0063), that of glucose concentration was significant only for *Ldha* (p=0.0037). The effect of time of incubation was significant (p<0.0001) for all genes except *Ldhb* (NS).

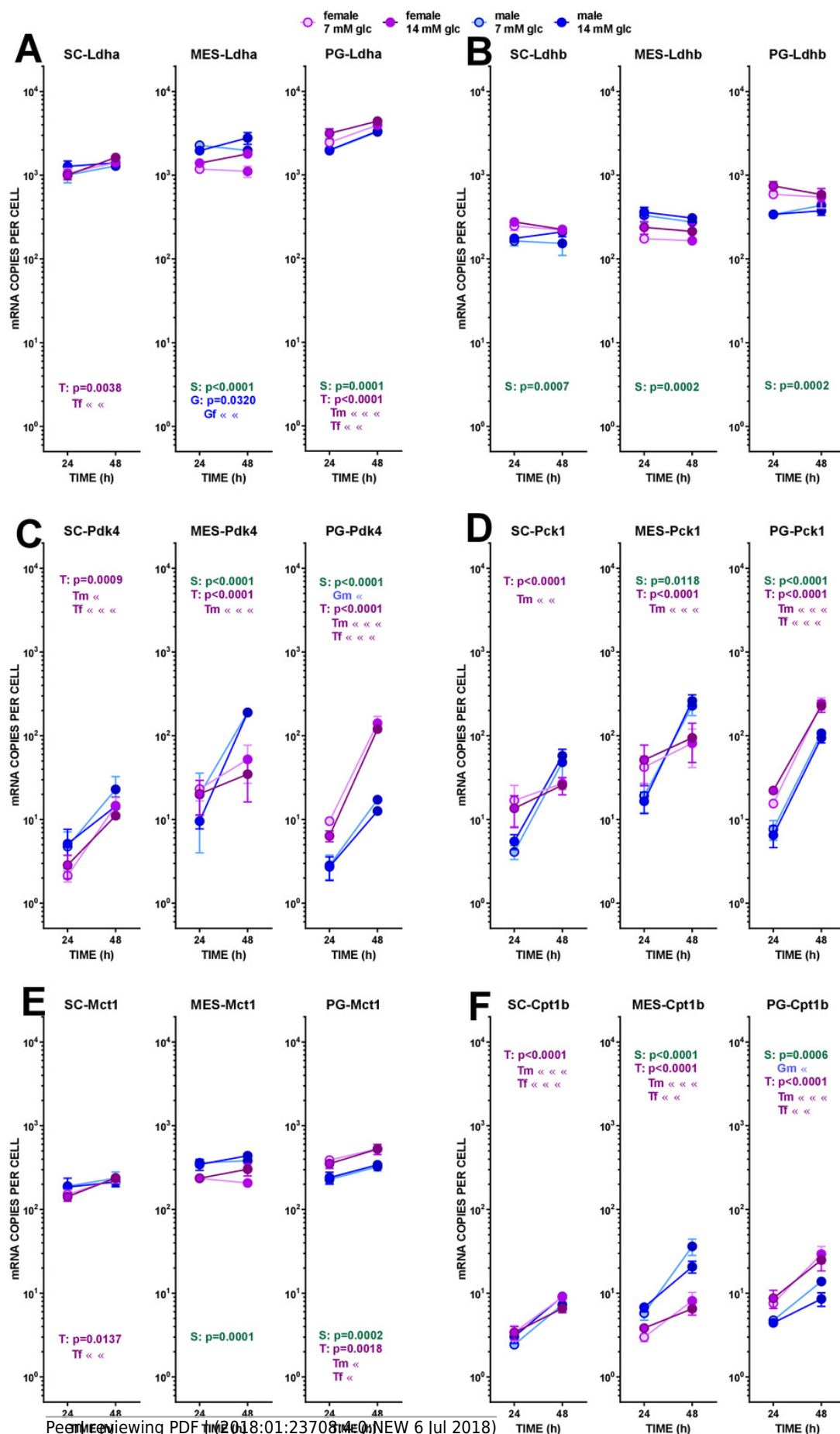



Figure 5

Expression of genes related with the metabolism of glucose, lipogenesis and 3C handling in adipocytes of male and female adult rats incubated in the presence of 7 mM or 14 mM glucose (III)

The data are the mean ± sem of 4 (or 3) groups of 2 rat-pools for sex and site, incubated for 24 or 48 h, and represent the number of mRNA copies per cell of each gene. The distribution figure set-up and other conventions are those described in Figure 4. [1] *Gdp1*, [B] *Gpam*, [C] *Pgp*, [D] *Gk*, [E] *Aqp7*, [F] *Cd36*. The results for 4-way ANOVAs were significant (p<0.0001) for time in all gene expressions (p=0.0030 for *Cd36* and p<0.0001 for all others). The effect of sex was significant only for *Pgp* (p<0.0001) and *Gk* (p=0.0001) Glucose concentration did not elicit any significant change of expression. The effect of time of incubation was significant (p<0.0001) for *Gpd1*, *Gpam*, *Gk* and *Cd36*.

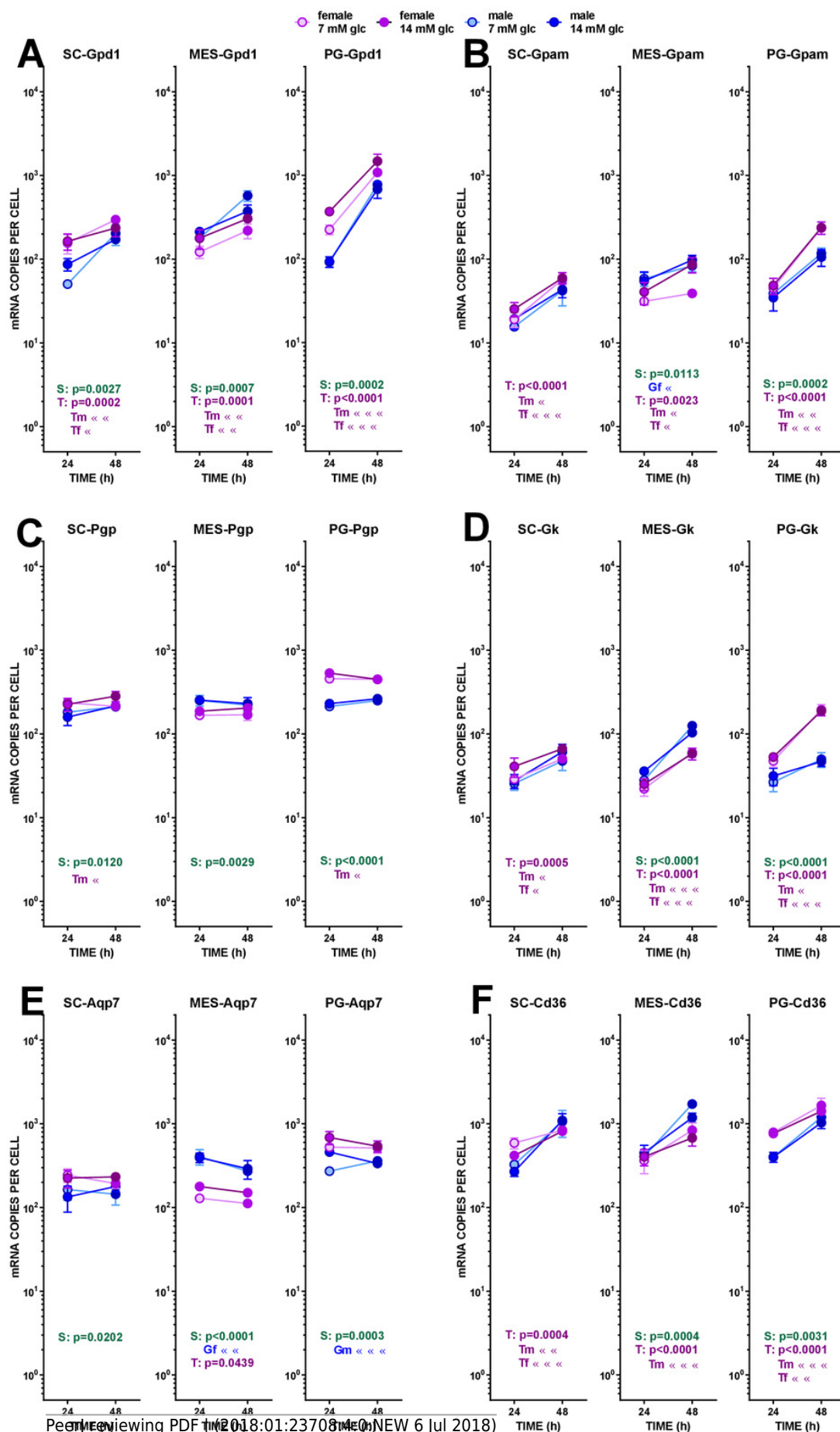



Figure 6

Expression of genes related with the metabolism of glucose, lipogenesis and 3C handling in adipocytes of male and female adult rats incubated in the presence of 7 mM or 14 mM glucose (IV)

The data are the mean ± sem of 4 (or 3) groups of 2 rat-pools for sex and site, incubated for 24 or 48 h, and represent the number of mRNA copies per cell of each gene. The distribution figure set-up and other conventions are those described in Figure 4. [1] *Acaca*, [B] *Fas*, [C] *Acly*, [D] *Lpl*, [E] *Hsl*, [F] *Atgl*. The results for 4-way ANOVAs were significant (p<0.0001) for time in all gene expressions. The effect of sex was significant for *Fas* and *Acly* (p<0.0001), *Lpl* (p=0.0001, *Acaca* (p=0.0020) and *Hsl* (p=0043); that of glucose concentration was significant only for *Fas* (p=0.0003) and *Acly* (p=0.0118). The effect of time of incubation was significant for *Lpl*, *Hsl*, *Atgl* (p<0.0001), and *Fas* (p=0-014).

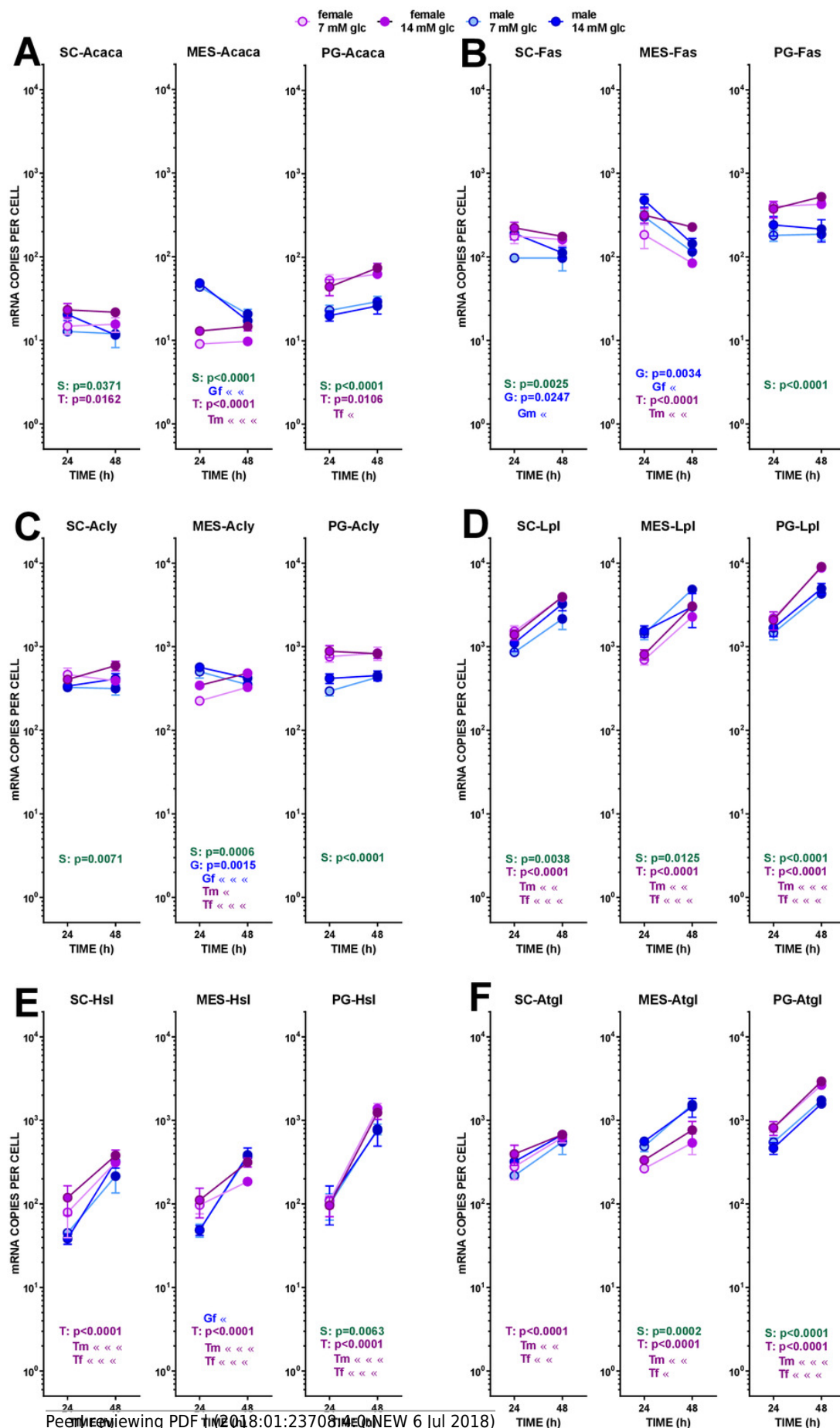
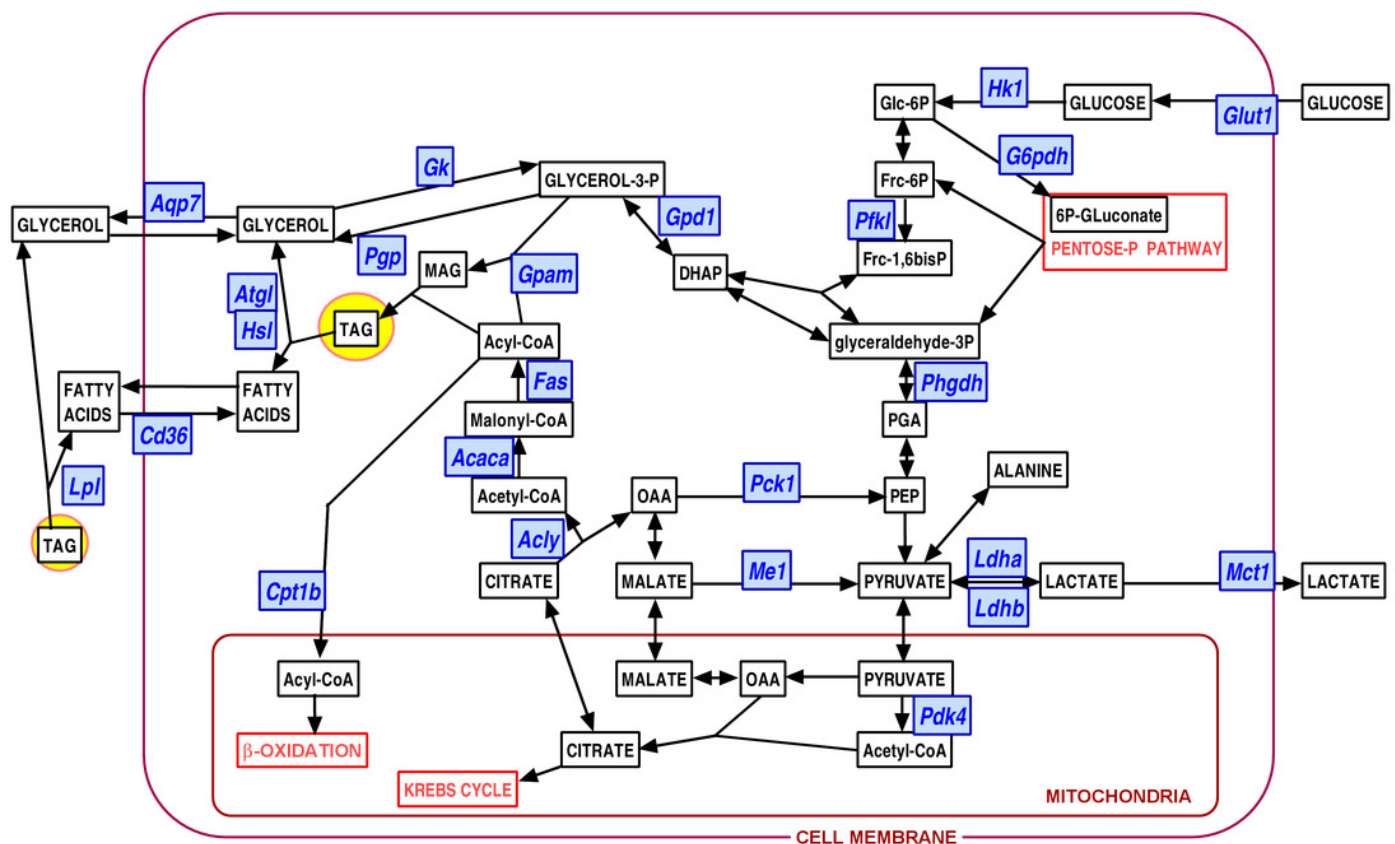



Figure 7

Diagram showing the position of the genes investigated on a scheme of the main adipocyte energy metabolism pathways

Blue: genes (for enzymes and transporters); white: metabolites.

List of primers used in the present study

1   TABLE 1   List of primers used in the present study

gene	protein	direction	sequences	bp
(Glut-1) *Slc2a1*	Glucose transporter type 1, erythrocyte/brain	5' >	GCTCGGGTATCGTCAACACG	97
		> 3'	ATGCCAGCCAGACCAATGAG	
Hk1	Hexokinase type 1	5' >	TGGATGGGACGCTCTACAAA	100
		> 3'	GACAGGAGGAAGGACACGGTA	
Pfkl	Phospho-fructokinase, liver, b-type	5' >	CAGCCACCATCAGCAACAAT	90
		> 3'	TGCGGTCACAACTCTCCATT	
Phgdh	Phospho-glycerate dehydrogenase	5' >	CTGAACGGGAAGACACTGGGAA	138
		> 3'	AACACCAAAGGAGGCAGCGA	
G6pdx	Glucose-6-phosphate dehydrogenase X-linked	5' >	GACTGTGGGCAAGCTCCTCAA	77
		> 3'	GCTAGTGTGGCTATGGGCAGGT	
Me1	NADP⁺-dependent malic enzyme	5' >	GGAGTTGCTCTTGGGGTAGTGG	143
		> 3'	CGGATGGTGTTCAAAGGAGGA	
Ldha	L-lactate dehydrogenase a	5′ >	AAAGGCTGGGAGTTCATCCA	96
		> 3'	CGGCGACATTCACACCACT	
Ldhb	L-lactate dehydrogenase b	5' >	GCGAGAACTGGAAGGAGGTG	145
		> 3'	GGGTGAATCCGAGAGAGGTTT	
Pdk4	Pyruvate dehydrogenase kinase, isoenzyme 4	5′ >	CTGCTCCAACGCCTGTGAT	142
		> 3'	GCATCTGTCCCATAGCCTGA	
Pck1	Phosphoenol-pyruvate carboxykinase, cytosolic	5′ >	CGGGTGGAAAGTTGAATGTG	142
		> 3'	AATGGCGTTCGGATTTGTCT	
Mct1	Monocarboxylate transporter	5′ >	CCCAGAGGTTCTCCAGTGCT	133
		> 3'	ACGCCACAAGCCCAGTATGT	
Cpt1b	Carnitine O-palmitoleoyl-transferase 1, muscle isoform	5′ >	TGCTTGACGGATGTGGTTCC	152
		> 3'	GTGCTGGAGGTGGCTTTGGT	
Gpd1	Glycerol 3-phosphate dehydrogenase	5′ >	CTGGAGAAAGAGATGCTGAACG	113
		> 3'	GCGGTGAACAAGGGAAACTT	
Gpam	Glycerol-3-phosphate acyl-transferase, mitochondrial	5′ >	GGTGAGGAGCAGCGTGATT	129
		> 3'	GTGGACAAAGATGGCAGCAG	
Pgp	Phosphoglycolate phosphatase	5′ >	CCTGGACACAGACATCCTCCT	100
		> 3'	TTCCTGATTGCTCTTCACATCC	
Gk	Glycerol kinase	5' >	ACTTGGCAGAGACAAACCTGTG	74
		> 3'	ACCAGCGGATTACAGCACCA	
Aqp7	Aquaporin 7	5' >	ACAGGTCCCAAATCCACTGC	127
		> 3'	CCGTGATGGCGAAGATACAC	
CD36	Platelet glycoprotein 4 [fatty acid transporter]	5' >	TGGTCCCAGTCTCATTTAGCC	154
		> 3'	TTGGATGTGGAACCCATAACTG	
Acaca	Acetyl-CoA carboxylase 1	5' >	AGGAAGATGGTGTCCGCTCTG	145
		> 3'	GGGGAGATGTGCTGGGTCAT	
Fas	Fatty acid synthase	5' >	CCCGTTGGAGGTGTCTTCA	117
		> 3'	AAGGTTCAGGGTGCCATTGT	
Acly	ATP citrate lyase	5' >	TGTGCTGGGAAGGAGTATGG	137
		> 3'	GCTGCTGGCTCGGTTACAT	

Lpl	Lipoprotein lipase	5' >	TGGCGTGGCAGGAAGTCT	116
		> 3'	CCGCATCATCAGGAGAAAGG	
(Hsl) Lipe	Lipase, hormone sensitive	5' >	TCCTCTGCTTCTCCCTCTCG	108
		> 3'	ATGGTCCTCCGTCTCTGTCC	
(Atgl) Pnpla2	Adipose triacylglycerol lipase	5' >	CACCAACACCAGCATCCAAT	120
		> 3'	CGAAGTCCATCTCGGTAGCC	
Arbp	0S acidic ribosomal phospho-protein PO [housekeeping gene]	5' >	CCTTCTCCTTCGGGCTGAT	122
		> 3'	CACATTGCGGACACCCTCTA	