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1 **The role of mitochondria linked fatty-acid uptake-driven adipogenesis in**
2 **Graves' Orbitopathy**

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13 **Short title:** The essential role of mitochondria in Graves' Orbitopathy

14 **Key words:** Graves' Orbitopathy; Orbital Adipose Tissue; fatty acid uptake;
15 adipogenesis; mitochondria OXPHOS; ATP production.

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23

24 **Abstract**

25 **Context**

26 Depot-specific expansion of orbital-adipose-tissue (OAT) in Graves' Orbitopathy (GO,
27 an autoimmune condition producing proptosis, visual impairment and reduced quality
28 of life) is associated with fatty-acid (FA) uptake-driven adipogenesis in
29 preadipocytes/fibroblasts (PFs).

30 **Objective**

31 A role for mitochondria in OAT-adipogenesis in GO.

32 **Design/Setting/Participants**

33 Confluent PFs from healthy OAT (OAT-H), OAT from GO (OAT-GO) and white-
34 adipose-tissue in culture-medium compared with culture-medium containing a mixed
35 hormonal-cocktail as adipogenic-medium (ADM); or culture-medium containing FA-
36 supplementation, oleate:palmitate:linoleate (45:30:25%) with/without different
37 concentration of mitochondrial bio-substrate ADP/GDP, AICAR (adenosine-analog)
38 or inhibitor oligomycin-A for 17 days.

39 **Main outcome measures**

40 Oil-Red-O staining and foci-count of differentiated adipocytes for in-vitro
41 adipogenesis; flow-cytometry, relative-QPCR, MTS-assay/ 10^6 cells, total cellular-
42 ATP detection kit and Seahorse-XFe96-Analyzer for mitochondria and
43 OXPHOS/Glycolysis-ATP production analysis.

44 **Results**

45 During early adipogenesis before adipocyte formation (day-0,4&7), we observed
46 OAT-specific cellular ATP-production via mitochondrial-OXPHOS in PFs from both
47 OAT-H/OAT-GO, and substantially disrupted OXPHOS-ATP/Glycolysis-ATP
48 production in PFs from OAT-GO, e.g. 40% reduction in OXPHOS-ATP and trend-

49 increased Glycolysis-ATP production on day-4&7 compared with day-0, which
50 contrasted with the stable levels in OAT-H.

51 FA-supplementation in culture-medium triggered adipogenesis in PFs from both
52 OAT-H/OAT-GO, which was substantially enhanced by 1mM GDP reaching 7-18%
53 of ADM-adipogenesis. The FA-uptake-driven adipogenesis was diminished by
54 oligomycin-A but unaffected by treatment with ADP or AICAR. Furthermore, we
55 observed significant positive correlation between FA-uptake-driven adipogenesis by
56 GDP and the ratios of OXPHOS-ATP/Glycolysis-ATP through adipogenesis of PFs
57 from OAT-GO.

58 **Conclusions**

59 Our study confirmed that FA-uptake can drive OAT-adipogenesis and revealed a
60 fundamental role for mitochondria-OXPHOS in GO development, which provides
61 potential for therapeutic interventions.

62

63 **Introduction**

64 Graves' Orbitopathy (GO), also called thyroid eye disease, is a disfiguring disease of
65 the orbit with a higher incidence in women (80%) (1,2). The uncontrolled expansion of
66 orbital adipose tissue (OAT) contributes to proptosis, double vision and in some cases
67 visual loss. GO develops mainly in the context of an autoimmune condition, Graves'
68 disease, in which thyrotropin receptor (*TSHR*) activation by thyroid stimulating
69 antibodies (TSAB) mimics the action of TSH producing hyperthyroidism (1,2). The
70 *TSHR* is also detected and increased in OAT in GO, and is an essential cellular target
71 for both GO and Graves' disease (3-6). Previous GO studies have examined the cross-
72 talk of signalling pathways via two cell-surface receptors, *TSHR* and insulin like growth
73 factor 1 receptor (*IGF1R*), focusing on disease targeted preadipocytes/fibroblasts (PFs)
74 embedded in OAT (7,8).

75 OAT-PFs are mesenchymal stem cell (MSC) with multi-differentiation potentials, as
76 has been described by ourselves and others (9-11). In GO patients, the excessive
77 adipogenesis via lineage-specific differentiation of PFs in OAT occurs rapidly (1,12). By
78 contrast, WAT (white adipose tissues) from the same individual typically shrinks in
79 Graves' disease due to hyperthyroidism (2). Previously we described a cell specific
80 signalling network (*PI3K/Akt/mTORC1/FOXOs*) in PFs from human OAT not present in
81 WAT (13,14). The identified pathways interact with *TSHR/IGF1R* signalling to play
82 essential roles in the depot-specific OAT expansion in GO (2). Our recent work has
83 demonstrated that OAT is a distinctive metabolic-quiescent fat depot which neither
84 stores additional triglyceride in obesity (15) nor burns fatty acid (FA) (11), in contrast
85 to WAT and BAT (brown adipose tissue)/BRITE (BRown in whITE), respectively. OAT
86 also displays a unique FA-uptake-driven adipogenesis mechanism, which occurs in
87 addition to the hyperplastic expansion (increased adipocyte number) of PFs in OAT

88 in GO (11). In particular, lower lipolytic activity with similar (low) FA-synthesis
89 accompanied by increased expression of a depot-specific FA-transporter (*SLC27A6*)
90 were observed in OAT from healthy individuals (OAT-H) and GO patients (OAT-GO)
91 compared with WAT (11).

92 Involvement of mitochondrial dysfunction in the orbital fat expansion in GO is
93 suggested by increased expression of the uncoupling protein *UCP1* in OAT from GO.
94 This has been observed both in human models - ex-vivo analysis of OAT-GO (11),
95 GO-targeted PFs by TSHR activation (16,17) - and a mouse model of TSHR-induced
96 GO (18). *UCP1* expression in mitochondria is a known feature of BAT, which dissipates
97 energy as heat by uncoupling mitochondrial oxidative-phosphorylation (OXPHOS) from
98 ATP production and also plays an important role in mitochondrial function (19).
99 Hyperplastic expansion of adipocytes in human BAT caused by mitochondrial
100 dysfunction via mutation of *MFN2* has recently been reported (20). Substantially
101 increased expression of *MFN2* has also been observed in OAT from GO patients
102 compared with OAT-H in our recent study (11). Overexpression of adiponectin which is
103 also a mitochondrial function modulator has been shown to induce expansion of both
104 OAT and BAT in a mouse model (21,22).

105 These factors, together with the identified specific molecular signatures of OAT (e.g.
106 *Sirtuin/Wnt/Ca+* signaling pathways) from our recent study suggest a role for
107 mitochondria in the development of GO (11). Apart from being a cellular 'power-
108 house', mitochondrial-OXPHOS and its bio-substrates, e.g. ATP/ADP, GTP/GDP,
109 play fundamental roles in the regulation of cell metabolism through interacting with
110 complex molecular cascades, e.g. cell proliferation, differentiation (23,24).

111 Our current study investigated the hypothesis that dysfunction of mitochondria plays a
112 role in the FA-uptake-driven adipogenesis in OAT expansion in GO. Our investigation

113 demonstrated mitochondrial dysfunction in PFs from OAT-GO, which linked with the
114 confirmed FA-uptake-driven adipogenesis from in vitro adipogenesis analysis.

115 **Materials and Methods**

116 All reagents were obtained from Sigma-Aldrich (U.K.) and tissue culture components
117 from Cambrex (U.K.) unless otherwise stated.

118 **Adipose Tissue Collection & Preparation**

119 Adipose Tissue was collected with informed written consent and local research
120 ethics committee approval. WAT (subcutaneous) was from 10 patients undergoing
121 elective open abdominal or breast surgery for non-metabolic conditions. OAT from
122 GO patients (n=13) were from 10 inactive GO patients with a CAS (clinical activity
123 score) <2 , 3 active GO patients with CAS ≥ 3 undergoing 2-wall or 3-wall orbital
124 decompression surgery. Most of the GO patients had carbimazole treatment, RAI
125 and/or thyroidectomy in the past; two GO patients received no anti-thyroid treatment
126 and two were receiving carbimazole treatment while the OAT samples were obtained.
127 OATs from non-GO patients (n=11) who were free of thyroid or other inflammatory
128 eye disease and were undergoing augmented upper eyelid blepharoplasty surgery.
129 OAT-PFs were obtained from adipose tissue explants and WAT-PFs were obtained
130 by collagenase digest, both as previously described (17). Cells were used at low
131 passage number (<5), hence not all samples were analyzed in all experiments.

132 **Preadipocytes/fibroblasts culture and in vitro adipogenesis**

133 Preadipocytes/fibroblasts (PFs) were cultured in DMEM/F12 10% FCS (complete
134 medium, CM). Adipogenesis was induced in confluent cells by replacing with ADM
135 (adipogenic medium) containing 10% FCS, biotin (33 μ M), panthothenate (17 μ M), T3
136 (1nM), dexamethasone (100nM), thiazolidinedione (1 μ M) and insulin (500nM) for 17

137 days. Adipogenesis was assessed by microscopic examination to detect the
138 characteristic morphological changes (cell rounding, accumulation of lipid droplets),
139 acquisition of lipid filled droplets (oil-red-O staining) and transcript measurement of
140 terminal adipogenic marker (lipoprotein lipase) by QPCR as described previously
141 (17).

142 For experiments using exogenous fatty acid (FA), a fatty acid mixture (200 μ M)
143 comprising oleate:palmitate:linoleate (45:30:25%) bound to BSA was added to CM
144 throughout the whole time culture as previous described (25), with/without ADP
145 (Adenosine 5'-diphosphate sodium salt), GDP (Guanosine 5'diphosphate sodium
146 salt), oligomycin (mitochondrial inhibitor) or AICAR (adenosine analog) for 17-19
147 days. Adipogenesis was analysed by foci of differentiation (groups of cells with lipid
148 droplets), which were counted in ten different fields for each experimental condition
149 as described before (16).

150 **Triglyceride (TAG) extraction from differentiated PFs and iLAB analysis**

151 Confluent PFs were cultured in ADM with/without the supplementation of FA,
152 oleate:palmitate:linoleate (45:30:25%), for 10 days in 24-well plate. Cellular TAG
153 from the differentiated PFs were analysed as previously described (25). In brief, cell
154 lysates were obtained using lysis buffer (1% IGEPAL CA-630, 150 mM NaCl and
155 50 mM Tris-HCl (pH8.0)) and sonication. Some of the lysates were used for protein
156 quantification using BCA assay (Bio-Rad, DC protein assay kit). The lysates used for
157 TAG analysis were heated at 95°C for 30 minutes and centrifuged at 12,000 g for 10
158 minutes after cooling. Cellular TAG concentration was measured using enzymatic
159 assay (TAG assay, Instrumentation Laboratory UK) with glycerol standards, and run
160 on an ILAB 650 clinical analyzer (Instrumentation Laboratory UK). The normalised

161 TAG content per unit protein were obtained using the following calculation: TAG (μM)
162 \div protein (mg / ml).

163 **NAD(P)H and ATP measurement**

164 Confluent PFs were cultured in CM or ADM for 4 days and changed to CM before
165 the following experiments. Cell number was counted using Cellometer from
166 Nexcelom. MTS assay was performed (indicating the production of NAD(P)H (26))
167 using CellTiter 96(R) AQueous One Solution Assay from Promega according to the
168 manufacturer's instructions, 490nm absorbance was measured after 2h incubation
169 and normalised by cell number. Cells were harvested after culture in CM or ADM for
170 4 days, total cellular ATP (μM) was measured using standard ATP dilutions by
171 luminescent ATP detection assay kit from Abcam according to the manufacturer's
172 instructions. Each condition had four repeats for the above experiments.

173 **Mitochondria number analysis by relative QPCR**

174 DNA was extracted from confluent PFs from OAT-H, OAT-GO or WAT using
175 standard protocol and QPCR was conducted using SYBR Green incorporation
176 measured on a Stratagene MX 3000 as previous described (16). Comparative QPCR
177 was measured and expressed relative to a reference DNA *RPL13A* for mitochondria
178 DNA cytochrome b (*Cytob*) detection to determine relative mitochondria number
179 using the primers as follows: *RPL13A*, forward 5'- CTCAAGGTCGTGCGTCTG-3'
180 and reverse 5'-TGGCTTTCTCTTTCCTCTTCT-3'; *Cytob*, forward 5'-
181 GCGTCCTTGCCCTATTACTATC-3' and reverse 5'-
182 CTTACTGGTTGTCCTCCGATTC-3' as described before (27).

183 **Flow cytometry analysis**

184 Confluent PFs were cultured in CM (as day 0), and changed to CM or ADM for 4
185 days. Cells number were counted by Cellometer from Nexcelom, and followed the

186 procedures using flow cytometry (FACS) (BD FACSCanto II systems) with
187 FACSDiva 6.0 software from Becton Dickinson and Co. (Mountain View, CA) as
188 described before (28). In brief, cells were fixed with 100% methanol and
189 permeabilized with 0.1% PBS-Tween20 for 20 min. Cells were then incubated with
190 10% normal goat serum/0.3M glycine to block non-specific protein-protein
191 interactions followed by primary antibody of mitochondrial-cytochrome-oxidase
192 (MtCO₂) (ab3298 from Abcam (RRID:[AB_303683](#)), 1µg/1x10⁶ cells) or isotype
193 control antibody (mouse IgG) for 30 min. The secondary antibody used Alexa Fluor
194 488 anti-mouse IgG (1:500 dilution) for 30 min, and then fluorescence emissions
195 were collected for 10,000 cells by FACS analysis. Flow cytometric intensity of positive
196 MtCO₂ staining referenced to negative control was analysed using FlowJo software
197 version 10.0.5 (Tree Star, Inc., Ashland, OR).

198 **Mitochondrial functional assays by Seahorse XFe96 Analyzer**

199 Basal Assay medium and XFe96 consumables were purchased from Agilent
200 Technologies; Draq5 was purchased from Abcam for DNA staining. Oxygen
201 consumption rate (OCR) and extracellular acidification rate (ECAR) were measured
202 with the Seahorse XFe96 (Agilent Technologies) using Mito Stress kit according to
203 the manufacturer instructions as described before (29). Briefly, cells were seeded at
204 1×10^4 per well in Seahorse plate. 48 h post-seeding, confluent PFs were then
205 transferred into non-buffered Seahorse Assay medium containing 17.5 mM glucose,
206 1.5 mM sodium pyruvate and 2.5mM of L-Glutamine with 3% FCS. Basal cellular
207 respiration rate was first measured followed by oligomycin A injection (1 µM) to
208 inhibit ATP synthase. Maximal respiration capacity was determined in 3.5 µM FCCP.
209 Finally, non-mitochondrial respiration rate was determined using a combination of
210 rotenone (0.5 µM) and antimycin A (1 µM). Measurements were performed with 3

211 cycles including 4 min of medium mixing followed by 3 min of measurements. For
212 data normalization, cells were fixed for 20 min with 4% PFA. Cell nuclei were then
213 stained for 1 h with Draq5 (ab108410 from Abcam) 1/10000 diluted in PBS +0.1%
214 Tween-20. Stained cells were then detected with Odyssey Scanner (Li-Cor).
215 Fluorescent intensity per well was used to normalize respiration values per well. The
216 average of the non-mitochondrial respiration measures was then subtracted from
217 each corresponding condition/time-point per well; all similar condition measurements
218 per well were then averaged. On day 0 (48 h post-seeding), PFs from OAT, OAT-GO
219 and WAT (n=4 for each) in CM were measured with 5 repeats of each condition from
220 one 96-well plate; day 4 (or day 7) PFs (n=4) in CM and ADM were measured from
221 two 96-well plates. PFs from WAT (n=4), OAT (n=8) and OAT-GO (n=9) were used,
222 and two sets of independent experiments were performed for OAT (n=4 & 4) and
223 OAT-GO (n=4 & 5), respectively.

224 Cellular ATP production rates by mitochondria-OXPHOS and glycolysis were
225 calculated using obtained OCR and ECAR from Seahorse analysis, taking into
226 account of the acidification rates due to mitochondrial CO₂ production as described
227 previously (30,31).

228 **Statistics**

229 Results were analysed using Prism 5 (version 5.02), data normality was initially
230 analysed using the Kolmogorov-Smirnov test. To compare groups we used the T-test
231 for variables normally distributed and the Mann Whitney test was used for non-
232 normal distributed data. Differences between groups were analysed using one way
233 ANOVA. We applied Dunnett's multiple comparison post hoc test for multiple
234 comparisons when identifying statistically significant differences. In all cases p<0.05
235 was considered significant. Data are presented as mean±SEM.

236 For correlation analysis, the percentage increased FA-uptake adipogenesis by GDP
237 and measured OXPHOS-ATP/Glycolysis-ATP of the cells were analysed for
238 Normality using the Kolmogorov-Smirnov test. All the data were normally distributed
239 and correlation was analysed using Pearson's correlation.

240 **Result**

241 **Depot-specific mitochondrial-ATP production by PFs from OAT in early** 242 **adipogenesis**

243 We induced in vitro adipogenesis using ADM (adipogenic medium) with a mixed
244 hormonal-cocktail in normal culture medium (CM) on human primary PFs for 17 days.
245 The differentiated cells displayed cell rounding and accumulation of lipid droplets
246 (triglyceride formation) with positive Oil-Red-O staining and the induction of LPL
247 expression (marker of late adipogenesis) as previously reported (17).

248 We compared the early stages of adipogenesis, prior to the formation of adipocytes,
249 in confluent PFs from WAT, OAT-H or OAT-GO cultured in CM (non-differentiating)
250 or ADM (differentiating) conditions. We analysed mitochondria numbers by relative
251 QPCR of mitochondrial DNA, cytochrome b (*Cytob*) to a reference genomic DNA,
252 *RPL13A*. Figure 1A demonstrates lower ($35.3\pm 3.2\%$) mitochondrial number in OAT-
253 H or OAT-GO, either in CM or ADM-condition on day 4 compared to PFs from WAT.
254 Interestingly, mitochondrial-cytochrome-oxidase (MtCO₂) levels were significantly
255 higher ($52.8\pm 2.5\%$) in OAT-H and OAT-GO compared with PFs from WAT using flow
256 cytometry analysis (Figure 1B). MtCO₂ is a necessary component of the respiratory
257 chain of mitochondria for OXPHOS-ATP production (32).

258 Furthermore, we detected a substantially higher production of NAD(P)H (1.23 ± 0.06
259 fold-increase) indicating increased mitochondrial-OXPHOS activity (MTS assay/ 10^6
260 cells (26), Figure 1C) and increased total cellular-ATP (1.97 ± 0.03 fold-increase) of

261 differentiating-PFs from OAT-H cultured in ADM compared with CM-condition (non-
262 differentiating-PFs) (Figure 1D), which was not observed in WAT. The increased
263 production of NAD(P)H and total cellular-ATP were also observed in differentiating-
264 PFs from OAT-GO compared with non-differentiating-PFs (supplemental Figure S1)
265 (33), however significantly lower fold-increases were observed when compared with
266 OAT-H. There was no significant difference in cell number between PFs cultured in
267 CM and ADM on day 4 (supplemental Figure S2) (33).

268 **Dysfunction of live ATP-production via mitochondrial-OXPHOS in PFs from**
269 **OAT-GO.**

270 We next explored live ATP-production rates by mitochondrial-OXPHOS or cellular-
271 Glycolysis according to oxygen consumption rate (OCR) and extracellular acidification
272 rate (ECAR) measured by 96-well Seahorse analyser, as previously described (30,31).
273 Analyses were performed at basal-day-0 in CM-condition or on day 4 and 7 in CM &
274 ADM conditions at early adipogenesis, i.e. before observation of any differentiated
275 adipocytes. All the obtained data were referenced to cell DNA staining at each time
276 point and condition.

277 During early adipogenesis, an increased OXPHOS-ATP level of differentiating-PFs was
278 observed compared to non-differentiating-PFs on day 4 from WAT, OAT-H and OAT-
279 GO, and further increased on day 7 from WAT and OAT-H, but not OAT-GO (Figure
280 2A). We then looked detail into changes in OXPHOS & Glycolysis-ATP levels in PFs
281 from OAT-H and OAT-GO separately.

282 From OAT-H, non-differentiating-PFs had substantially decreased Glycolysis-ATP level
283 on day 4 compared with day 0 and day 7 (Figure 2B), accompanied by unchanged
284 levels of OXHPOS-ATP (Figure 2A). Consequently, there was a higher OXPHOS-
285 ATP/Glycolysis-ATP ratio on day 4 compared with day 0 and day 7 in non-

286 differentiating-PFs (Figure 2C). Interestingly, in differentiating-PFs (through
287 adipogenesis), significantly higher levels ($28.8\pm 11.7\%$; $46.9\pm 15.3\%$) of OXPHOS-ATP
288 production (Figure 2A) and OXPHOS-ATP/Glycolysis-ATP ratio ($56\pm 10.8\%$; $68.9\pm 23\%$)
289 (Figure 2C) with unchanged Glycolysis-ATP (Figure 2B) were observed on day 4 or 7
290 when compared with day 0, respectively.

291 From OAT-GO, non-differentiating-PFs showed a trend to increased Glycolysis-ATP
292 levels (Figure 2B) contrasting to a sharp and consistent drop ($39.7\pm 7.5\%$; $40.8\pm 10.7\%$)
293 of OXPHOS-ATP levels (Figure 2A) on day 4 or day 7 when compared with day 0,
294 respectively. Consequently, there was a trend to decreased ratio of OXPHOS-
295 ATP/Glycolysis-ATP on day 4 & 7 compared with day 0 in non-differentiating-PFs
296 (Figure 2C). Through adipogenesis (Differentiating PFs) in OAT-GO, unchanged levels
297 of OXPHOS-ATP/Glycolysis-ATP & its ratio on day 0, 4 & 7 were observed contrasting
298 with our findings in OAT-H (Figure 2).

299 In summary, there is a reduction in ATP production by OXPHOS in OAT-GO non-
300 differentiating-PFs, which recovers somewhat in differentiating-PFs. By contrast this
301 reduction of OXPHOS-ATP production is absent in healthy OAT non-differentiating-PFs
302 and levels increase as PFs differentiate.

303 **FA-uptake-driven adipogenesis and the link with mitochondrial in OAT**

304 Cells with lipid droplets and positive Oil-Red-O staining were identified as
305 differentiated adipocytes, and a foci count was performed as described before (16).

306 1) In order to investigate FA-uptake-driven adipogenesis in vitro, we cultured
307 confluent PFs from OAT-H or OAT-GO with extra free FA using a mixture of three
308 major triglyceride FA, oleate:palmitate:linoleate (45:30:25%) as described in the
309 investigation of FA-uptake in PFs from WAT (25). As expected, differentiated
310 adipocytes were apparent in ADM after 17 days full-differentiation protocol with

311 adipogenesis hormonal-cocktail, while no adipogenesis was observed in CM-
312 condition (Figure 3A). Addition of the FA-supplement to ADM, led to the
313 differentiated adipocytes as early as day 10, which had about 3-fold higher cellular
314 triglyceride per protein unit compared with adipocytes in ADM alone which were still
315 at the early-middle stage of adipogenesis (supplemental Figure S3) (33).

316 The effect of FA and the role of mitochondria in OAT-adipogenesis were further
317 investigated by culturing confluent PFs from OAT-H and OAT-GO with FA-
318 supplement in CM without adipogenesis hormonal-cocktail and with addition of
319 different concentrations of mitochondrial bio-substrates, ADP (0.05, 0.1, 0.2mM) and
320 GDP (0.1, 0.5, 1mM) for 17 days. To our surprise, we observed differentiated
321 adipocytes by feeding FA only in CM for 17 days to both OAT-H and OAT-GO PFs
322 (OAT-H n=1 and OAT-GO n=4, Figure 3A). This type of FA-uptake-driven
323 adipogenesis was substantially enhanced by 1mM GDP in CM reaching to 7-18% of
324 ADM-hormonal-cocktail induced adipogenesis (Figure 3A). In contrast, no significant
325 changes in the FA-uptake-driven adipogenesis were observed by ADP-supplement
326 in PFs from both OAT-H and OAT-GO (Figure 3A).

327 2) We then analysed the effect of the optimal 1mM concentration of GDP and FA
328 supplement in FA-uptake-driven adipogenesis in additional samples. We also tested
329 the effect of ADP and chemical adenosine-analogue/inhibitor of mitochondria
330 (AICAR/oligomycin, respectively). The results are summarised in Figure 3B; we
331 observed differentiated adipocytes from OAT-H (n=1 out of 4) and OAT-GO PFs
332 (n=7 out of 8) by feeding FA in CM. Furthermore, substantially increased FA-uptake-
333 driven adipogenesis in PFs from all OAT-H and OAT-GO was observed upon
334 addition of 1mM GDP (Figure 3B). In contrast, addition of the adenosine-analogue
335 AICAR or ADP had no effect on FA-uptake-driven adipogenesis, and the use of

336 different concentrations of mitochondrial inhibitor, oligomycin, abolished the induced
337 FA-uptake-driven adipogenesis of all PFs from OAT-H and OAT-GO (Figure 3B).

338 3) We have replicated the effects of GDP, ADP with FA-supplement in CM on OAT-
339 PFs in further experiments (supplemental Figure S4) (33).

340 In summary, FA-triggered adipogenesis was observed in OAT-H PFs from 4 out of 5
341 individuals and 8 out of 10 OAT-GO PFs samples from GO patients. In all cases, this
342 was substantially enhanced by addition of GDP-supplement.

343 **Correlation of FA-uptake-driven adipogenesis by GDP with increased** 344 **OXPHOS/Glycolysis capacity of PFs from OAT-GO**

345 In 6 OAT-GO samples we had data from both seahorse analysis (Figure 2) and FA-
346 induced adipogenesis (Figure 3). The further enhanced FA-uptake-driven
347 adipogenesis by GDP treatment (percentage increase versus FA-feeding only)
348 significantly and positively correlated with the measured OXPHOS-ATP levels ($r =$
349 0.946 , $p = 0.004$) (Figure 4A), no significant correlation with the levels of Glycolysis-
350 ATP ($r = -0.75$, $p = 0.086$) (Figure 4B) and significantly and positively correlated
351 OXPHOS-ATP/Glycolysis-ATP ratio ($r = 0.840$, $p = 0.04$) (Figure 4C) in non-
352 differentiating-PFs at basal-day-0. The further enhanced FA-uptake-driven
353 adipogenesis by GDP treatment had no significant correlation with the measured
354 levels of OXPHOS-ATP ($r = 0.096$, $p = 0.857$) (figure 4D), but significantly correlated
355 negatively with the levels of Glycolysis-ATP ($r = -0.966$, $p = 0.002$) (Figure 4E) and
356 positively with OXPHOS-ATP/Glycolysis-ATP ratio ($r = 0.857$, $p = 0.03$) (Figure 4F)
357 in differentiating-PFs on day 4.

358 **Discussion**

359 Our study demonstrated increased cellular ATP-production via mitochondrial-
360 OXPHOS during adipogenesis of PFs from OAT but not WAT. Mitochondrial

361 dysfunction was observed with disrupted levels of OXPHOS-ATP/Glycolysis-ATP
362 and its ratio in PFs from OAT-GO compared with OAT-H. Furthermore, FA-
363 supplementation was able to trigger in vitro adipogenesis in PFs from both OAT-H
364 and OAT-GO, which was substantially enhanced by addition of GDP and diminished
365 by mitochondrial inhibitor. The enhanced FA-uptake-driven adipogenesis by GDP
366 was significantly and positively correlated with ratios of OXPHOS-ATP/Glycolysis-
367 ATP at basal non-differentiating-PFs or early-adipogenesis of differentiating-PFs
368 from OAT-GO. Taken together, these observations suggested that an essential role
369 of mitochondrial OXPHOS-ATP production and its bio-substrate GDP in the depot-
370 specific FA-uptake-driven adipogenesis of OAT-H and exacerbated in GO by
371 mitochondrial dysfunction.

372 Our investigation employed a well-established cell model, heterogeneous population
373 (PFs) derived from the entire stromal-vascular fraction of human orbital (OAT) and
374 WAT (10,34). The key findings of this study were confirmed by using more than one
375 independent technique and the observations from our recent ex vivo OAT analysis
376 (11) as discussed below. The low availability of human orbital PFs precludes the use
377 of a model comprising a homogeneous cell type, although this would be preferable.

378 We and others have described the multi-differentiation MSC potential of adipose
379 tissue derived-PFs (9-11,35). Classically, in vitro adipogenesis is triggered by ADM
380 with a mixed hormonal cocktail to activate key transcriptional factors, e.g. *PPAR*,
381 *CEBP*, etc. (36,37), as routinely used in our lab (17). Our current study demonstrated
382 that FA alone were able to induce in vitro adipogenesis in PFs from OAT-H and
383 OAT-GO. This confirms the depot-specific FA-uptake-driven adipogenesis in OAT,
384 which was suggested by ex vivo analysis of human adipose tissue from our recent
385 study (11). Furthermore, we previously demonstrated an abundant expression of the

386 FA-transporter, *SLC27A6*, with limited FA-synthesis/lipolysis in OAT in GO
387 supporting the excessive FA-uptake-driven adipogenesis in GO (11). Thyroid
388 hormone, *PPAR γ* ligand and cytokines (e.g. TNF or IL6) play important roles in the
389 pathogenesis of GO reviewed in (2), which may also contribute to OAT adipogenesis
390 through its role in the regulation of lipid metabolism via FA transporter (38,39).
391 Further investigation is needed to dissect the important role of the FA transporter
392 system, in the specific context of the MSC phenotype of OAT-PFs, in the FA-uptake-
393 driven adipogenesis in GO (9-11).

394 Our study revealed that inhibition of mitochondrial-OXPHOS by oligomycin abolished
395 the FA-uptake-driven adipogenesis of OAT, which supports the important function of
396 mitochondria in adipogenesis as reported in other fat depots (20,40). However
397 AICAR (adenosine-analog) or ADP, the activator of AMP kinase, had no effect on the
398 FA-uptake-driven adipogenesis of OAT thereby eliminating the AMPK-pathway,
399 which plays important roles in other fat depots (41). By contrast, our current study
400 demonstrated that the depot-specific FA-uptake-driven adipogenesis was
401 substantially enhanced by supplementing GDP in PFs from both OAT-H and OAT-
402 GO. The important role of GDP in mitochondrial function and cellular metabolism has
403 been reported in OXPHOS-ATP production (42) or via GTP/GDP exchange (43). Our
404 study suggested that the reduced mitochondrial-OXPHOS results in more available
405 GDP to form a feedback loop to regulate the FA-uptake-driven adipogenesis in OAT.
406 The current study demonstrated that mitochondrial function in OAT-PFs during
407 adipogenesis is linked with an inducible total cellular-ATP production via
408 mitochondrial-OXPHOS. It contrasts with WAT-PFs, or other human stem cells, that
409 have unchanged or even decreased levels of total cellular ATP, apart from increased
410 OXPHOS-ATP through adipogenesis as observed in this study and by others (40,44).

411 Our investigation observed a depot-specific higher level of mitochondrial
412 cytochrome-C oxidase (32) with low mitochondria number in PFs from OAT
413 compared with WAT suggesting increased mitochondrial activity rather than numbers.
414 Furthermore, in OAT-H, early adipogenesis (days 0-4-7) is accompanied by a U-
415 shaped distribution (high, low, high) of Glycolysis-ATP and unchanged OXPHOS-ATP
416 levels. In contrast, levels of OXPHOS-ATP fell sharply with compensated high level of
417 Glycolysis-ATP from non-differentiating-PFs from OAT-GO indicating sustained
418 mitochondrial dysfunction of PFs in GO.

419 Our recent study highlighted the key role of enhanced proliferation of PFs synergized
420 with FA-uptake-driven adipogenesis in the hyperplastic expansion of OAT in GO (11).
421 Studies have shown that the Warburg phenotype of proliferating cells is important to
422 have enhanced Glycolysis with suppressed mitochondrial-OXPHOS, however non-
423 proliferating cells display higher OXPHOS-ATP production with inhibited Glycolysis
424 (45,46). Our data clearly demonstrate OXPHOS-ATP levels falling sharply with
425 compensated high-level of Glycolysis-ATP production in non-differentiating-PFs from
426 OAT-GO, which may trigger the proliferation of PFs in GO (45,46). Once proliferation
427 is induced, we see higher levels of OXPHOS-ATP and low levels of Glycolysis-ATP
428 through adipogenesis in differentiating-PFs from OAT-H reported here, which may in
429 turn lead to inhibition of proliferation and drive PFs to differentiate (45,46). This
430 study provides further support in the positive correlation noted between FA-uptake-
431 driven adipogenesis by GDP and the ratios of OXPHOS-ATP/Glycolysis-ATP
432 through adipogenesis of PFs from OAT-GO. We hypothesise that the levels of
433 mitochondrial OXPHOS-ATP and metabolites (GDP) play central roles in triggering
434 PFs proliferation and excessive FA-uptake-driven adipogenesis of OAT in GO. The
435 uncorrected dysfunction of mitochondria in PFs from OAT-GO leads to maintained

436 levels of low-OXPHOS/high-Glycolysis causing excessive proliferation, and more
437 available GDP promoting FA-uptake-driven adipogenesis in OAT expansion in GO.
438 The OAT depot-specific cell signalling cascades (13,14) play a central role in the
439 pathogenesis of OAT expansion in GO through the interplay of *TSHR/PKA*,
440 *IGF1R/PI3K/Akt*, *mTORC1*, and the downstream nucleus factor, *FOXOs*, (7,47-50),
441 which are also important in mitochondrial function (43). More work is needed to
442 clarify how *TSHR/IGF1R* interfere with mitochondria via GDP and OXPHOS-ATP
443 production in triggering proliferation and FA-uptake-driven adipogenesis of OAT, e.g.
444 via pathological activation of *UCP-1* (11), or interference with the adenylate cyclase
445 system of GTP/GDP exchange to allow the activation of GO targeted *TSHR/IGF1R*
446 (51,52). However, we hypothesise that the resultant mitochondrial dysfunction in
447 association with FA-uptake mechanism drives OAT expansion in GO (summarized in
448 Figure 5).

449 **Ethical approval**

450 Human Adipose Tissue was collected with informed written consent and approved by
451 the South East Wales Research Ethics Committee (30 May 2006) with registry
452 number (06/WSE03/37).

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457 this study.

458 **Author contributions**

459 LZ designed the study, performed experiments, prepared the figures, and drafted the
460 manuscript; PR, SM and DMT supported LZ for Seahorse technique and analysis;
461 SM supported LZ for OXPHOS-ATP/Glycolysis-ATP calculation using OCR/ECAR;
462 MSD, DAR, ASH, DM supported sample collections with ethical approval and
463 obtained consent forms from patients; ART supported LZ for relative QPCR analysis
464 of mitochondria number; LZ, ML, DMT & CMD interpreted data and finalised the
465 manuscript; all authors revised the manuscript and approved the submitted version.

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651 **Supplementary data** (4 supplemental figures) 10.6084/m9.figshare.14578311
652 Figure S1 Fold-increase on day 4 ADM-adipogenesis compared with CM condition.
653 Figure S2 Cell number count on day 4 adipogenesis.
654 Figure S3 Increased adipogenesis by FAs supplement in ADM.
655 Figure S4 Induced adipogenesis by FAs and GDP treatment in CM.

656 **Figure 1. Mitochondria analysis & cellular ATP production of OAT-PFs in ADM-**
657 **adipogenesis compared with WAT.** Confluent PFs from OAT-H, OAT-GO or WAT
658 were cultured in complete medium (CM) or ADM (adipogenic medium) for 4 days. **A)**
659 DNA extracted from OAT (n=3) & OAT-GO (n=3) or WAT-PFs (n=4) on day-4, relative
660 QPCR of cytochrome b (*Cytob*, mitochondrial DNA) to *RPL13A* (reference genomic
661 DNA) was performed. **B)** MtCO₂ antibody (mitochondria cytochrome oxidase) was
662 analysed by FACS from confluent OAT (n=4), OAT-GO (n=4) & WAT-PFs (n=7) at day-
663 0 in CM or day-4 in CM & ADM, the percentage of flow cytometric (FL) intensity, positive
664 MtCO₂ staining, was shown referenced to negative control. **C)** MTS assay (indicating
665 the production of NAD(P)H and mitochondria-OXPHOS activity [26]) was performed on
666 PFs from OAT-H (n=3) & WAT-PFs (n=4) on day 4 in CM & ADM, 490nm absorbance
667 normalized by cell number. **D)** Total level of cellular ATP (Luminescent ATP detection
668 assay kit) was measured from OAT-H (n=7) & WAT-PFs (n=4) on day 4 in CM & ADM.
669 Histograms = mean±SEM of all samples studied. T-test was used for statistical
670 analysis. ***p≤0.001, ****p<0.0005.

671 **Figure 2. Live ATP production from OXPHOS/Glycolysis in differentiating- or**
672 **non-differentiating PFs from WAT, OAT-H & OAT-GO.** Confluent PFs were cultured
673 in 96-well plates, Oxygen Consumption Rate (OCR) as OXPHOS-ATP and
674 extracellular acidification rate (ECAR) as Glycolysis-ATP were measured using Mito
675 Stress kit from WAT (n=4), OAT (n=8) and OAT-GO (n=9) using Seahorse analyzer.
676 OXPHOS-ATP (**A**), Glycolysis-ATP (**B**) were measured as pmol ATP/min on day (D)0
677 plate in complete culture medium (CM, white square), or after PFs in CM (non-
678 differentiating) or adipogenic medium (ADM, black square) (differentiating-PFs) for 4
679 or 7 days and referenced to DNA staining. **C)** Percentage ratio of OXPHOS vs
680 Glycolysis on D0, 4 & 7 with CM or ADM condition were presented. Basal-day-0

681 levels of ATP production by OXPHOS (4.5 pmol ATP/min), Glycolysis(16.6 pmol
682 ATP/min) and its ratio (31.4%) of PFs from OAT-H were displayed and indicated with
683 dash lines. Histograms = mean±SEM of all samples studied. Data were normally
684 distributed, a one-way ANOVA with Dunnett's multiple comparison test or t-test was
685 used to compared D4 or D7 time points with D0 (^); or t-test was used to compared
686 between CM and ADM on D4 and D7 (*). *p<0.5; **p≤0.01; ***p<0.005; ****p<0.0005.

687 **Figure 3. The link of mitochondrial-OXPHOS in FA-uptake-driven adipogenesis**

688 **of PFs from OAT. A)** Confluent cells from OAT-H (n=1) and OAT-GO (n=4),

689 passage 2-4, were cultured in CM or ADM (black square), or with FA supplement in

690 CM (square with dot) for 17 days with treatment of ADP (0.05mM, 0.1mM or 0.2mM),

691 or GDP (0.1mM, 0.5mM or 1mM); **B)** Confluent cells from OAT-H (n=4, white column)

692 or OAT-GO (n=8, black dot column), passage 2-3 were cultured in CM with FA

693 supplement for 19 days, with/without 10ng/ml oligomycin-A (Olig-10), 100ng/ml

694 oligomycin-A (Olig-100), 0.1mM ADP, 0.1mM AICAR, 1mM GDP. Cells were then

695 fixed and stained using oil-red-O technique. Differentiated adipocytes (with lipid

696 droplets) were observed and counted in ten different fields. Representative photos

697 were shown with arrows indicating differentiation adipocytes (x10 magnification).

698 Histograms = mean±SEM of all samples studied. Normal (t-test) or non-normal

699 (Mann Whitney test) distributed data was analysed accordingly. *p<0.05, ***p<0.005.

700 **Figure 4. Correlations of FA-uptake-driven adipogenesis by GDP treatment**

701 **with levels of OXPHOS/Glycolysis-ATP in PFs from OAT-GO.** The scatterplot

702 showing the relationship between percentage induced FA-uptake-driven

703 adipogenesis by GDP+FA vs FA-only, and levels of **A)** OXPHOS-ATP (dot), **B)**

704 Glycolysis-ATP (triangle) and **C)** the percentage ratio of OXPHOS/Glycolysis (square)

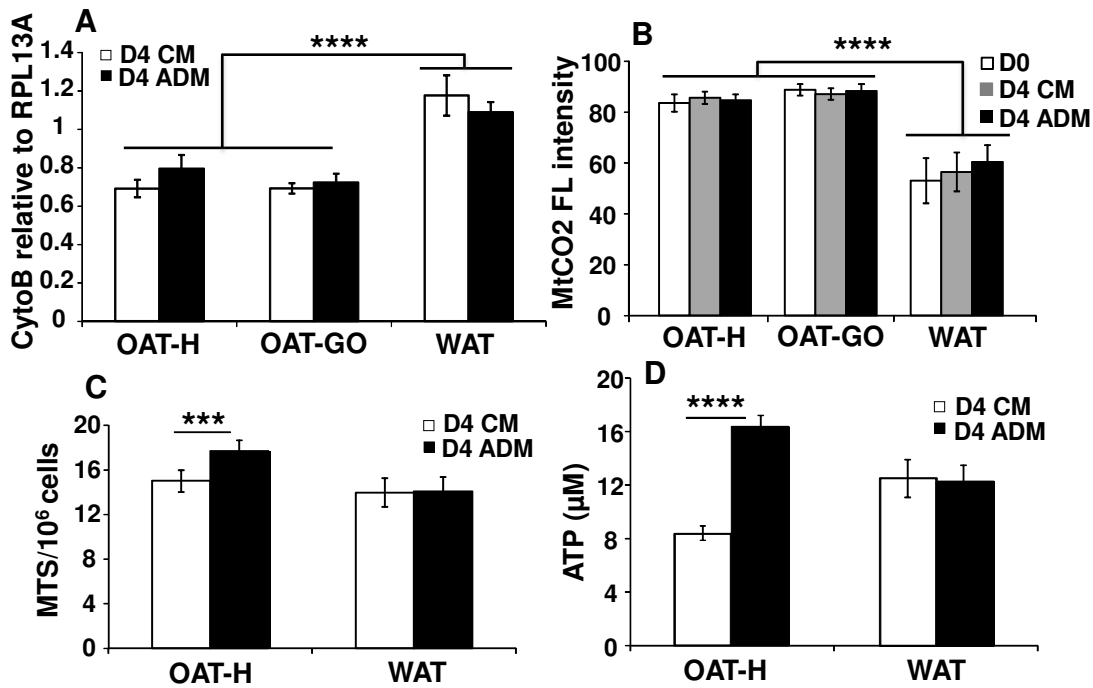
705 in non-differentiating-PFs at basal day 0; or **D)** OXPHOS-ATP levels, **E)** Glycolysis-

706 ATP levels and **F**) the percentage ratio of OXPHOS/Glycolysis of differentiating-PFs
707 on day 4 adipogenesis. Statistically Pearson's correlations for the normally
708 distributed data have shown (significant correlations were underlined).

709 **Figure 5. The essential role of mitochondrial-OXPHOS for FA uptake-driven**
710 **adipogenesis in the hyperplastic OAT expansion.** Our study demonstrated a
711 depot specific cellular ATP production via mitochondrial-OXPHOS through
712 adipogenesis in OAT-PFs. Dysfunction of mitochondria (dys-Mt) with disrupted
713 OXPHOS-ATP production was observed in PFs from OAT-GO due to GO factors, e.g.
714 *TSHR/IGF1R* etc. (11,17,18,21,53-55). **Hypothesis:** Mitochondrial OXPHOS-ATP
715 production through adipogenesis is important in maintaining OAT stability by forming a
716 beneficial relationship with the available GDP in PFs to maintain a healthy (low) level of
717 proliferation/adipogenesis in OAT-H (highlighted in dash blue-square). The pathological
718 fall in OXPHOS-ATP with compensated high-level of Glycolysis-ATP by GO factors
719 triggered the proliferation of PFs in OAT in GO, the kept low-level of mitochondrial-
720 OXPHOS and consequent higher GDP availability drive excessive fatty-acid uptake-
721 driven adipogenesis in OAT in GO (highlighted in red-square). Dashed lines indicate
722 proposed mechanism scheme.

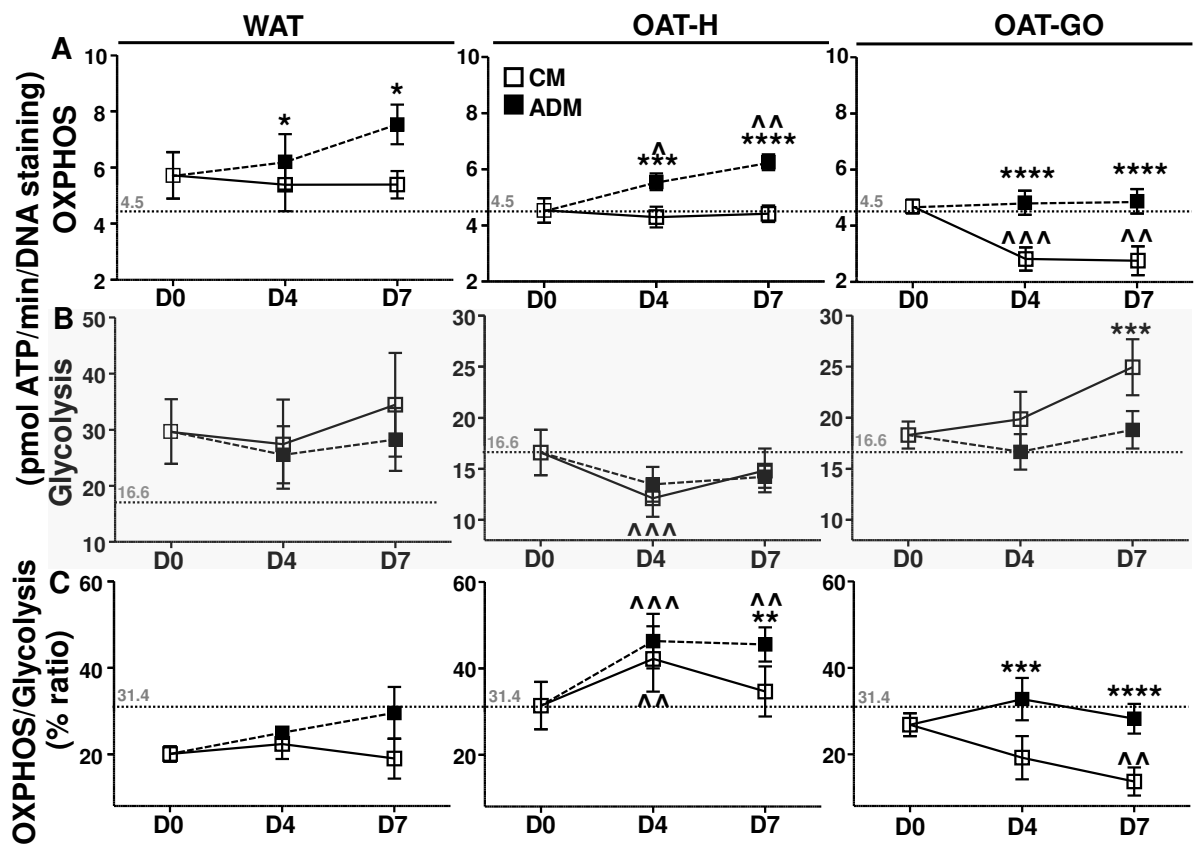
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724 **Figure 1**



725

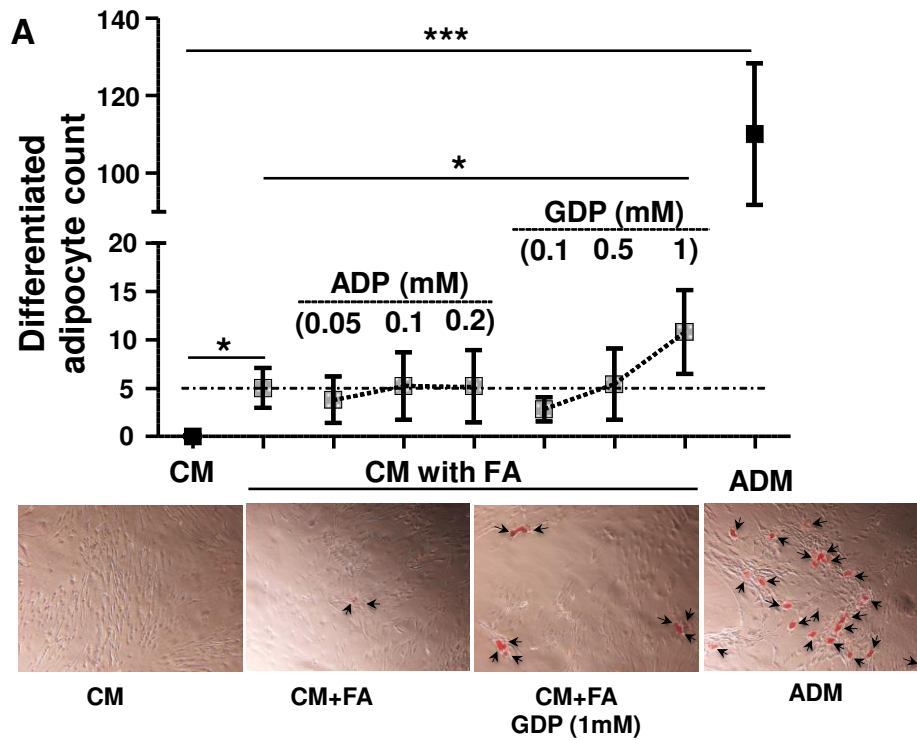
726 **Figure 2**



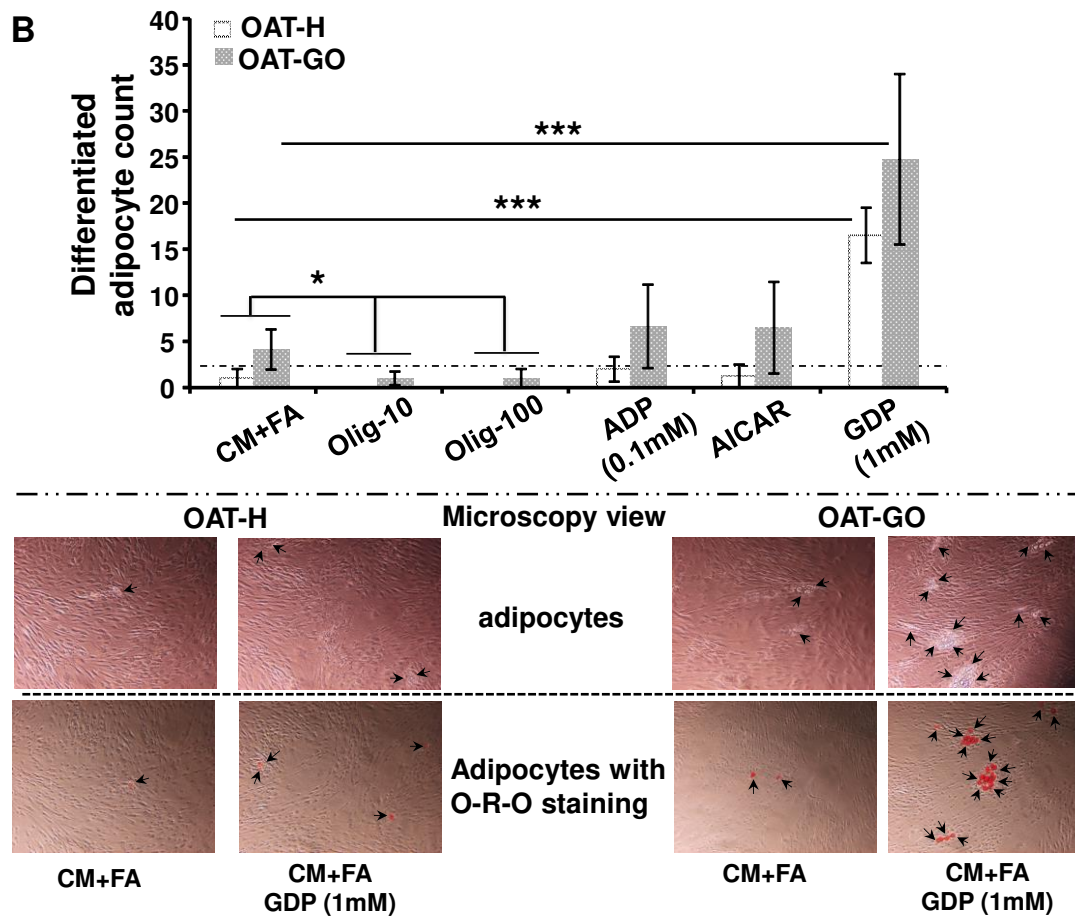
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729 **Figure 3**

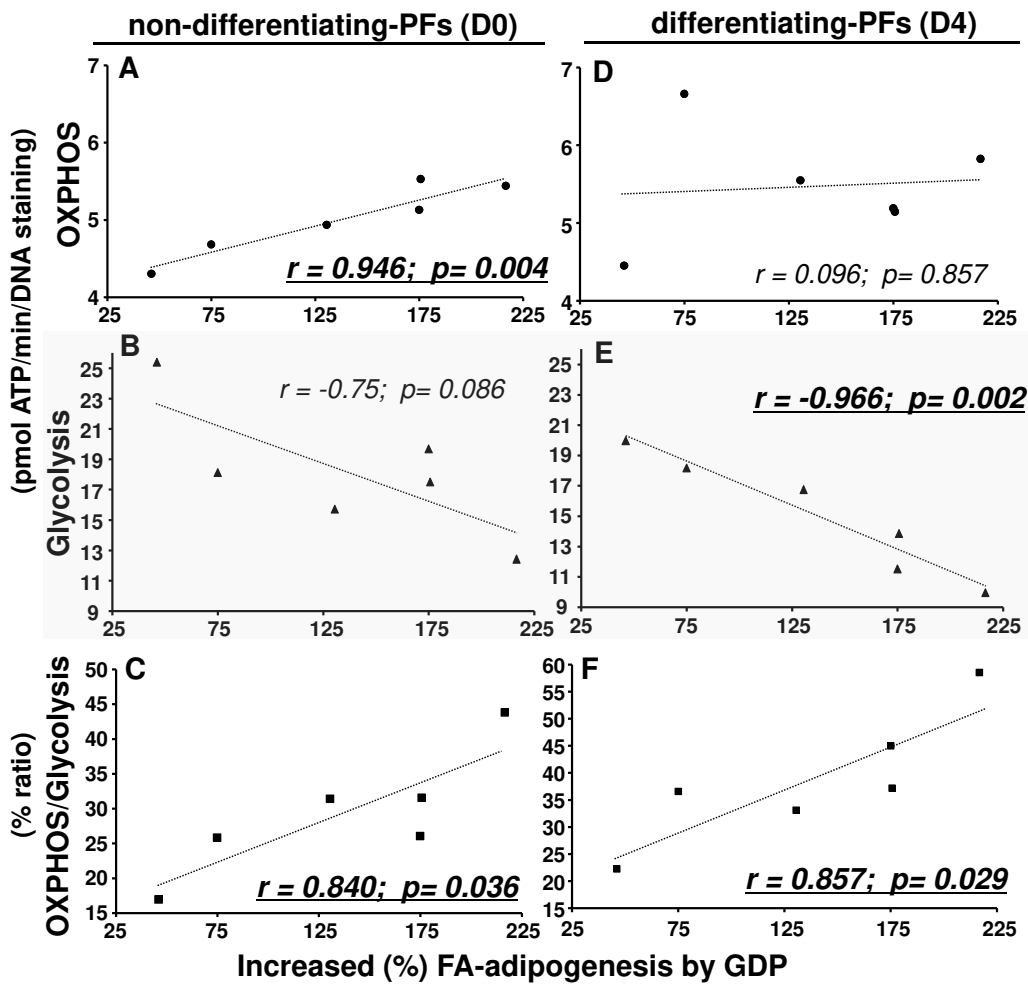


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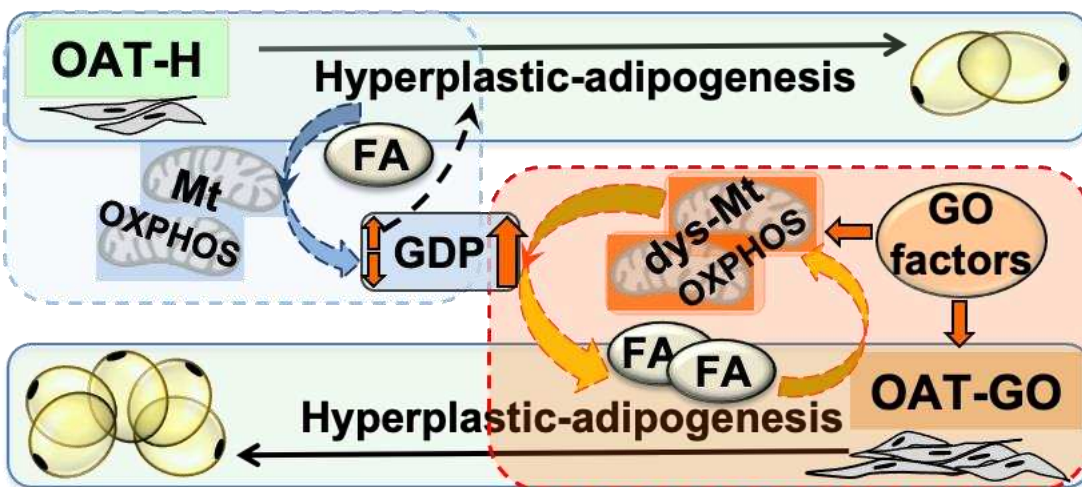
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733 **Figure 4**



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735 **Figure 5**



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