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1 **Integration of metabolomic and transcriptomic profiling to compare**
2 **two protocols of differentiation of human induced pluripotent stem**
3 **cells into hepatocytes**

4
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33 **Abstract:**

34 Human hepatocyte-like cells derived from human induced pluripotent stem cells
35 (hiPSC) may provide an unlimited supply of cells for in vitro liver models. However,
36 hiPSC differentiation remains a major challenge due to immaturity of the hepatocytes
37 obtained and the high cost of differentiation protocols currently proposed. Here, we
38 studied the efficacy of new protocol, with reduction of growth factors, for the
39 generation of hepatocyte-like cells from hiPSC. We performed metabolomic and
40 mRNA analysis by RTqPCR and nanoCAGE processing to identify and understand
41 key metabolisms during differentiation. By reducing the change frequency of the
42 culture medium in the new protocol, we successfully generated hepatocyte-like cells
43 producing albumin, urea, and CYP3A4 positive. The metabolomic analysis
44 successfully extracted both signatures, common and specific, for each differentiation
45 step. Integrating the metabolomic data with transcriptomic contributed to explaining
46 the kinetics of carbohydrate, lipid and nitrogen metabolism throughout differentiation.
47 The information extracted during differentiation showed that the cells moved from an
48 aerobic-like respiration pattern to a mitochondrial oxidative respiration pattern in both
49 protocols. Reducing culture medium renewal led to reduced glucose consumption,
50 followed by fructose production and significant extracellular lipogenesis throughout
51 differentiation. We believe that the overall dataset can provide information on the
52 sequence of process.

53

54

55 **Keywords:** metabolomic, nanoCAGE, hepatocyte-like cells, induced pluripotent stem
56 cells

57

58 1. Introduction

59

60 The maturation of induced pluripotent stem cells (iPSC) into hepatocytes is
61 one of the technological challenges for producing adult cells with functions
62 comparable to primary tissues. Several protocols are proposed in the literature to
63 derive iPSC (and more generally stem cells, including embryonic) into hepatocyte-
64 like cells. It is thus possible to generate liver-like tissue with albumin-secreting cells
65 and positive CYP450 tissue [1-6]. However, even if CYP450 activities have been
66 achieved, the CYP450 functionality is still limited when compared to *in vivo* levels [7-
67 9]. In addition, iPSC displayed genomic instability and wide variability in the results
68 depending of the choice of cell lines and differentiation protocols [10]. Furthermore,
69 the choice of certain basic and fundamental culture parameters, such as the initial
70 cell density for differentiation and the sequence of growth factors, are still unclear [11,
71 12].

72

73 In this context, several genomic, proteomic and metabolomic profiles were
74 generated to understand the behavior of iPSC and hepatocyte-like cells when
75 compared either to human primary cells or various iPSC lines [10, 13-17]. In order to
76 contribute to the knowledge of hepatic maturation, we proposed a first protocol
77 consisting of a step-by-step differentiation process from definitive endoderm, to
78 hepatic specification, followed by a hepatic progenitor step and finally reaching
79 hepatic-like cells (based on Si Tayeb et al., protocol [4]). The step-by-step
80 transcriptomic and gene promoter analysis illustrated the passage from the epithelial-
81 mesenchymal transition (EMT) state to the mesenchymal-epithelial transition (MET)
82 state throughout differentiation (Tauran et al., submitted). It also extracted specific

83 networks involving complex sequences on extracellular matrix reorganization, BMP
84 signaling, and EGF signaling with hepatic development markers (Tauran et al.,
85 submitted). These findings were consistent with other analyses, showing the
86 importance of *FGF2*, *BMP2*, *CDK1*, *HNF4A* and *EGF* [13]. In parallel, using a
87 metabolomic dataset we demonstrated that IPS cells, from their definitive endoderm
88 status to their final hepatocyte-like cells, followed a switch from glycolytic to oxidative
89 phosphorylation respiration. Furthermore, we found a switch in the lipid metabolism,
90 leading to high glycerol levels and low palmitoleic, azelaic, elaidic and lauric acids in
91 hepatocyte-like cells when compared to definitive endoderm cells. We also monitored
92 the kinetic signature of urea and nitrogen metabolism (Jellali et al., submitted).

93

94 In order to reduce the cost of hepatocyte-like cell production, it is important to
95 find economically sustainable cell differentiation protocols. In this context, we
96 investigated a second protocol in which we tried to reduce the quantity of growth
97 factor during the differentiation sequence [12]. Growth factors such as activine A,
98 OSM, HGF and BMP4 were reduced by 40%. As the protocol inspired from Si Tayeb
99 work [4], this protocol led to albumin secreting cells and to immunostaining positive
100 *CYP3A4* tissue. Furthermore, both protocols led to activation of *HNF1A* and *HNF1B*
101 promoters and to specific modulation of *WT1*, *MTF1*, *HCFC1*, and *PGC α 1* regulation.
102 However, higher functionality and expression of hepatic genes were found in second
103 protocol (although primary hepatocytes levels were not yet achieved). The
104 comparison also revealed a modulation of targets involved in glucose homeostasis
105 and lipid pathways [12].

106

107 In the present paper, in order to refine and extend those analyses, we propose
108 integrating and comparing the metabolomic and transcriptomic profiles of both
109 protocols. The aim is to confirm the potential of each protocol but also to understand
110 the key events involved in the production process of hepatocyte-like cells.

111

112 **2. Material and methods**

113

114 **2.1. hiPSC differentiation**

115

116 TkDN-4M human iPS cells were obtained from the stem cell bank of the
117 Institute of Medical Science of the University of Tokyo [18]. Cells were cultivated on
118 standard tissue culture polystyrene (TCPS) dishes following two protocols: protocol 1
119 (P1) previously published by Si Tayeb et al., [4] and protocol 2 (P2) derived from the
120 literature [5]. The two protocols differ in the frequency of culture medium renewal,
121 which was reduced in protocol 2 to decrease the amount of growth factors needed for
122 the differentiation of hiPS cells into hepatocytes. The protocol 1 and 2 sequences are
123 presented in Fig. 1. The details of the protocol are given in our previous work and in
124 supplementary file 1 [12].

125

126 **2.2. Metabolomic analysis**

127

128 Metabolomic analysis was performed with culture medium collected at the
129 end of each step using gas chromatography (Agilent 7890B) coupled with mass
130 spectrometry (Agilent 5977A, GC-MS). The column was an Rxi-5SiIMS from Restek
131 (30 m with 10 m Integra-Guard column - ref 13623-127). Sample preparation and

132 metabolite extraction were performed according to our previous work [19, 20]. All
133 steps in the GC-MS analyses were carried out as described in Fiehn et al., [21, 22].
134 The datafiles obtained were analyzed with AMDIS software
135 ([http://chemdata.nist.gov/mass-
136 spc/amdis/](http://chemdata.nist.gov/mass-spc/amdis/)). Peak areas were determined with the
137 Masshunter Quantitative Analysis (Agilent) and normalized to ribitol. Metabolite
138 contents are expressed in arbitrary units. The complete protocol for the metabolomic
139 analysis is detailed in supplementary file 1.

140 The metabolomic multivariate data analysis was performed using
141 XLSTAT.2016 software (Addinsoft) and MetaboAnalyst [23, 24]. Supervised Partial
142 Least Squares-Discriminant Analysis (PLS-DA) was carried out in order to discover
143 significant variations between the groups. Discriminating metabolites were identified
144 using the Variable Importance for the Projection (VIP) scores. Univariate statistics
145 was performed with Student's t-test, and a *P* value of less than 0.01 was considered
146 statistically significant. Metabolites with significant changes in the groups (*P* value <
147 0.01 and VIP > 1) were selected as biomarkers. Pathway analysis of potential
148 biomarkers was performed using MetaboAnalyst [23, 24].

150 **2.3. CAGE analysis**

151
152 NanoCAGE libraries were generated and sequenced as previously described
153 by Poulain et al. [25] using pseudo-random primers to reduce the amount of
154 sequencing reads mapping to ribosomal DNA [26]. Total RNA extractions, library
155 preparation steps and sequencing were performed as detailed in Lereau-Bernier et al.
156 [12].

157 NanoCAGE sequencing data were processed for CAGEscan analysis as
158 detailed in Lereau-Bernier et al. [12]. Custom R scripts using the CAGEr package
159 were used to produce expression tables that were uploaded on the iDEP server
160 (<http://ge-lab.org:3838/idep/>) for differential gene expression and pathway analysis
161 [27, 28]. Datasets were deposited at Zenodo (FASTQ files DOI:
162 10.5281/zenodo.1014009 and MOIRAI output DOI: 10.5281/zenodo.1017276).

163

164 **2.4. RT-qPCR**

165

166 RT-qPCR experiments were performed as previously described [12]. The
167 primer sequences used in this study are shown in Table S1 (supplementary file 1).
168 ACTB (β -Actin) was used as the reference gene and undifferentiated hiPS cells as
169 the reference sample for the normalization of gene expression data.

170

171 **2.5. Albumin measurements**

172

173 Albumin production was quantified by ELISA sandwich assays using anti-
174 Human Albumin IgG (Bethyl, Japan, capture antibody) and anti-Human Albumin IgG
175 coupled with peroxidase (Bethyl, Japan, detection antibody) [12]. After peroxidase
176 revelation by H₂O₂/OPD mixture, the plate was read at 490 nm using an iMark
177 Microplate reader (Bio-Rad).

178

179

180

181

182 **2.6. CYP3A4 immunostaining**

183

184 CYP3A4 immunostaining was performed as described by Danoy et al., [29]. The
185 primary and secondary antibodies used were anti-cytochrome P450 3A4/CYP3A4
186 antibody (rabbit, ab135813, abcam) and anti-rabbit Alexa Fluor 647 (goat, ab150129,
187 abcam), respectively. Observations were made with an Olympus IX-81 confocal
188 laser-scanning microscope.

189

190 **3. Results**

191

192 **3.1. Cell differentiation into hepatocyte-like cells**

193

194 The differentiation into hepatocyte-like cells was confirmed in both protocols.
195 The morphologies at the end of the differentiation are shown in Fig. 2A. The hepatic
196 phenotype was illustrated by the presence of albumin in the culture at the end of
197 differentiation (Fig. 2D). The immunostaining revealed the presence of a *CYP3A4*
198 positive subpopulation in both protocols (Fig. 2B). Interestingly, we found higher
199 numbers of cells positive to CYP3A4 in protocol 2 than in protocol 1 (Fig. 2B).

200

201 To determine whether iPSC-derived hepatocytes in both protocols displayed
202 the mature characteristics of a hepatic lineage, we measured the gene expression of
203 *OCT3/4* (pluripotency marker) and various hepatic markers (*ALB*, *AFP*, *FOXA2*,
204 *CYP3A4* and *HNF4A*) by means of RTqPCR analysis. As shown in Fig. 2C, mRNA
205 levels measured for the *ALB*, *AFP*, *CYP3A4*, *FOXA2* and *HNF4A* genes in both
206 protocols were significantly higher in the iPSC-derived hepatocytes than in

207 undifferentiated iPSC. Furthermore, these genes were more highly expressed in
208 protocol 2 (when compared with protocol 1). The expression of *OCT3/4* was clearly
209 downregulated at the end of differentiation in protocols 1 and 2 (Fig. 2C). This overall
210 set of data confirmed the success of the differentiation into a hepatocyte-like cell
211 lineage.

212

213 **3.2. Metabolomics analysis**

214

215 *3.2.1. Multivariate metabolomics contribute to extracting common metabolite* 216 *signatures at each differentiation step*

217

218 The metabolomic treatment led to the identification of 105 metabolites in the
219 culture medium (supplementary file 2). As the culture media were not the same in the
220 different steps, especially in step 4, we performed a normalization for each
221 compound (using the level of each compound in the basal culture media, the basal
222 media being the control media without cell exposure). The PLS-DA analysis applied
223 to the overall dataset led to several separate steps in different clusters, as shown in
224 Fig. 3A. We found a clear separation between the steps 1 in each protocol, indicating
225 distinct metabolic profiles. In addition, both steps 4 were clustered in the same area
226 of the PLDS-DA, but still separately. These results were confirmed by PLS-DA
227 performed with samples from step 1 and step 4 only (Fig. 3B). However, the clusters
228 of steps 2 and 3, for both protocols, were almost superimposed.

229

230 The heatmap of the top 25 metabolites in this analysis is provided in Fig. 3C.
231 In step 1, we found common signatures in both protocols, including high levels of

232 glyceric, palmitoleic and aspartic acids, spermidine, and O-phosphocolamine when
233 compared to basal culture medium. In parallel, the specificity of each protocol was
234 illustrated in protocol 1 by high levels of 3 phosphoglyceric and malic acids, and by
235 high levels of glucose and galacturonic acid in protocol 2.

236

237 The heatmap also contributed to extracting common patterns characterizing
238 steps 2 to 4 in protocols 1 and 2. Step 2 was characterized by high levels of
239 ketoisocaproic, citric and gluconic acids, histidine and O-phosphocolamine.
240 Concerning step 3, we observed high levels of histidine, ketoisocaproic acid, glucose
241 and galacturonic acid. Finally, both steps 4 were characterized by high levels of uracil,
242 urea, glycerol, putrescine, glutamine, phosphoric, oxalic, pyroglutamic and threonic
243 acids.

244

245 This general analysis demonstrated that both protocols led globally to the
246 similar metabolic profiles after the end of step 2. In order to characterize the
247 difference in the kinetics of each protocol more specifically, and to refine the
248 difference in the differentiation process more particularly, we then ran a step-by-step
249 differential analysis.

250

251 3.2.2. *Differential analysis of step 1*

252

253 PLS-DA analysis contributed to clearly distinguishing the two protocols in step
254 1 of differentiation, as shown in Fig. 4A. More particularly, the differential analysis
255 contributed to extracting 56 metabolites differentially expressed between the two
256 protocols in step 1, with a *P* value of less than 0.01 (Fig. 4B and supplementary file

257 3). The heatmap of the 25 most significant metabolites at the end of step 1 is shown
258 in Fig. 4C. The levels of carbohydrates such as glucose, fructose and galacturonic
259 acid appeared higher in the second protocol. The first protocol exhibited high levels
260 of Krebs cycle intermediates (fumaric, citric, succinic and malic acids) and glycolysis-
261 related compounds such as 3 phosphoglycreic acid, lactic acid and gluconic acid.
262 Extended to the top 50 metabolites, we found higher levels of hexanoic, palmitoleic,
263 caprylic and pyroglutamic acids in protocol 2 (supplementary file 3). Table S2
264 (supplementary file 1) also summarizes the fold change in 56 significant metabolites,
265 in comparison with the initial basal media (for both protocols).

266

267 The metabolite set enrichment using MetabolAnalyst with those 56
268 compounds (P value < 0.01) revealed that the Warburg effect, urea cycle,
269 gluconeogenesis, glutamate metabolism and ammonia recycling were the top 5
270 modulated pathways. Of the top 10 pathways, we also found glycine-serine
271 metabolism and the glycoase alanine cycle (Fig.S1, supplementary file 1).

272

273 *3.2.3. Differential analysis of steps 2 and 3*

274

275 PLS-DA treatments performed with step 2 and step 3 samples contributed to
276 distinguishing protocol 1 from protocol 2 for each step (Fig. 5A and D). However, only
277 a few metabolites were differentially expressed in step 2 when compared to the step
278 1 situation. In fact, only 8 compounds with a P value of less than 0.01 were extracted
279 (Fig.5B and C). Nevertheless, we found 23 metabolites modulated between both
280 protocols, with a P value of less than 0.05 (volcano plot in Fig. 5B and supplementary
281 file 3). The variations in these metabolites (fold change) in comparison with basal

282 culture media are presented in Table S2 (supplementary file 1). The metabolite
283 enrichment pathway in MetaboAnalyst (using 23 metabolites with a P value < 0.05)
284 highlighted the glycerol phosphate shuttle, beta oxidation of fatty acids, vitamin B6
285 metabolism and glutathione metabolism (Fig.S2, supplementary file 1).

286

287 Similarly, in step 3, we found only five metabolites differentially expressed
288 between both protocols, with a P value of less than 0.01 (13 metabolites with a P
289 value of less than 0.05; Fig.5E and F). Using these 13 metabolites for pathway
290 analysis with MetaboAnalyst software, we found that the pathways modulated
291 significantly were related to propanoate metabolism, glycerol phosphate shuttle, beta
292 oxidation of fatty acids and linoleic acid metabolism (Fig.S3, supplementary file 1).

293

294 3.2.4. Differential analysis of step 4

295

296 The two protocols were distinguished in step 4 as two distinct clusters were
297 formed to group each protocol (PLS-DA score plot in Fig.5G). However, step 4 was
298 still characterized by a low number of metabolites significantly modulated between
299 protocols 1 and 2 (12 metabolites with a P value of less than 0.01; Fig.5H and
300 supplementary file 3). It included fatty acids and lipids such as caprylic acid,
301 palmitoleic acid, and carbohydrates such as fructose, glucose and galacturonic acid.
302 All these metabolites were higher in protocol 2 (Fig.5I). We also found higher levels
303 of pyroglutamic acid in protocol 2, whereas urea was higher in protocol 1. The top 20
304 metabolites (with a P value of less than 0.05) are presented in the heatmap in Fig.S4
305 (supplementary file 1). Of them, we monitored higher levels of azelaic acid, O-
306 phosphocolamine and cholesterol in protocol 2, and higher levels of uracil and

307 aspartic acid in the first protocol. The metabolite set enrichment with these 20 top
308 molecules revealed the modulation of glutathione metabolism, the transfer of acetyl
309 groups into mitochondria and nitrogen metabolism (urea cycle, ammonia recycling,
310 arginine-proline metabolism), as shown in Fig.S5 (supplementary file 1).

311

312 **3.3. Transcriptomic characterization**

313

314 In order to complete the metabolomic profiling, we integrated genomics
315 analysis of the cells. We focused particularly on steps 1 and 4 of differentiation as a
316 starting and end point, respectively. For step 1, a selection of target genes was
317 analyzed by RTqPCR at the end of the step in both protocols. As shown in Fig.6A,
318 RTqPCR confirmed higher levels of glycolytic genes in protocol 1 at the end of step 1
319 for *PFKM*, *PGAM*, *ENO1*, *G6PD* and *LDHA* genes. The levels of *IDH2* (TCA) were
320 also higher in protocol 1. *ACLY* (lipogenesis) and *HIF1A* (hypoxia) mRNA levels
321 remained similar in both protocols.

322

323 Concerning step 4, we used transcriptomics data obtained from nanoCAGE
324 sequencing of the cells at the end of the differentiation protocol (step 4). For that
325 purpose, we firstly post processed the transcriptomic data with a false discovery rate
326 (FDR) of 0.1 and a fold change of 1.1 using iDEP software (version 8.1). This led to
327 the extraction of 270 genes differentially modulated between both protocols (Fig.6B
328 and Fig.S6 in supplementary file 1). The full list of differentially expressed genes is
329 provided in supplementary file 4.

330

331 The pathway analysis, run with an FDR of 0.2, using the KEGG database,
332 extracted the glycolysis/gluconeogenesis, the metabolism of various carbohydrates
333 (fructose, mannose, galactose), ferroptosis (iron metabolism), HIF signaling and
334 arginine-proline metabolism among the top modulated pathways (Fig.6C). The full
335 figures and modulated genes are given in supplementary file 5. The GO molecular
336 function also illustrated phospholipid- and steroid-related pathways, and cellular
337 remodeling and modulations (via integrin binding, cadherin binding and cell adhesion
338 binding activities; Fig.S7 in supplementary file 1). A selective list of lipid genes
339 (*APOA2*, *HMGCR*, *HMGCS1*, *ACLY*, *SCD*, *SQLE*) and carbohydrate genes (*ENO1*,
340 *PGK1*, *GAPDH*, *GPI*, *HK2*, *LDHA*, *PKM*, *ALDOA*) are presented in Table 1. All these
341 genes were downregulated in protocol 2 when compared with protocol 1. We also
342 included the mRNA ratio of HIF obtained by RTqPCR, which was found to be
343 upregulated by 4.5 times in protocol 2.

344

345 Using the STRING protein database clustering tool, with the Kmeans method
346 applied to the 270 genes, we extracted 8 typical interconnected networks with 106
347 genes (Fig.S8 in supplementary file 1). Those networks were related to ECM and
348 tissue remodeling (*via* genes such as *LUM*, *COL1A1*, *COL4A5*, *COL14A1*, *COL3A1*,
349 *ACTB*, *CTNNA3* and *VIL1*), the glycolytic pathway (*via* genes such as *ENO1*, *PGK1*,
350 *GAPDH*, *GPI*, *HK2*, *LDHA*, *PKM* and *ALDOA*), lipid- and steroid-related metabolisms
351 (*via* genes such as *APOA2*, *HMGCR*, *HMGCS1*, *ACLY*, *SCD* and *SQLE*), some
352 transporters (*SLC1A3*, *SLC16A1*, *SLC2A3* and *VDAC2*), and ribosomal, RNA and
353 DNA processing (*RPL3*, *RPS3*, *EIF5* and *EEF2*). Furthermore, specific liver
354 progenitor markers were extracted (*ALB*, *NR3C2*, *TF*, *CK19* and *RBP4*). In those
355 networks, the important nodes appeared to be the genes *ACLY*, *APOA2*, *HSP90AA1*,

356 *EEF2*, *RPL3*, *RPS3*, *GADPH* and *TPI1* linking the subnetworks. We also found the
357 presence of chaperone protein-related genes, such as *CCT2*, and calcium-
358 dependent genes (*CALU*, *CADM1* and *RYR2*).

359

360 **3.4. Metabolomic and transcriptomic integration of step 4 data**

361

362 The 270 genes and the top 20 metabolites from step 4 were integrated into
363 metaboanalyst software. The list of enriched pathways and the corresponding genes
364 and metabolites are given in supplementary files 6 and 7. The integration principally
365 identified glycolysis and gluconeogenesis as the main enriched pathway (9 hits). Of
366 the others, we also found the metabolism of nitrogen (5 hits) and glutathione
367 metabolism (5 hits). The network explorer option connected 7 metabolites (glycine,
368 glutamic acid, aspartic acid, Palmitoleic acid, citric acid, 4 hydroxybenzoic acid and
369 N-acetyl-L aspartic acid) and 10 genes (*ANPEP*, *EEF2*, *CACNA2D1*, *GRID2*, *MITF*,
370 *PAICS*, *FDPS*, *SND1*, *ACLY*, *FST*). The network map is provided in Fig.S9 in
371 supplementary file 1.

372

373 **Discussion**

374

375 In the present work, we investigated metabolomic and transcriptomic
376 signatures of hiPS cells during their differentiation using two culture protocols. The
377 first protocol is the conventional protocol adapted from Si-Tayeb et al. [4]. In protocol
378 2, the quantity of growth factors used was reduced by 40% (when compared to the
379 first protocol). Our aim was to confirm the potential of each protocol to generate
380 hepatocyte-like cells, and to obtain a better understanding of the key events involved

381 in hiPSC differentiation. The hepatic phenotype was confirmed at the end of
382 differentiation in both protocols by albumin and urea production, and positive
383 immunostaining for CYP3A4. We also demonstrated that hepatocyte-like cells
384 derived through these two protocols express markers of hepatocytes such as *ALB*,
385 *AFP*, *CYP3A4*, *FOXA2* and *HNF4A*. Further studies would be required to confirm
386 those results on other iPSC sources.

387

388 During protocol 1, the culture medium sequence was changed every day. We
389 expected an adequate supply of nutrients to the tissue, such as glucose or fatty acids
390 as potential sources of energy. In non-differentiated iPSC, the respiration patterns
391 were reported as leading to glycolytic respiration (in contrast to oxidative
392 phosphorylation respiration in mature and differentiated tissue) [30]. When comparing
393 the two protocols, metabolomic profiling revealed significant and very complex
394 modulation of the cell signature at the end of step 1. More particularly, the two
395 protocols differed in terms of the high levels of lipids such as palmitoleic, caprylic and
396 hexanoic acids in protocol 2, revealing lower consumption of fatty acids. However,
397 the *ACLY* gene (lipogenesis process) was not significantly modulated between the
398 two protocols. Furthermore, glucose levels were higher in protocol 2 and lactate
399 levels higher in protocol 1 at the end of step 1. These tendencies illustrated intense
400 energy demand in protocol 1 and probable higher fatty acid beta oxidation and
401 glycolysis. This was consistent with the activation of the TCA cycle in protocol 1 as
402 demonstrated by high levels of typical intermediates such as succinic, malic, fumaric
403 and citric acids. At the mRNA levels, these results were accompanied by higher
404 mRNA levels of glycolytic and TCA genes in protocol 1. In protocol 2, the medium
405 was not changed every day leading to the creation of a potential glucose shortage.

406 Consistently, it has been reported in the literature that starvation results in lower
407 levels of glycolytic genes [31]. In addition, in the present study, this downregulation
408 was independent of the *HIF* mRNA levels in step 1, which remained closed to each
409 other for both protocols (*HIF* being involved in glycolysis and lipid regulation) [32, 33].
410

411 Lipolysis is reported to be one source of carbohydrates in a starvation state
412 [34]. As a result, we expected lower lipid levels in protocol 2, which was not the case
413 in step 1. Furthermore, high levels of fructose are said to induce lipogenesis and only
414 limited amounts of lipids can be used to generate glucose [35, 36]. These results
415 may reflect the behavior observed in protocol 2, in which high lipid levels were
416 correlated with high levels of fructose. A second alternative source of glucose during
417 starvation is amino acids (amino acids are usually extracted from protein degradation
418 in muscle). As we observed higher levels of amino acids in protocol 2, including
419 isoleucine, cystine, serine, lysine, tyrosine and tryptophan, additional analysis is
420 required if we are to identify whether or not protocol 2 led to reduced amino acid
421 consumption or to amino acid production as a result of protein degradation for
422 generating glucose.

423
424 After step 1, the metabolomic profiles were very similar in steps 2 and 3 for
425 both protocols. Nevertheless, small differences appeared when considering *P* values
426 of less than 0.05. In step 2, it was mainly fatty acid, glycerol and lipid metabolisms
427 that were highlighted due to lower levels of heptadecanoic acid and moderately
428 higher levels of glycerol-1 phosphate, cholesterol, phosphoric, capric and caprylic
429 acids in protocol 2 (fold change of 0.62, 1.13, 1.16, 1.06, 1.06 and 1.16 respectively).
430 This tendency was also observed in step 3, in which we observed higher lipid levels

431 (linoleic, lauric and elaidic acids) in the culture medium of protocol 2. In parallel, for
432 both steps, we detected high levels of kynurenine and low levels of tryptophan.
433 Interestingly, L-kynurenine is said to decrease cell membrane fluidity (in liver cells)
434 and to reduce linoleic acid incorporation into phospholipids [37]. It led in parallel to an
435 increase in cholesterol levels, which appeared consistent with our findings [37]. A
436 slight difference in vitamin B6 metabolism was finally extracted in step 2 (higher
437 levels of pyridoxine in protocol 2). Vitamin B6 deficiency is linked to an altered fatty
438 acid profile and impaired fatty acid synthesis, which may also be the illustration of
439 overall complex crosstalk in the fatty acid metabolism in steps 2 and 3 [38].

440

441 A difference in both protocols was detected concerning lipid and fatty acid
442 levels in step 4. The high levels of palmitoleic, caprylic and azelaic acids in protocol 2
443 revealed a difference in the lipolysis/lipogenesis processes (and consistently with
444 their kinetics from steps 1 to 3) when compared to protocol 1. These high levels of
445 lipids were correlated with high levels of fructose and glucose in protocol 2 (as in step
446 1). In addition, the transcriptomic analysis confirmed modulation at the gene level of
447 lipid and steroid metabolisms (in protocol 2, downregulation of *APOA2*, *HMGCR*,
448 *HMGCS1*, *ACLY*, *SCD*, *SQLE*), glycolysis and carbohydrate metabolism (in protocol
449 2, downregulation of *ENO1*, *PGK1*, *GAPDH*, *GPI*, *HK2*, *LDHA*, *PKM*, *ALDOA*, as in
450 step 1) and modulation of HIF levels (upregulation of *HIF* by RTqPCR in protocol 2).

451

452 A potential underlying mechanism and transcription factor could thus be the
453 hypoxia and HIF-related signaling, given that several upstream and downstream
454 targets were modulated in our study. mRNA levels of HIF1A in step 4 confirmed
455 significant upregulation of HIF1A in protocol 2. Under hypoxia, HIF1A induced

456 glycolysis and also increased glucose uptake [32]. In parallel, it is said to induce fatty
457 acid uptake and lipid storage, but HIF-1 also inhibits de novo fatty acid synthesis in
458 the liver [32, 33]. HIF, as a pivotal lipid glucose compound in energy metabolism, is
459 also reported, mainly by triggering a switch from mitochondrial oxidative
460 phosphorylation to anaerobic glycolysis in hypoxic conditions [32]. In our study, we
461 found higher levels of glucose and lipids in the culture medium of protocol 2. In
462 addition, the glycolytic genes were not upregulated. As a result, it seems that we did
463 not face a HIF signaling governing process in step 4 of protocol 2. The process in
464 step 4 appeared similar to step 1, in which fructose- and starvation-like dependent
465 control seemed to govern the phenomena. However, as the metabolomic signature
466 was closer in step 4 between both protocols (8 metabolites with a *P* value of 0.01
467 compared to 65 in step 1), it also appeared that the predominance of this
468 phenomenon could be attenuated when compared to the situation in step 1. Although
469 we can postulate that liver-like cells start to play their role in metabolism homeostasis,
470 additional analysis is needed to clarify the status of the situation in protocol 2.

471

472 Finally, step 4 was characterized by a common signature, illustrating liver
473 differentiation, as shown by the level of urea in both protocols when compared to
474 step 1. Although urea and glutamine were produced in both protocols in step 4, the
475 urea, glutamic acid and aspartic acid levels were higher in protocol 1. At the
476 transcriptomic level, it was related to moderate upregulation, in protocol 1, of typical
477 urea cycle genes such as *ARG1*, *ARG2*, *GOT1*, *GOT2* and *OAT*.

478

479 We also observed high levels of pyroglutamic acid for both protocols,
480 illustrating greater oxidative phosphorylation in step 4 when compared to step 1. This

481 process is consistent with other iPSC differentiation reports presenting a switch from
482 ATP production via glycolysis in the pluripotent stem stage, to mitochondrial oxidative
483 phosphorylation (OXPHOS) respiration, which leads to an increase in reactive
484 oxygen species (ROS) levels (ROS are produced by OXPHOS) [30, 39].
485 Pyroglutamic acid production illustrates the activation of glutathione metabolism in
486 order to detoxify the ROS produced by OXPHOS. Glutathione metabolism activation
487 was also illustrated by the consumption of the main glutathione precursors in step 4
488 of both protocols (glycine, cysteine and glutamic acid; fold change between 0.4 and
489 0.9 when compared to basal culture media) [38].

490

491 The results presented in this work demonstrate that the reduction of growth
492 factors by 40% (protocol 2) efficiently generate hepatocyte-like cells similar to those
493 generated by the classical protocol (protocol 1). Integration of metabolomic and
494 transcriptomic data highlighted several key metabolic pathways during differentiation
495 process and contributed to identify the effect of frequency of the culture medium
496 renewal and growth factors reduction on iPSC metabolism. However, additional
497 analysis such as the repetition of those protocols of differentiation on other iPSC
498 sources, evaluation of the protocol stability and their key events, cell sorting to
499 quantify the population heterogeneity, proteomic characterisation, and benchmark
500 with both fetal and primary hepatocyte cultures, are required (i) to clarify the real
501 benefit of our protocols (ii) to understand the metabolic effects observed. In addition,
502 precise hepatocyte quantification would be required to normalise the hepatocytes
503 productions/consumptions regarding the overall tissue. Nevertheless, we believe that
504 those results provide knowledge on the behavior of iPSC during hepatic
505 differentiation.

506 **Conclusion**

507

508 We compared the metabolomic and transcriptomic signatures of human
509 induced pluripotent stem cells during their differentiation into hepatocyte-like cells.
510 Two differentiation protocols were tested. By reducing the frequency of culture
511 medium change in one protocol, we managed to produce hepatocyte-like cells
512 producing albumin and urea. The metabolomic signature demonstrated a major
513 difference at the start of differentiation, leading to 65 differentially-expressed
514 metabolites. The difference became weaker once the differentiation reached hepatic
515 specification. At the end of both protocols, only 8 metabolites remained differentially-
516 expressed (including fructose, palmitoleic and caprylic acid). During the process as a
517 whole, the reduction in culture medium change led to a metabolic signature with high
518 levels of fructose and lipids in the culture medium. This was accompanied by
519 downregulation of glycolytic genes. The present study clearly reveals the potential of
520 our new protocol to generate hepatocyte like-cells from hiPSC, with a reduced cost.
521 Our analysis could also contribute to a better understanding of differentiation and
522 suggest novel targets to improve iPSC maturation into functional hepatocytes.

523

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525

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532 **Conflict of interest**

533

534 The authors declare no conflict of interests

535

536 **References**

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