1 **Histone deacetylase 9 promoter hypomethylation associated with adipo-**

2 **cyte dysfunction is a statin-related metabolic effect**

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Abstract

Background

Adipogenesis, the process whereby preadipocytes differentiate into mature adipo-cytes, is crucial for maintaining metabolic homeostasis. Cholesterol lowering statins increase type 2 diabetes (T2D) risk possibly by affecting adipogenesis and insulin resistance but the (epi)genetic mechanisms involved are unknown. Here, we charac-terised the effects of statin treatment on adipocyte differentiation using *in vitro* human preadipocytes cell model to identify putative effective genes.

Results

Statin treatment during adipocyte differentiation caused a reduction in key genes in-volved in adipogenesis, such as *ADIPOQ, GLUT4* and *ABCG1.* Using Illumina's Infin-ium '850K' Methylation EPIC array, we found a significant hypomethylation of cg14566882, located in the promoter of the histone deacetylase 9 (*HDAC9)* gene, in response to two types of statins (atorvastatin and mevastatin), which correlates with an increased *HDAC9* mRNA expression. HDAC9 is a transcriptional repressor of the cholesterol efflux *ABCG1* gene expression, which is epigenetically modified in obesity and prediabetic states. Thus, we assessed the putative impact of *ABCG1* knockdown in mimicking the effect of statin in adipogenesis. *ABCG1* KD reduced the expression of key genes involved in adipocyte differentiation and decreased insulin signalling and glucose uptake. In human blood cells from two cohorts, *ABCG1* expression was impaired in response to statins, confirming that *ABCG1* is in vivo targeted by these drugs.

Conclusions

We identified an epigenetic link between adipogenesis and adipose tissue insulin re-sistance in the context of T2D risk associated with statin use, which has important

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51   implications as HDAC9 and ABCG1 are considered potential therapeutic targets for

52   obesity and metabolic diseases.

53   **Keywords**

54   Adipogenesis, methylation, ABCG1, HDAC9

55   **1   Background**

56   Adipose tissue plays a crucial role in regulating insulin sensitivity and glucose ho-

57   meostasis (1). In obesity, adipose expansion occurs as a result of cellular hypertro-

58   phy, *i.e.*, the increase in size of the adipocyte, and/or *de novo* adipogenesis, which is

59   the production of new mature adipocytes from residing preadipocytes (2–4). Dysregu-

60   lation in the adipogenic process is associated with metabolic diseases and insulin

61   resistance (5) and is an independent risk factor for type 2 diabetes (T2D) (6). In con-

62   trast, appropriate adipocyte expansion is protective against T2D in the context of

63   obesity (7,8). Adipogenesis occurs as a result of metabolic cues that trigger the in-

64   duction of key differentiation regulators, such as ADIPOQ, FASN, PPARg, ABCG1

65   and GLUT4 (9–12). Epigenome wide association studies (EWAS) have found that

66   hypermethylation within one of these genes involved in adipogenesis, *ABCG1,* was

67   associated with increased body mass index (BMI), insulin resistance and T2D risk

68   (13–16), opening avenues in the elucidation of the links between adipogenesis and

69   metabolic diseases.

70   One of the most common drugs known to modulate adipogenesis are statins (17).

71   The clinical use of statin is associated with an increased risk of insulin resistance and

72   T2D risk (18), but the molecular mechanisms involved remain poorly understood. We

73   hypothesised that statin treatment modulates adipogenesis by modifying the adipo-

74   cyte epigenome. In this study, we confirmed the inhibitory effects of statin treatment

75   in human preadipocytes and investigated the whole methylome to identify potential

76   regulators that may be involved in adipogenesis.

2   Results

2.1   Statin treatment reduced adipogenesis and insulin signalling

The Simpson-Golabi-Behmel syndrome (SGBS) human preadipocyte cell line was

used in this study as an *in vitro* model for adipocyte differentiation. In SGBS cells,

lipid droplet formation occurred by 12-14 days of differentiation together with an in-

crease in the expression of key adipogenic markers (19). We retrieved adequate

SGBS cell morphology modification and formation of lipids droplets by day 12 (Addi-

tional File 1: Figure S1a), and observed that the expression of key genes involved in

adipocyte differentiation and maturation was accordingly up-regulated (Additional File

1: Figure S1b).

For statin treatment, SGBS cells were differentiated for 6 days and then treated with

atorvastatin and mevastatin for an additional 6 days until final maturation (Figure 1a).

We observed a clear decrease in lipid-droplet formation in statin-treated SGBS cells

(both atorvastatin and mevastatin) when compared to DMSO-vehicle controls (Figure

1b). We also found that statin-treatment induced a significant down-regulation of

many key genes associated with adipogenesis reported above (*ABCG1, LEPTIN* and

GLUT4), with the particular exclusion of *PPARG*, a gene known to play a role only in

the early stages of adipocytes differentiation (Figure 1c). We then measured the ef-

fects of statin on downstream regulators of insulin signalling and found decreased

efficiency of insulin to activate ERK and AKT (Figure. 1d). Taken together, the data

support the inhibitory effects of statin in the human adipocyte differentiation and insu-

lin signalling, a similar effect reported in statin-treated 3T3-L1 mouse adipocyte cells

(17).

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101   2.2   Whole methylome analysis of statin-treated SGBS cell line

102   To identify potential regulators involved in statin-induced adipocyte dysregulation, we

103   performed an unbiased whole methylation analysis in statin-treated SGBS cells using

104   Illumina's Infinium '850K' Methylation EPIC arrays (Additional File 1: Figure S2). We

105   filtered differentially methylated positions (DMPs) located in the promoter region, an-

106   notated as TSS200 or TSS1500 (within 200-1500 base pairs from the transcription

107   start site), in order to identify DMPs that were likely to have a biological effect. The

108   most significant DMP was cg14566882, located in the promoter of the histone deace-

109   tylase (*HDAC9*) gene, in mevastatin-treated cells (β = 8.28 %; p = 5.55 x 10^{-6}) (Figure

110   2a). This DMP was also significantly hypomethylated in response to atorvastatin

111   treatment, compared to DMSO-vehicle controls (β = 5.53 %; p = 1.35 x 10^{-3}) (Figure

112   2a, b).

113   Additionally, 87 DMPs were shared between the mevastatin and atorvastatin-treated

114   groups (Additional File 2: Table 1) and of the hypomethylated DMPs, cg14566882 in

115   *HDAC9* remained the top candidate in both atorvastatin and mevastatin treatments

116   (Additional File 1: Figure S3). A significant differentially methylated region (DMR)

117   overlapping this promoter region was also found in response to both treatments (Ad-

118   ditional File 1: Figure S4; False discovery rate < 0.05). In order to validate the effect

119   of cg14566882 hypomethylation on the expression of the *HDAC9* gene, we per-

120   formed qPCRs in statin-treated SGBS cell lines and found significant up-regulation of

121   the *HDAC9* gene at the mRNA level (p < 0.05; atorvastatin: 14-fold; mevastatin:11-

122   fold) (Figure 2c).

123   ABCG1 has been reported to be regulated by HDAC9-mediated changes in acetyla-

124   tion (20,21) and may be targeted by the *HDAC9* epigenetic alteration. We confirmed

125   that ABCG1 protein expression is indeed down-regulated in response to mevastatin

126   and atorvastatin treatment (Figure 2d). We confirmed that in response to statin, this

127   effect was independent of the previously reported hypermethylation in cg06500161

128   (p-value > 0.5) and cg27243685 (p-value > 0.5) found to be associated with in-

129   creased BMI and T2D incidence (Additional File 1: Table 2) (15,22–24).

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131   2.3   Knockdown of *ABCG1* in SGBS preadipocytes reduced adipocyte differentiation

132   We performed an *ABCG1* knockdown (KD) in SGBS cells to address whether re-

133   duced *ABCG1* expression mimics the effect of statins in adipogenesis. SGBS preadi-

134   pocytes were stably transfected with a shRNA targeting *ABCG1* mRNA and followed

135   them quantitatively through maturation and differentiation and this data was com-

136   pared with cells transfected with a non-targeting shRNA (control). A similar protocol

137   for stably knockdown *Abcg1* via shRNA has previously been achieved and described

138   in mouse 3T3-L1 preadipocytes (25). We initially analysed the expression of ABCG1

139   protein in normal adipocytes to show that it is positively associated with adipogenesis

140   as ABCG1 starts to become expressed at day 6 of differentiation (Figure 3a). The

141   efficient silencing of *ABCG1 (ABCG1* KD) was confirmed at the protein level (Figure

142   3b). *ABCG1* KD in SGBS cells was accompanied by a significant reduction in the lipid

143   content (20 % reduction, p<0.05; Figure 3c), along with the down-regulation of the

144   following adipocyte differentiation markers *FASN, PPARG* and *PLIN1* and key adipo-

145   cyte maturation markers *ADIPOQ* and *GLUT4* (Figure 3d). As a consequence of im-

146   paired adipogenesis, the *ABCG1* KD led to a significant reduction in glucose uptake

147   stimulated by insulin (65 % reduction, p < 0.001; Figure 3e). In addition, we found a

148   decreased efficiency of insulin to activate AKT in those cells (Figure 3f). As a whole,

149 this data indicates that A*BCG1* levels are pivotal for the control of human adipocyte

150 differentiation and glucose metabolism.

151 2.4   *ABCG1* is down-regulated in response to statin in human blood samples

152 We next explored whether *ABCG1* was also dyregulated in samples from human

153 subjects. We analyzed reported transcriptomic data from blood samples from two

154 cohorts. The first consisted of a total of 57 individuals from the ECLIPSE cohort, of

155 which 13 were statin users (26). A significant reduction in the expression of *ABCG1*

156 in the statin group ($p = 1.41 \times 10^{-5}$) was found, compared to non-users (Figure 4a). In

157 addition, we also analysed data from the YELLOW II study (27), a retrospective study

158 following 85 individuals before and following an extensive 8-12 week statin therapy.

159 In peripheral blood mononuclear cells obtained from blood samples, *ABCG1* expres-

160 sion was significantly decreased following statin treatment, compared to baseline

161 levels, for two *ABCG1* probes (ILMN_1794782 $p = 2.76 \times 10^{-5}$; ILMN_2329927 $p =$

162 4.28×10^{-4}) (Figure 4b). Taken together, this demonstrates that *ABCG1* reduction in

163 response to statin is indeed reflected in human blood samples. Of note, no data on

164 *HDAC9* expression was available in the ECLIPSE case control study, and no signifi-

165 cant change in *HDAC9* expression was reported in the intervention YELLOW II study,

166 maybe due to the lack of sufficient statistical power of this study.

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168 **3   Discussion**

169 A recent 15 year prospective study found a staggering 38 % increased incidence of

170 T2D in statin users, regardless of the type of statin used (28). Here, we report that

171 two statins, atorvastatin and mevastatin, hamper the differentiation process in the

172 SGBS human preadipocyte cell line and decreased insulin sensitivity.

173   We focused our analysis on promoter DMPs, which are normally inversely correlated

174   with expression (29,30). Therefore, not surprisingly, given the inhibitory effect of

175   statin, our whole methylome analysis revealed that most DMPs were hypermethy-

176   lated. This includes the *IDI1* gene, which encodes the isopentenyl diphosphate isom-

177   erase, a component of the cholesterol synthesising pathway (31,32).

178   We report for the first time that statin treatment was associated with a significant hy-

179   pomethylation of *HDAC9* promoter, which is inversely correlated with *HDAC9* gene

180   expression. These findings are of particular significance in light of several studies that

181   demonstrated the key role of HDAC9 in adipocytes function: overexpression of

182   *Hdac9* in 3T3-L1 preadipocyte mouse cell lines suppressed adipogenesis and in-

183   versely, preadipocytes isolated from *Hdac9* knockout mice had an accelerated adipo-

184   cyte differentiation (33). Furthermore, *Hdac9* knockout mice showed improved meta-

185   bolic homeostasis and were protected from adipose tissue dysfunction in mice fed on

186   a high fat feeding (34). These studies clearly indicate the deleterious role of HDAC9

187   in maintaining adipocytes homeostasis both *in vitro* and *in vivo*.

188   *HDAC9*-deficient macrophages and monocytes directly increased the accumulation

189   of total acetylated H3 and H3K9 at the promoter of the *ABCG1* gene (20,21), thereby

190   inducing the transcription of the *ABCG1* gene, indicating that HDAC9 mediates the

191   expression of *ABCG1* through promoter-mediated acetylation. This is of particular

192   interest, as several studies have reported a role of ABCG1 in obesity, insulin resis-

193   tance and T2D. Elevated *ABCG1* expression is associated with increased fat mass

194   from obese individuals, suggesting that *ABCG1* is also involved in human adipo-

195   genesis (25). Although genome-wide association studies have not found any single

196   nucleotide polymorphisms (SNPs) within or nearby *ABCG1* associated with increased

197   T2D risk, several EWAS have found that hypermethylation in the *ABCG1* gene was

198 associated with fasting glucose, HbA1C levels, lipid metabolism, fasting insulin, T2D

199 risk and BMI (15,16,22,35–38). Additional observations in mouse models have shown

200 that Abcg1$^{-/-}$ mice were protected from high fat diet-induced glucose intolerance (39).

201 A recent study found that *ABCG1* expression is reduced in both subcutaneous and

202 visceral adipose tissue in morbidly obese patients with metabolic syndrome com-

203 pared to those without metabolic syndrome, providing further evidence for a role of

204 *ABCG1* in the maintenance of metabolic homeostasis in adipocytes (40). In addition,

205 two studies showed that *ABCG1* expression was decreased in blood white human

206 cells in response to statins. As ABCG1 was down-regulated in response to statin, we

207 hypothesised that ABCG1 plays a role in statin-induced adipocyte dysregulation.

208 Indeed, we showed that *ABCG1* expression increases during human SGBS adipo-

209 cyte differentiation and through *ABCG1* silencing, confirm that the level of *ABCG1*

210 expression is crucial for the appropriate expression of lipid metabolism markers,

211 which include *FASN, FABP4, PLIN1* and *PPARG*, for correct human adipocyte differ-

212 entiation. Our findings are consistent with previous data showing variation in these

213 four genes following *Abcg1* silencing in mouse 3T3-L1 pre-adipocyte cells (41). The

214 down-regulation of *GLUT4* in *ABCG1* KD suggested a decrease in insulin-induced

215 glucose uptake. Indeed, we confirmed a down-regulation of phosphorylation of AKT

216 and ERK. Collectively this data indicates that normal ABCG1 function is required for

217 adipogenesis and insulin signalling. In addition, we have confirmed using two sepa-

218 rate datasets that statin use was indeed correlated with a reduction in *ABCG1* ex-

219 pression in human blood samples. Other studies have reported a link between

220 ABCG1 downregulation and diabetes incidence (42) and high fasting glycaemia (43).

221 Taken together, our human cellular data is consistent with human observational stud-

222 ies, in which the inhibition of *ABCG1* expression was deleterious for metabolism in

223 adipose tissue.

Conclusions

225 The model proposed based on our data from statin-induced insulin resistance is hy-

226 pomethylation of the *HDAC9* promoter induces *HDAC9* gene expression, which in

227 turn blocks *ABCG1* expression and thereby adipocyte differentiation and metabolic

228 dysfunction (Figure 5). Adipocyte turnover by adipogenesis is crucial for the mainte-

229 nance of metabolic homeostasis and insulin sensitivity (44). Our data provides a

230 novel epigenetic link between adipogenesis dysfunction and insulin resistance, medi-

231 ated by statins. The increased understanding of adipogenesis provides a promising

232 new avenue for the treatment of metabolic disease in obesity (9,44). Both HDAC9 and

233 ABCG1 have been proposed as therapeutic targets for patients with obesity in sepa-

234 rate previous studies (34,41), however, our data support a mechanistic pathway link-

235 ing them to metabolic diseases.

4   Methods

237 4.1   Cell culture and differentiation of SGBS cell line

238 SGBS human preadipocyte cell line was kindly provided by Prof. Dr. M. Wabitsch

239 (University of Ulm, Germany) and maintained in DMEM/F12 supplemented with 10 %

240 foetal bovine serum and 0.01 % penicillin/streptomycin (15140-122 - Life Technolo-

241 gies), as previously described (19). Confluent preadipocytes were differentiated un-

242 der serum-free culture conditions by washing twice with phosphate buffered saline

243 (PBS) and then exposing to DMEM/F12 supplemented with 2 µmol/l rosiglitazone, 25

244 nmol/l dexamethasone, 0.5 mmol/l methylisobuthylxantine, 0.1 µmol/l cortisol, 0.01

245 mg/ml transferrin, 0.2 nmol/l triiodotyronin, and 20 nmol/l human insulin for 4 days.

246 The cells were then cultured for a further 8 days in fresh DMEM/F12 supplemented

247   with 0.1 μmol/l cortisol, 0.01 mg/ml transferrin, 0.2 nmol/l triiodotyronin, and 20 nmol/l

248   human insulin. Microscopic images of SGBS cells were taken under a microscope

249   (IT404; VWR) using the Motic Image plus version 2.0 (Motic Europe).

250

251   4.2   Treatment with statins

252   At 6 days of differentiation, SGBS cells were treated with 10 μM mevastatin (M2537,

253   Sigma Aldrich) or atorvastatin (PZ0001, Sigma Alrich) and compared to a Dimethyl

254   sulfoxide (DMSO)-vehicle control (D2650, Sigma Aldrich). The cells were then incu-

255   bated for a further 6 days. On day 12 of differentiation, cells collected for further

256   analysis.

257   4.3   Whole methylome analysis

258   DNA was extracted from SGBS cells treated with atorvastatin and mevastatin at day

259   6 for 6 days and collected the cells at day 12. DNA was extracted using the Nucleo-

260   Spin Tissue kit (Takara Bio). Bisulfite conversion of 500 ng genomic DNA was per-

261   formed using the EZ-96 DNA Methylation kit (Zymo Research) following the manufac-

262   turer's protocol. Bisulfite-converted DNA was subjected to genome-wide DNA methy-

263   lation analysis using Illumina's Infinium '850K' Methylation EPIC array to identify dif-

264   ferentially methylated positions (DMPs). The resulting DNA methylation IDAT files

265   were imported using the *minfi* R package for further processing and quality control

266   (45). The following CpG probes were excluded from further analysis: probes on sex

267   chromosomes, cross-hybridising probes, non-cg probes and probes that lie near sin-

268   gle nucleotide polymorphisms (SNPs). Probe-design biases and batch effects were

269   normalised using R packages *ENmix* (46) and *SVA* (ComBat) (47), respectively.

270   To identify DMPs, the R package *limma* was used (48). The model included treat-

271   ment (atorvastatin, mevastatin or DMSO-vehicle) as a categorical variable and repli-

272   cates / day of experiment as a covariate. Methylation levels denoted by beta-values,

273   where 0 indicates 0 % methylation and 1 indicates 100 % methylation, were trans-

274   formed to M-values (49). To identify differentially methylated regions (DMRs), the R

275   package *DMRcate* was used (50). *DMRcate* ranks the differentially methylated re-

276   gions across the genome using Gaussian kernel smoothing based on the DMPs.

277   4.4   RNA extraction, cDNA conversion and RT-PCR

278   Total RNA was extracted from cultured cells using a RiboPure RNA Purification Kit

279   (AM1924; Invitrogen), according to the manufacturer's instructions and quantified on

280   a NanoDrop Spectrophotometer (Thermo Scientific). RNA was reverse transcribed

281   using a High-Capacity RNA-to-cDNA Kit (4387406; Applied Biosystems), according

282   to the manufacturer's instructions. qPCRs were conducted on an Applied Biosystems

283   7900HT Fast Real-Time PCR System and quantitative expression levels were ob-

284   tained using the SDS v2.3 Software (Applied Biosystems) using Taqman Gene Ex-

285   pression Assays (ThermoFisher Scientific). The following probes were used: *ABCG1*

286   (Hs01555193_m1), *CEBPB* (Hs00270923_s1), *LPL* (Hs00173425_m1), *ACACA*

287   (Hs01046047_m1) *FABP4* (Hs01086177_m1), *GLUT4* (Hs00168966_m1), *ACACB*

288   (Hs00153715_m1), *PPARG* (Hs01115513_m1), *CEBPA* (Hs00269972_s1), *ADIPOQ*

289   (Hs00605917_m1), *FASN* (Hs01005622_m1), *SREBF1* (Hs01088691_m1) and

290   *PLIN1* (Hs00160173_m1) (Life Technologies). Each reaction was normalised to a

291   *beta-2-microglobulin* (*B2M*) control (Hs00984230_m1; Applied Biosystems). For

292   quantifications using SYBR-green, qPCRs were performed using (SsoAdvanced Uni-

293   versal SYBR Green Supermix, BioRad) using the BioRad CFX96 Real-Time PCR

294   Detection System (Biorad). *HDAC9* gene primer sequences were forward:

295   AGTGGCAGAGAGGAGAAGCA and reverse: CAGTTCTCCAGGCTCTGGTC. At

296   least three biological replicates were performed and the data represents the means ±

297 SEM. A two-tailed t-test was performed using GraphPad Prism (GraphPad software

298 Inc., La Jolla, USA) and p-value < 0.05 was considered to be statistically significant.

299 4.5   Lipid quantification

300 Culture medium was removed and the cells were washed twice with PBS and then

301 fixed with 10 % formalin for 30 min. Fixed cells were washed twice with water and

302 incubated in 60 % isopropanol for 2 min. The alcohol was discarded and the cells

303 were then incubated in an Oil Red O (Sigma) and water solution (3:2) for 5 min. Cells

304 were rinsed four times with water and then 100 % isopropanol was added to extract

305 the red oil. The absorbance was measured at 540 nm on a VERSAmax ELISA mi-

306 croplate reader (Molecular Devices) and analysed using the SoftMax Pro Software

307 (Molecular Devices).

308 4.6   Western blotting

309 Cells were washed twice in ice-cold PBS and harvested in RIPA buffer (Ther-

310 moFisher Scientific) supplemented with protease inhibitors. Cell lysates were centri-

311 fuged at 20,000 x g for 20 min at 4°C and the total protein lysate was quantified using

312 Bradford Reagent (B6916; Sigma). Then, 40 µg of total protein lysate was separated

313 on a 4-12 % Bis-Tris Plus Gel (Life Technologies) and transferred to a nitrocellulose

314 membrane using the iBlot2 Gel Transfer Device (Life Technologies). Membranes

315 were blocked in 0.5 % non-fat dry milk and probed with anti-ABCG1 (1:1000;

316 ab52617, abcam) and antinuclear matrix protein p84 (1:5,000; ab487, abcam) pri-

317 mary antibodies anti-rabbit IgG (1:20,000; Ab205718, abcam) and anti-mouse IgG

318 (1:5,000; A4416, Sigma Aldrich) secondary antibodies for 1 h at room temperature.

319 Membranes were exposed using Clarity Western ECL Substrate (Bio-Rad) and pro-

320 tein bands were detected on a LI-COR Imaging system with C-DiGit Image Studio 4.0

321 software (LI-COR Biosciences, Ltd., UK).

322   4.7   Phosphorylated AKT analysis

323   Differentiated cells cultured in DMEM/F12 medium were serum starved overnight and

324   then washed with PBS and stimulated with or without 200 nM insulin for 1 h in

325   DMEM/F12 (without glucose or serum) at 37°C in 5 % CO_2. The total protein was

326   harvested in RIPA buffer supplemented with protease and phosphatase inhibitors, as

327   described above. The primary antibodies (all used at 1:1,000 dilution unless other-

328   wise stated) used were anti pAKT (S473; Cell Signaling) and anti Akt (9272 - 1:5,000

329   dilution; Cell Signaling) and the secondary antibody used was goat pAb to Rb igG

330   (Ab205718 - 1:20,000 dilution; Abcam). Protein expression studies were also per-

331   formed by WES, an automated capillary-based size separation and nano-

332   immunoassay system (ProteinSimple, San Jose CA, USA - a Bio-Techne Brand),

333   according to manufacturer's protocol, for analysis performed using the Compass for

334   Simple Western software v.4.0. The wes was performed on SGBS samples (1:100)

335   for anti pAKT (S473; Cell Signaling), anti Akt (9272 - 1:100 dilution; Cell Signaling)

336   anti pERK (9102; Cell Signaling) and anti ERK (9101 - 1:100 dilution; Cell Signaling).

337   4.8   Glucose uptake assay

338   A Glucose Uptake-Glo Assay (J1342; Promega) was used to measure glucose up-

339   take in differentiated cells (day 12), according to the manufacturer's protocol. A total

340   of 20,000 cells in 100 µl media were plated in each well of a 96-well white plate. Dif-

341   ferentiated cells were cultured overnight in DMEM/F12 media with no serum. On the

342   day of the assay, the media was replaced with 100 µl DMEM/F12 (without glucose or

343   serum) supplemented with or without 1 µM insulin and incubated for 1 h at 37°C in

344   5 % CO_2. Cells were washed with PBS and 50 µl 1 mM 2-Deoxy-D-glucose (2DG)

345   was added to each well and incubated for 10 min at room temperature. Next, 25 µl

346   Stop Buffer was added followed by 25 µl Neutralization Buffer per well. Finally, 100 µl

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347   2DG6P Detection Reagent was added and incubated for 4.5 h at room temperature.

348   The luminescence was recorded on a Mithras LB 940 luminometer (Berthold Tech-

349   nologies) and analysed using the MicroWin Software (Berthold Technologies).

350   4.9   *ABCG1* silencing using shRNA lentiviral vector

351   Undifferentiated SGBS cells were plated at 50 % confluence in 6-well plates and in-

352   fected with commercial lentiviral particles targeting either human *ABCG1*

353   (TRCN0000420907; Sigma) (TAGGAAGATGTAGGCAGATTG) or non-target controls

354   (SHC202) (CCGGCAACAAGATGAAGAGCACCAACTC) and (TRCN0000158395;

355   CCTACAGTGGATGTCCTACAT) (Sigma Aldrich). The transduced cells were se-

356   lected in media containing 1 ug/ml puromycin for 6 days. Stable *ABCG1* KD and con-

357   trol cells were then cultured and differentiated into mature adipocytes, as described

358   above.

359   4.10   Analysis of transcriptomic data from human samples

360   Transcriptomic data (Affymetrix Human Gene 1.1 ST Array) from the GSE71220

361   dataset was downloaded from the GEO database. The subjects analysed were the

362   57 control samples from the Evaluation of COPD Longitudinally to Identify Predictive

363   Surrogate Endpoints (ECLIPSE) study, of which 13 were statin-users (26). In addi-

364   tion, transcriptomic data (Illumina HumanHT-12 WG-DASL V4.0 R2 expression

365   beadchip) from peripheral blood mononuclear cells from the YELLOW II retrospective

366   study (GSE86216) was also downloaded from the GEO dataset. This included blood

367   samples from a total of 85 patients that were analysed before and after an extensive

368   8-12 week statin treatment (27). For both datasets, the data was downloaded and

369   analysed using the R packages GEOquery (45) and limma (48).

370

371   <u>List of abbreviations</u>

372   **B2M**: beta-2-microglobulin

373   **BMI**: body mass index

374   **DMP**: differentially methylated position

375   **DMR**: differentially methylated region

376   **DMSO**: dimethyl sulfoxide

377   **EWAS**: epigenome wide association study

378   **HDAC9**: histone deacetylase 9

379   **KD**: knockdown

380   **SGBS**: Simpson-Golabi-Behmel syndrome

381   **SNP**: single nucleotide polymorphism

382   **T2D**: type 2 diabetes

383

384   Declarations

385   **Ethics approval and consent to participate**

386   Not applicable

387   **Consent for publication**

388   Not applicable

389   **Availability of data and materials**

390   The datasets generated and/or analysed during the current study are available in the

391   Gene Expression Omnibus (GEO) repository, under GSE139211

392   https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE139211. Access to data-

393   sets will remain private during period of manuscript review. Reviewers can access

394   data using the following secure token: yzmzwwkaprgpdoj

395   **Competing interests**

396   The authors declare that they have no competing interests.

Authors' contributions

AK, AA, TA, FT and PF designed the project. AK, FT, SM, RB and HC have performed the experiments. SL performed the methylome wet lab experiments and MC performed the methylation analysis. AK and MC prepared the figures. AK and PF wrote the manuscript. All authors edited the paper.

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567

568   Figure Legends

569   **Figure 1: Response of SGBS cell line to statin treatment.** (a) The method used in

570   treating the SGBS cell line at day 6 of differentiation for 6 days. (b) Microscope imag-

571   es showing lipid droplets in statin-treated cells at day 12 of differentiation following

572   statin treatment for atorvastatin, mevastatin and DMSO controls (x10 magnification -

573   scale bar 10 μm). (c) Expression of key adipose genes for statin-treated cells com-

574   pared to time-matched DMSO controls (normalised to housekeeping gene B2M). * p

575   < 0.05; ** p < 0.01 (d) Protein expression of insulin signalling proteins pAkt and pErk

576   in statin-treated cells compared to controls using WES.

577 **Figure 2: Whole methylome analysis of statin-treated SGBS cells.** (a) Volcano

578 plots of whole methylome results for statin-treated cells (grey indicates log2 fold

579 change < 1). (b) The hypomethylation of the cg14566882 CpG within the *HDAC9*

580 gene in atorvastatin and mevastatin-treated cells compared to vehicle-treated DMSO

581 cells in the 4 biological replicates (raw β-values shown). (c) The mRNA expression

582 level of *HDAC9* in mevastatin and atorvastatin-treated SGSB cell line. * $p < 0.05$; ** p

583 < 0.01 (d) The protein expression of ABCG1 compared to housekeeping gene p84

584 shows a reduced expression in atorvastatin and mevastatin-treated SGBS cells.

585 **Figure 3: Adipogenesis changes in stably transfected *ABCG1* KD cells.** (a)

586 Western Blot protein expression of ABCG1 (a) during differentiation (b) after silencing

587 in SGBS cell lines. (c) Lipid content analysed by red oil of *ABCG1* KD compared to

588 controls. (d) Expression of key adipose genes at day 12 differentiation in KD *ABCG1*

589 cells compared to shRNA controls, normalised to housekeeping gene *B2M* and com-

590 pared to DMSO-vehicle controls. Experiments were performed at n = 4 biological rep-

591 licates. (e) Glucose uptake in *ABCG1* KD compared to controls stimulated with or

592 without 1 µM insulin for 1 hour. Fold change in KD and control cells compared to cells

593 not treated with insulin. (f) Analysis of insulin signalling in SGBS *ABCG1* KD cell line

594 through protein expression of phosphorylated AKT at day 12, stimulated with or with-

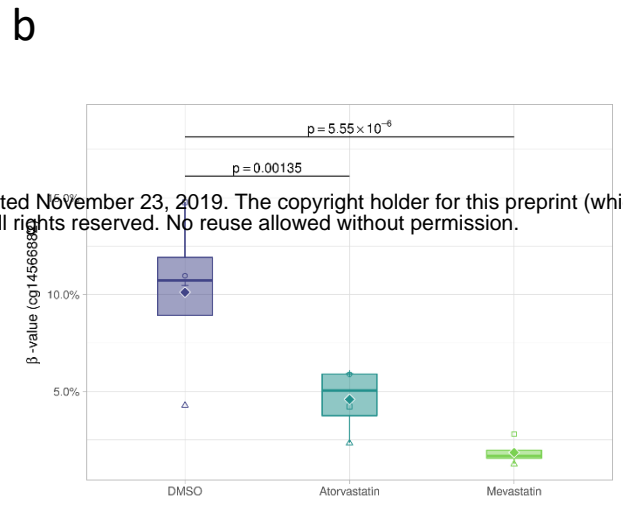595 out 200 nM insulin for 1 hour, using western blot analysis. * $p < 0.05$; ** $p < 0.001$; ***
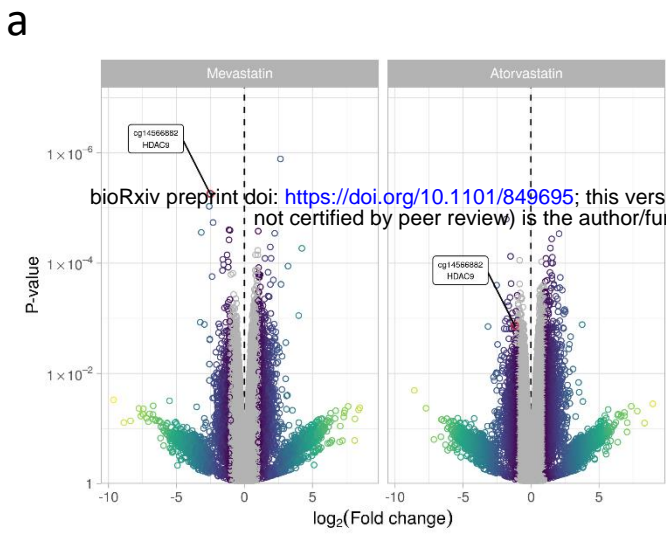
596 $p < 0.0001$.

597 **Figure 4: The expression of *ABCG1* in human samples.** (a) *ABCG1* expression

598 was reduced in 13 statin-treated individuals compared to control non-users using

599 transcriptomic data. (b) Data from a total of 85 samples, after extensive statin treat-

600 ment for 8-12 weeks, there was a reduction in *ABCG1* expression compared to base-

601 line levels in two probes.

22

602 **Figure 5: A schematic representation of the role of adipocyte turnover in health**

603 **and disease.** In healthy individuals, preadipocytes differentiate into mature adipo-

604 cytes, which have a role in maintaining insulin sensitivity. However, in response to

605 statins, epigenetic changes in *HDAC9* cause acetylation changes in *ABCG1* and

606 other crucial adipogenesis genes, which in-turn an obstruction of differentiation and
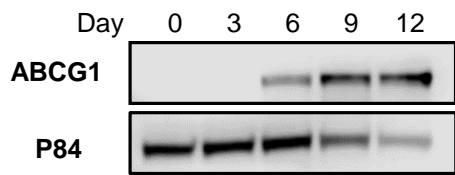
607 metabolic dysfunction.
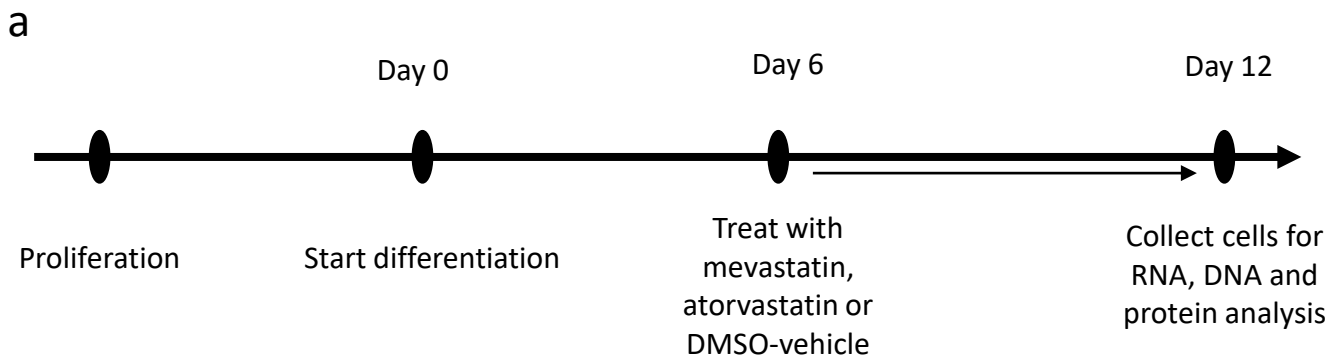
a

Day 0 — Day 6 — Day 12

Proliferation — Start differentiation — Treat with mevastatin, atorvastatin or DMSO-vehicle — Collect cells for RNA, DNA and protein analysis

b

Control Atorvastatin Mevastatin

c

Fold-change

DMSO
ATORV
MEVA

PPARg GLUT4 ADIPOQ ACACA ACACB LPL SREBF1 ABCG1 LEPTIN

d

DMSO ATORV MEVA

Insulin - + - + - +

ERK

pERK

AKT

pAKT

a

b

c

d

a

Day 0   3   6   9   12

ABCG1

P84

b

KD Control

ABCG1

P84

c

Lipid content in ABCG1 KD day 12

Fold-change

1.5

1.0

0.5

0.0

Control   KD

*

d

***ABCG1* KD in adipocytes**

Fold-change

1.5

1.0

0.5

0.0

■ Control
■ ABCG1 KD

PPARg GLUT4 ADIPOQ ACACA ACACB FASN FABP4 PLIN1 LPL

***   **   ***   *   ***   ***   ***   ***   ***

c

Fold-change

4

3

2

1

0

■ No Insulin
■ Insulin

KD   Control

**

d

KD   Control

Insulin   -   +   -   +

Akt

P84

Adipogenesis

Metabolic Homeostasis
↑GLUT4/glucose uptake
Insulin sensitive

↑HDAC9

Statins

HDAC9

p300

↓ABCG1
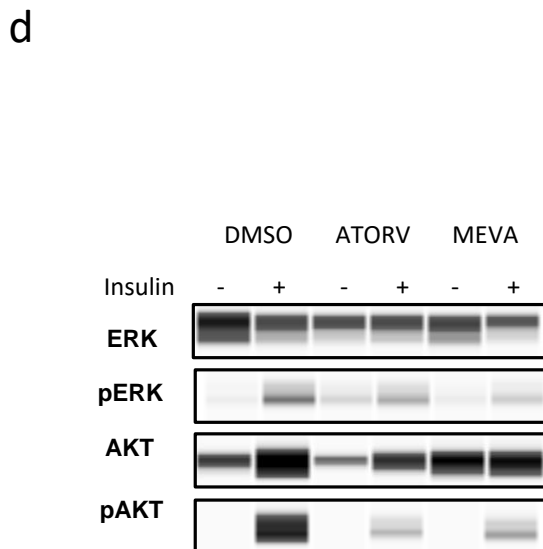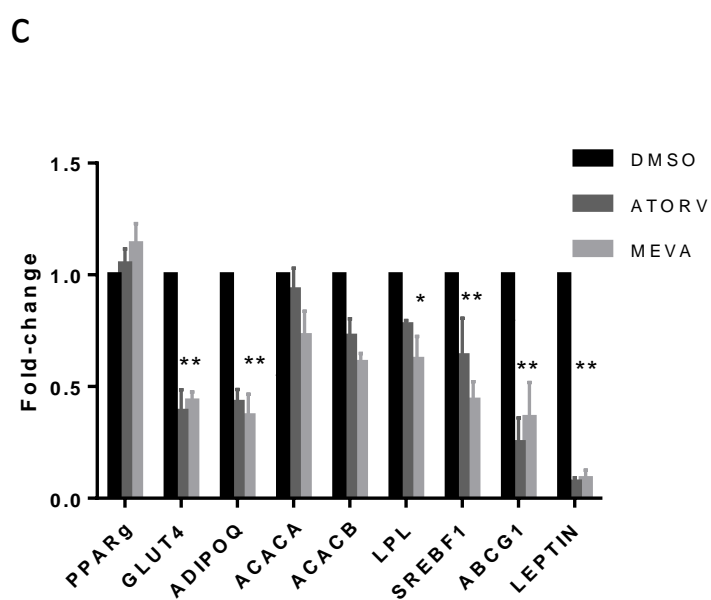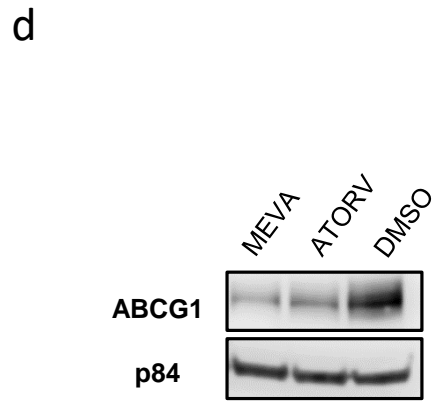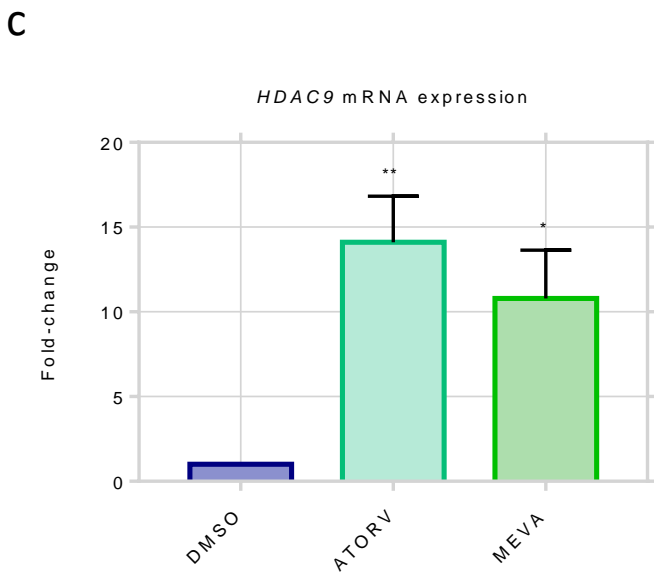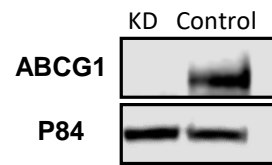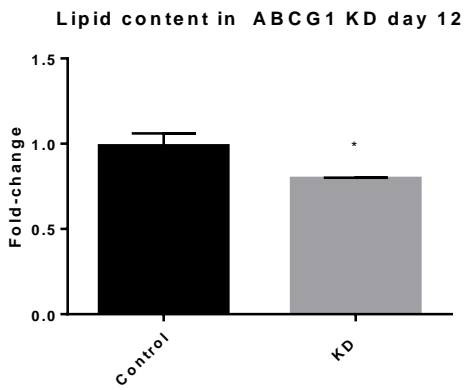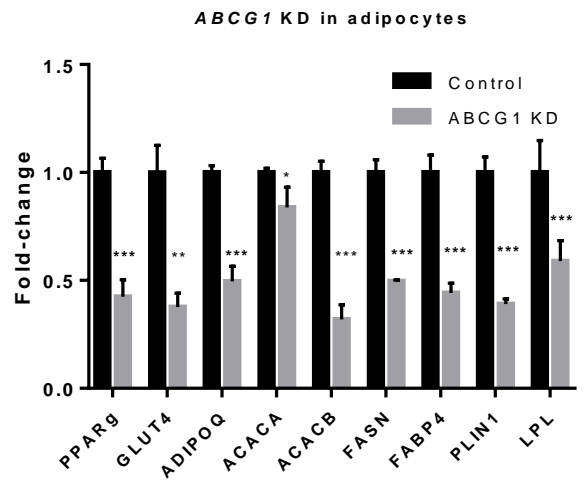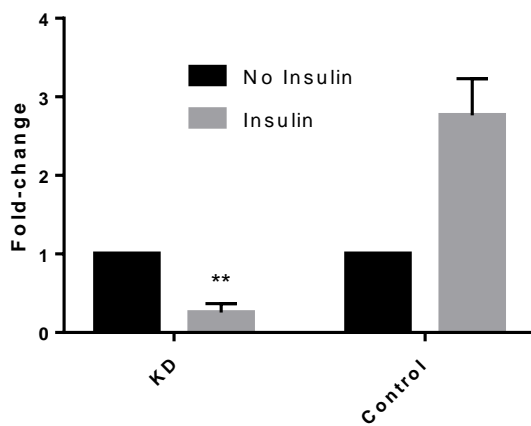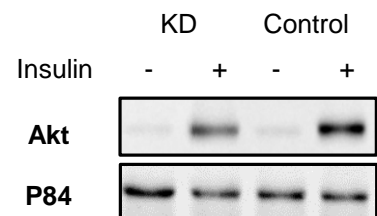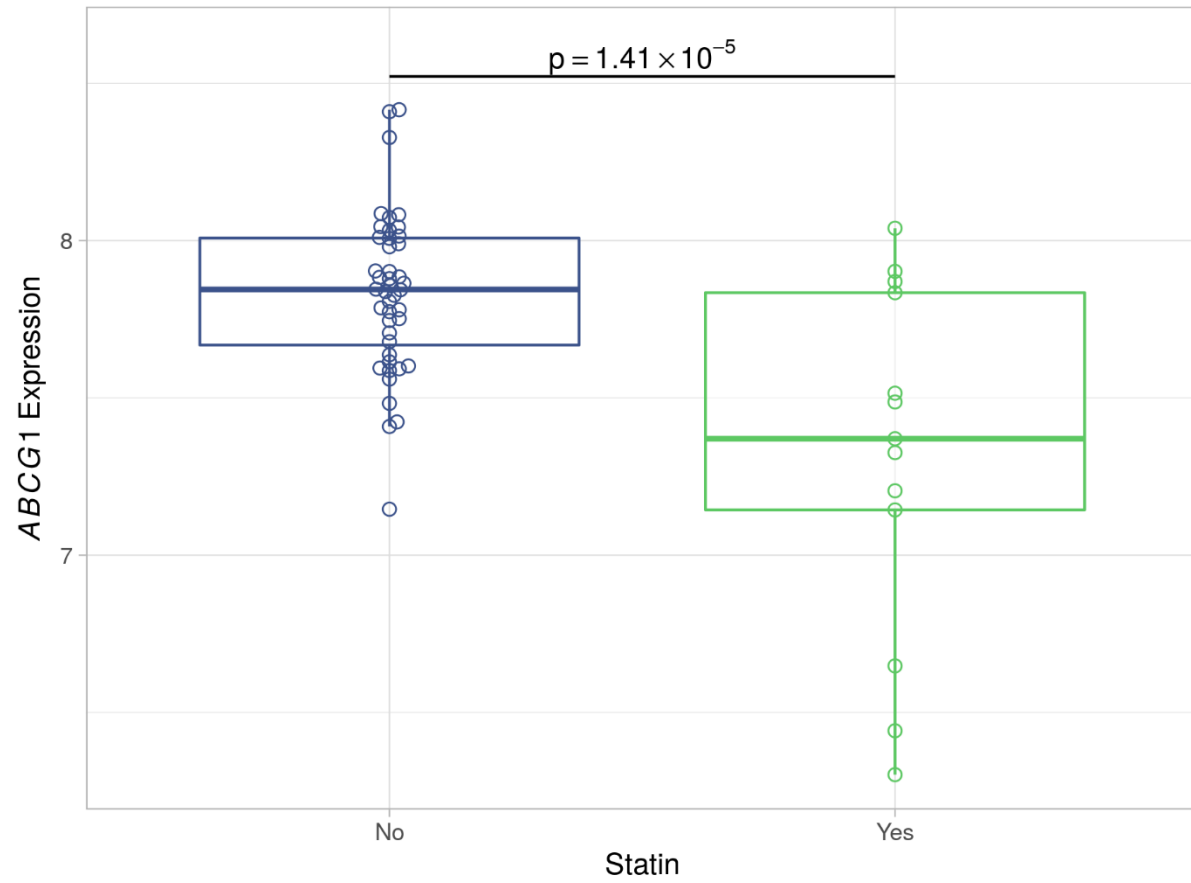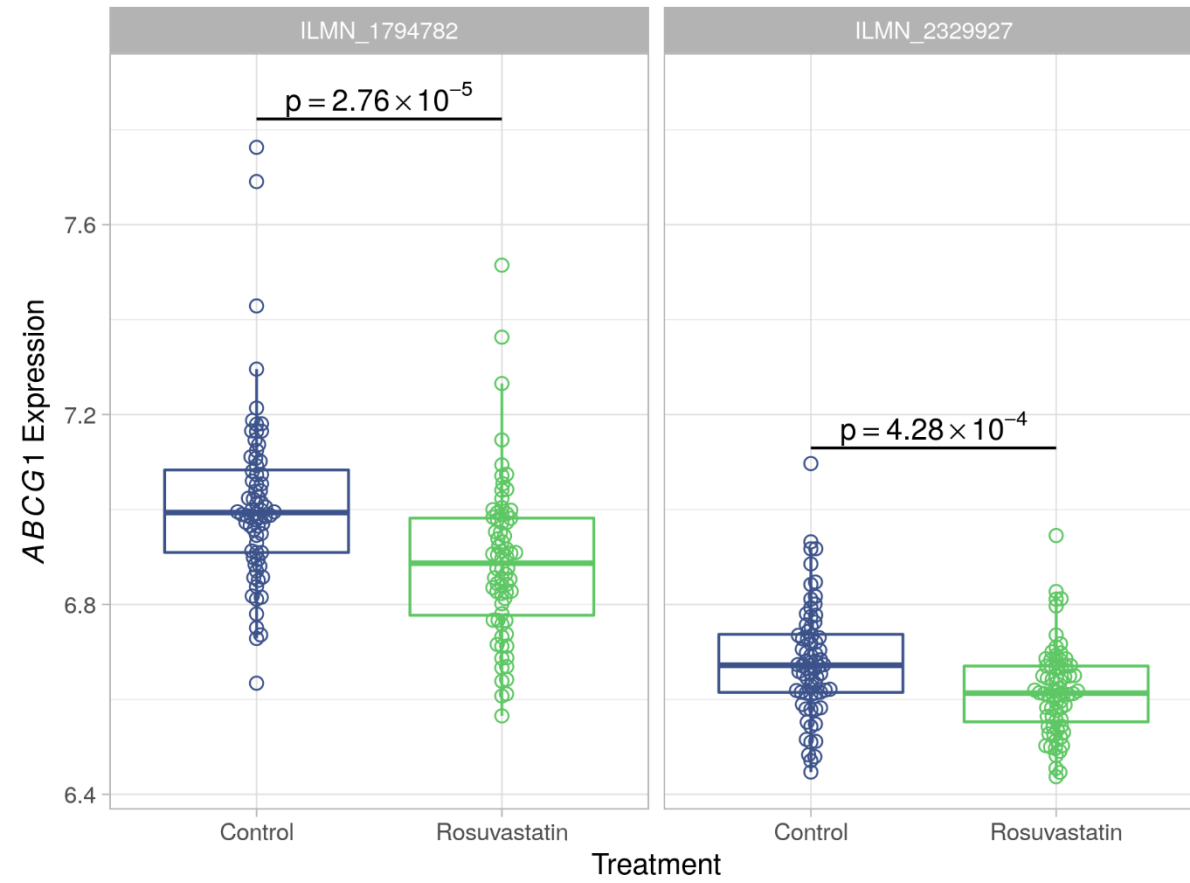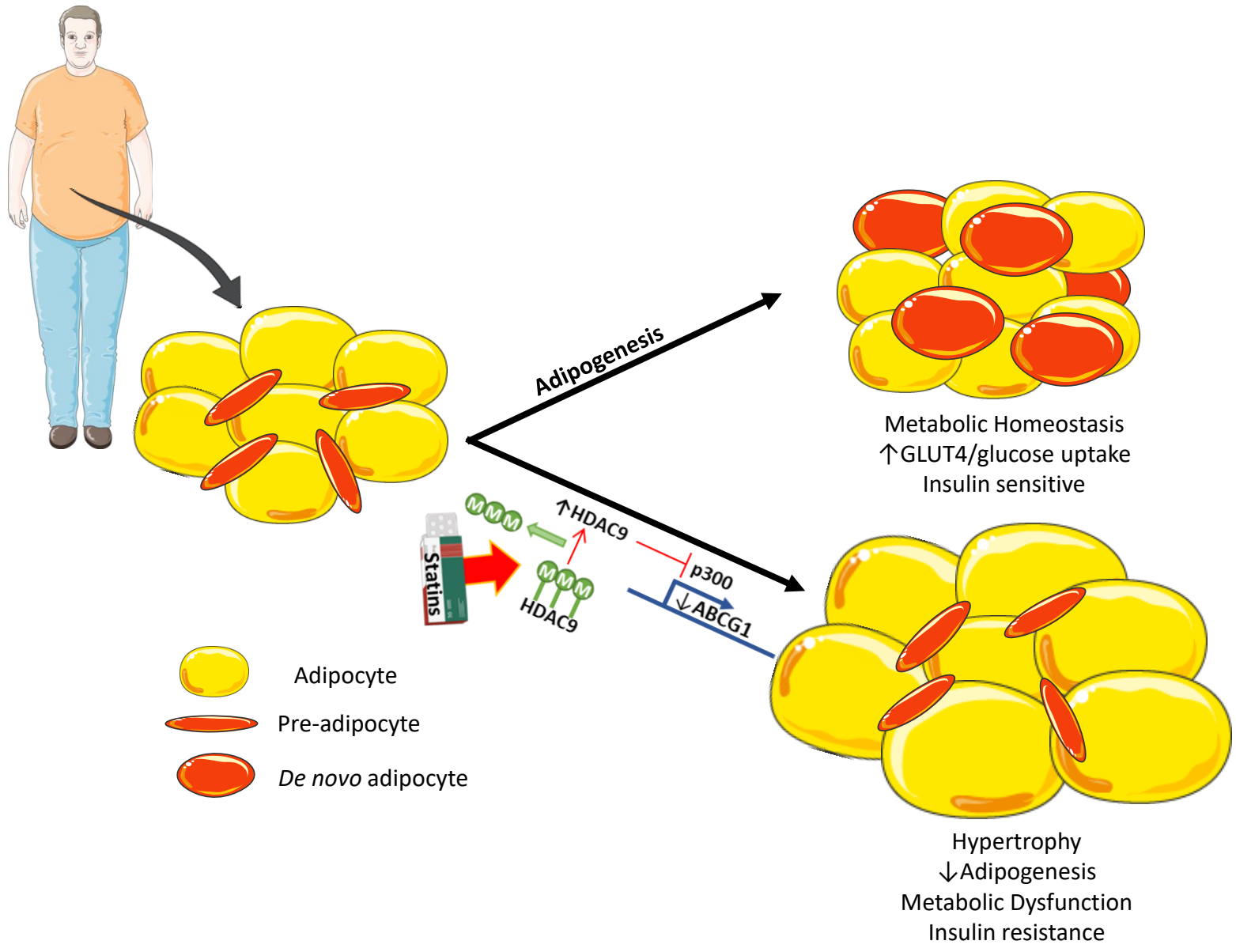
Adipocyte

Pre-adipocyte

De novo adipocyte

Hypertrophy
↓Adipogenesis
Metabolic Dysfunction
Insulin resistance