

1 **Statins reduce intratumor cholesterol affecting adrenocortical cancer growth**

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33
34 **Short title:** Statins reduce ACC growth

35 36 **Abbreviations**

37 ACC: adrenocortical cancer;
38 HMGCR: 3-Hydroxy-3-Methylglutaryl-CoA Reductase
39 ER α : estrogen receptor alpha,

40 COXIV: cytochrome c oxidase

41 OCR: oxygen consumption rate

42

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44 **Abstract**

45

46 Mitotane causes hypercholesterolemia in ACC patients. We suppose that cholesterol increases within the
47 tumor and can be used to activate proliferative pathways. In this study, we used statins to decrease
48 intratumor cholesterol and investigated the effects on ACC growth related to ER α action at the
49 nuclear and mitochondrial levels. We first used microarray to investigate mitotane effect on genes
50 involved in cholesterol homeostasis and evaluated their relationship with patients' survival in ACC TCGA.
51 We then blocked cholesterol synthesis with simvastatin and determined the effects on H295R cell
52 proliferation, estradiol production and ER α activity in vitro and in xenograft tumors. We found that mitotane
53 increases intratumor cholesterol content and expression of genes involved in cholesterol homeostasis, among
54 them INSIG, whose expression affects patients' survival. Treatment of H295R cells with simvastatin to
55 block cholesterol synthesis decreased cellular cholesterol content and this affected cell viability. Simvastatin
56 reduced estradiol production and decreased nuclear and mitochondrial ER α function. A mitochondrial target
57 of ER α , the respiratory complex IV (COX IV) was reduced after simvastatin treatment, which profoundly
58 affected mitochondrial respiration activating apoptosis. In vivo experiments confirmed the ability of
59 simvastatin to reduce tumor volume and weight of grafted H295R cells, intratumor cholesterol content, Ki-
60 67 and ER α , COX IV expression and activity and increase TUNEL positive cells. Collectively these data
61 demonstrate that a reduction in intratumor cholesterol content prevents estradiol production, inhibits
62 mitochondrial respiratory chain inducing apoptosis in ACC cells. Inhibition of mitochondrial respiration by
63 simvastatin represents a novel strategy to counteract ACC growth.

64

65 **Introduction**

66 Adrenocortical carcinoma (ACC) is a rare but aggressive cancer with a very poor prognosis. At
67 present the only valuable option for ACC therapy is an early prognosis followed by surgical
68 resection of the tumor. Mitotane (1,1-dichloro-2-(o-chlorophenyl)-2-(p-chloro-phenyl)-ethane or o,p-
69 DDD), an inhibitor of steroid synthesis with adrenolytic activity, alone or combined with cytotoxic
70 drugs such as etoposide, doxorubicin, and platinum agents, is the only specific treatment for ACC
71 (1). Overall survival rate at 5-years is 16-38%, but in the case of metastatic disease (stage IV),
72 survival rate at 5 years drops to less than 10 % (2). Because mitotane treatment has a relatively low
73 response rate and carries significant systemic toxicity, better treatment methods are critically
74 needed for more effective targeting and inhibition of ACC.

75 Mitotane works by inhibiting cytochrome P450s involved in steroid synthesis and by inhibiting
76 SOAT1, an enzyme involved in cholesterol esterification, leading to an increase in free cholesterol
77 toxic to the cell. Mitotane serum concentrations above 14 mg/l are required for its therapeutic
78 effects (3). However, even with administration of high doses, effective mitotane serum
79 concentrations are achieved in only half of patients and are never reached in others (4). Doses
80 below 14 mg/l are less effective in inhibiting SOAT1, but still able to induce 3-hydroxy-3-
81 methylglutaryl-coenzyme A reductase (HMGCR) activity in the liver (5), favoring an increase in
82 serum cholesterol levels, a side effect that ACC patients experience during mitotane treatment (6).
83 Possibly, the increase in serum cholesterol will allow the adrenal tumor to have a higher uptake.
84 Alternatively, a direct effect of mitotane on adrenal HMGCR, impacting de novo synthesis, cannot
85 be excluded, since the adrenal can synthesize cholesterol in the endoplasmic reticulum (7). Both
86 uptake or de novo synthesis will increase cholesterol availability within the tumor cells, favoring
87 activation of proliferative mechanisms.

88 Our previous studies have demonstrated that ACC is characterized by aromatase over-expression
89 (8) and insulin-like growth factor II (IGF-II) (resulting overexpressed in 90% of ACCs and
90 activating an autocrine mitogenic effect) can induce aromatase transcription (9). Then, it is possible
91 that in ACC patients, despite normal circulating estrogen levels, a higher local estrogen production
92 can occur, allowing estrogens, through estrogen receptor α (ER α), to foster ACC progression.

93 A study performed on 152 ACC patients showed that increased intra-abdominal fat is associated
94 with tumor worsening and decreased survival (10). The rise in fat deposition observed in mitotane-
95 treated patients can also be responsible for increased estradiol production, since the adipose tissue
96 has high aromatase expression, which can convert steroid precursors into estrogens (11).
97 Importantly, it has been recently suggested that adipose tissue may contain the steroidogenic
98 machinery necessary for the initiation of de novo steroid biosynthesis from cholesterol (12). The

99 increase in cholesterol and body fat is also responsible for lowering hematic mitotane concentration,
100 since the drug is a lipophilic compound and accumulates into circulating lipoprotein fractions and
101 high-lipid-containing tissues (13).
102 A drug capable of reducing cholesterol synthesis both at hepatic and intratumor level would be
103 effective in preventing ACC growth. In this study we propose statins, drugs that target HMGCR,
104 largely used to reduce hypercholesterolemia, as a valid treatment for ACC. By reducing cholesterol
105 synthesis within the tumor cells, statins could be a reliable mean to prevent estrogen production and
106 then action through ER α in ACC.

107 **Materials and Methods**

108 Detailed experimental information is provided in the Supplemental Experimental Procedures.

109

110 **Cell cultures and tissue**

111 H295R, SW13 and Y1 cells were purchased from ATCC. H295R were cultured as previously
112 described (14). SW13 were maintained in DMEM/F-12 with 10% fetal bovine serum (FBS). Y1
113 cells were maintained in DMEM/F-12 with 2.5% FBS and 15% horse serum. Cell monolayers were
114 subcultured into 6 well plates for protein and RNA extraction (4×10^6 cells/plate) and 12 multi-well
115 for colony formation assay (1×10^3 cells/well) and grown for 14 days. Cells were treated with statins
116 or mitotane (Sigma) in DMEM/F-12 containing 10% FBS.

117 Fresh-frozen samples of adrenocortical tumors, removed at surgery, were collected at the hospital-
118 based Divisions of the University of Padua (Italy). Tissue samples were obtained with the approval
119 of local ethics committees and written informed consent from patients. Studies were conducted in
120 accordance with the Declaration of Helsinki guidelines as revised in 1983 and approved by the
121 institutional review board of the University of Padua. Diagnosis of malignancy was performed
122 according to the histopathological criteria proposed by Weiss *et al.* (15) and the modification
123 proposed by Aubert *et al.* (16). Patients included in the mitotane-treated group received the drug for
124 at least 4 months at the dose of 4-6 g/day.

125

126 **MTT assay**

139 The effect of simvastatin on cell viability was measured using 3-[4,5-dimethylthiazol-2-yl]-2,5-
140 diphenyltetrazoliumbromide (MTT) assay as previously described (17). Briefly, cells were cultured
141 in complete medium in 48 well plates (1×10^4 cells/well) for 48 h, then treated in 10% FBS medium
142 for 24, 48 or 72 h. Fresh MTT (Sigma), resuspended in PBS, was then added to each well (final
143 concentration 0.33 mg/mL). After 2h incubation, cells were lysed with 200 μ l of DMSO and optical
144 density was measured at 570 nm in a multi plate reader (Synergy H1, BioTek, Agilent).

145

146 **Intracellular cholesterol extraction and colorimetric cholesterol assay**

147 Cholesterol was measured using a colorimetric cholesterol assay kit (Cell Biolabs). Intracellular
148 cholesterol was extracted from cells using a mixture of chloroform, isopropanol and NP-40 (7: 11:
149 0.1). The same mix was added to tumor samples of known weight, and lysed using stainless steel
150 beads in the Bullet Blender Tissue Homogenizer (Next Advance, Inc.; Troy, NY USA). Purified
151 water was then added to lysed samples, and upon centrifugation, the organic, bottom phase was
152 taken and dried by vacuum centrifugation. The resulting lipid pellet was resuspended in 200 μ l of

153 1X cholesterol assay buffer. Then, 50 μ L of sample were processed according to manufacturer's
154 instruction.

155

156 **ELISA for Estradiol**

157 The H295R cells were kept in complete medium for 48h in multi-wells of 12 (1×10^5 cells/well) and
158 treated in DMEM F-12 enriched with 5% DCC-FBS (FBS treated with Dextran coated in order to
159 repair steroids) with increasing doses of simvastatin (2.5-5-10 μ M). After 48 hours of treatment the
160 contents of 17β -estradiol (E2) was measured by means of ELISA (enzyme-linked immuno-
161 absorbent assay) (NovaTec) following manufacturer's instruction.

162

163 **Spheroids culture**

164 A single cell suspension was prepared using enzymatic (1X Trypsin-EDTA, Sigma Aldrich,
165 #T3924), and manual disaggregation (25 gauge needle) (18). Cells were plated at a density of 500
166 cells/cm² in spheroids medium (DMEM-F12/B27/EGF (20ng/ml)/ Pen-Strep) in non-adherent
167 conditions, in culture dishes coated with (2-hydroxyethylmethacrylate) (poly-HEMA, Sigma,
168 #P3932). Cells were grown for 5 days and maintained in a humidified incubator at 37°C at an
169 atmospheric pressure in 5% (v/v) carbon dioxide/air. After 5 days of culture, spheres $>50 \mu$ m were
170 counted using an eye piece graticule, and the percentage of cells plated which formed spheres was
171 calculated and is referred to as percentage spheroids formation, and was normalized to one (1 =
172 100% TSFE, tumor-spheres formation efficiency). Cells were directly seeded on low-attachment
173 plates in the presence of treatments.

174

175 **Colony formation**

176 The NCI-H295R cells were plated in 12-well plates (1×10^3) and allowed to attach. Treatment
177 commenced for 24 hours with drug alone. Untreated or simvastatin-treated cells were controls. The
178 medium was changed and surviving cells were allowed to grow colonies of ≥ 50 cells for 2 weeks,
179 washed, fixed, and stained with Coomassie blue, and counted. Total colony numbers were
180 normalized to untreated controls.

181

182 **Protein extraction and Western-blotting**

183 H295R cells were cultured in complete medium for 48 hours in 100 mm plates (2×10^6 cells) before
184 being treated in complete medium with simvastatin for 48 hours and then used for cytosolic and
185 mitochondrial protein extraction. The extracts were then analyzed by western blotting (WB).

186 Total proteins were prepared using RIPA buffer. Equal amounts of proteins were subjected to WB
187 analysis. Blots were incubated overnight at 4 °C with primary antibodies. Membranes were
188 incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham) and
189 immunoreactive bands were visualized with the ECL (Amersham).

190

191 **Xenograft experiments**

192 All animal procedures approved by the Ethics Research Committee University animals from
193 Calabria (protocol No. 1077/2016-PR from the Ministry of Health to Dr. Sirianni) were performed
194 in female Foxn1nu mice (Harlen Envigo) mice. Following H295R xenograft establishment, mice
195 received 4 mg/kg/d of simvastatin in the water for 24 days, and tumors were harvested and weighed.
196 The water with the treatment has been replaced every week. The dose was chosen to equal the
197 therapeutic dose used for patients of 20 mg/d (based on the equivalence of body surface area) (19).

198

199 **Immunohistochemistry**

200 IHC experiments were performed using 8 mm thick paraffin-embedded sections of H295R
201 xenograft tumors from mice treated with vehicle or simvastatin. Slides were deparaffinized and
202 dehydrated and incubated over-night at 4°C with Aromatase (MBL International Corporation,
203 Woburn, MA, USA, MCA2077S, 1:50), COXIV (Abcam, ab14744, 1:200), Ki-67 (DAKO, M7240,
204 1:100), CCNE (Bethyl, IHC-00341, 1:100), ER α (Santa Cruz, sc-8002, 1:50), TOM20 (Santa Cruz,
205 sc-17764, 1:100) primary antibodies. Then, a horse biotinylated anti-mouse/rabbit IgG was applied
206 for 1h at RT, to form the avidin biotin horseradish peroxidase complex (Vector Laboratories).
207 Immunoreactivity was visualized by DAB (Vector Laboratories). For ER α detection was used a
208 FITC-conjugated secondary antibodies (Santa Cruz) for 1h at RT. Fluorescent images were
209 collected on Olympus fluorescent microscope.

210

211 **Oil red O staining**

212 H295R cells (1×10^6) were plated on glass coverslips for 48h and then treated for 48h with
213 simvastatin (10 μ M). The cells were washed with cold phosphate-buffered saline (PBS) and fixed
214 with 4% paraformaldehyde for 30 min at RT. Then, the cells were stained with 0.5% Oil Red O
215 (Sigma-Aldrich, St. Louis, MO, USA) solution for 20 min at RT and counterstained with
216 hematoxylin for 2 min, followed by PBS washes and microscopic evaluation.

217

218 **Cytochrome C oxidase (COX)/complex IV activity**

219 Cryostat sections (8 μ m) were prepared and stored at -80°C until use. For the COX activity staining,
220 frozen sections were brought to RT, washed for 5 min with 25 mM sodium phosphate buffer, pH
221 7.4, and then incubated for 0.5, 1 or 2 h at 37°C with the COX incubation mixture. The COX
222 solution consisted of 10 mg Cytochrome C (cat# C7752, Sigma-Aldrich), 10 mg 3,3'-
223 diaminobenzidine tetrahydrochloride hydrate (cat# D5637, Sigma-Aldrich) and 2 mg catalase (cat#
224 C1345, Sigma-Aldrich) dissolved in 10 ml of 25 mM sodium phosphate buffer. The solution was
225 filtered after preparation and the pH was adjusted to 7.2-7.4 with 1 N NaOH.

226

227 **HMGCR activity assay**

228 The HMGCR activity in H295R lysates was measured with HMGCR Activity Assay Kit (CS1090,
229 Sigma, USA) according to manufacturer's instructions. The assay is based on the
230 spectrophotometric measurement of the decrease in absorbance at 340 nm, which represents the
231 oxidation of NADPH by the catalytic subunit of HMGCR in the presence of the substrate HMG-
232 CoA. Cells were lysed in RIPA buffer containing protease inhibitors. Two microliters of cell lysate
233 were used to measure HMGCR activity. One unit converts 1.0 μ mol of NADPH to NADP⁺ per 1
234 min at 37°C . The unit specific activity is defined as μ mol/min/mg protein (units/mg P).

235

236 **Detection of apoptosis by TUNEL assay**

237 The induction of apoptosis was assessed by the TUNEL assay (Terminal deoxynucleotidyl
238 transferase dUTP Nick End Labeling), a method that evaluates the fragmentation of DNA. The
239 click-it® TUNEL Alexa Fluor® Imaging Assay kit (Invitrogen) was used, following the
240 manufacturer's instructions. Sections of vehicle- and simvastatin-treated tumors from paraffin-
241 embedded H295R xenografts were cut to a thickness of 5 μ m, deparaffinized and dehydrated and
242 then used for the assay.

243

244 **Seahorse XFe96 metabolic flux analysis**

245 Real-time oxygen consumption rates (OCR) for H295R cells treated with simvastatin or vehicle
246 (control) were determined using the Seahorse Extracellular Flux (XF96) analyzer (Seahorse
247 Bioscience, MA, USA). H295R cells were maintained in DMEM supplemented with 10% fetal
248 bovine serum (FBS), 2 mM GlutaMAX, and 1% Pen/Strep. 7×10^4 cells were seeded per well into
249 XF96-well cell culture plates (Seahorse Bioscience, MA, USA), and incubated overnight at 37°C in
250 a 5% CO₂ humidified atmosphere. After 24h, cells were treated with simvastatin (2.5, 5 and 10 μ M)
251 for 48h. At the end of treatment, cells were processed as previously published (20).

252

253 **Microarray**

254 H295R cells (1×10^5) were plated on 60 mm dishes for 48h and then treated for 24h with Mitotane
255 (25 μ M). RNA was extracted using PureLink™ RNA Mini Kit (Thermo Fisher). The quality of total
256 RNA was first assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA).
257 Biotin-labeled cDNA targets were synthesized starting from 150 ng of total RNA. Double stranded
258 cDNA synthesis and related cRNA was performed with GeneChip® WT Plus Kit (Affymetrix,
259 Santa Clara, CA). With the same kit was synthesized the sense strand cDNA before to be
260 fragmented and labeled. All steps of the labeling protocol were performed as suggested by
261 Affymetrix, starting from 5.5 μ g of ssDNA. Hybridization was performed using the GeneChip®
262 Hybridization, Wash and Stain Kit. A single GeneChip® Clariom S was then hybridized with each
263 biotin-labeled sense target. GeneChip arrays were scanned using an Affymetrix GeneChip®
264 Scanner 3000 7G using default parameters. Affymetrix GeneChip® Command Console software
265 (AGCC) was used to acquire GeneChip® images and generate .DAT and CEL files, which were
266 used for subsequent analysis with proprietary software.

267

268 **RNA extraction, reverse transcription and real time PCR**

269 Following total RNA extraction, 1 μ g of total RNA was reverse transcribed and then used for PCR
270 reactions were performed in the iCycler iQ Detection System (Bio-Rad Laboratories S.r.l., Milano,
271 Italia). Final results were expressed as n-fold differences in gene expression relative to 18S and
272 calibrator, calculated using the $\Delta\Delta$ Ct method as previously shown (14).

273

274 **Patients' data analysis**

275 Gene expression and survival data were obtained using two independent cohorts of adrenocortical
276 tumors were used: Expression Cohort included 33 ACC, 22 ACA (adrenocortical adenoma) and 10
277 NA (normal adrenal) (GEO dataset GSE33371) and TCGA cohort included 78 ACC
278 (<https://portal.gdc.cancer.gov/legacy-archive>).

279

280 **Statistics**

281 All experiments were performed at least three times. Data were expressed as mean values +
282 standard error), statistical significance between control and treated samples was analyzed using
283 GraphPad Prism 5.0 (GraphPad Software, Inc.; La Jolla, CA) software. Control and treated groups
284 were compared using t-test or the analysis of variance (ANOVA). Significance was defined as $p <$
285 0.05. Microarray data analysis was performed using Partek Genomics Suite software (PGS), version
286 6.6 (6.16.0812 for Mac). Affymetrix CEL-files were extracted, normalized and summarized using

287 RMA algorithm (CEL file imported by Partek on Wed Feb 21 10:25:17 2018; Probes to Import:
288 Interrogating Probes; Probe filtering: skip; Algorithm: RMA; Background Correction: RMA
289 Background Correction; Normalization: Quantile Normalization; Log Probes using Base: 2;
290 Probeset Summarization: Median Polish) (21-23). Genes differentially expressed were identified
291 using a t-test.

292 **Results**

293 Mitotane increases intratumor cholesterol content by affecting expression of genes involved in the 294 regulation of cholesterol homeostasis.

295 Here we wanted to determine if mitotane can also increase cholesterol content in the tumor. As it
296 can be seen in **Fig. 1A**, cholesterol is increased in ACC from patients treated with mitotane
297 compared to tumors from ACC patients that underwent surgery prior to any treatment. A key
298 enzyme in cholesterol synthesis is HMGCoA reductase (HMGCR). We conducted a retrospective
299 analysis of publicly available microarray data from ACC patients' cohorts. Expression levels of
300 HMGCR are higher in ACC when compared to the normal adrenal (NC) (**Fig. 1B**). However, its
301 expression does not affect patients' survival (**Fig. 1C**). We evaluated HMGCR protein expression
302 and activity in H295R cells after 2 and 14 days of mitotane treatment. As previously demonstrated
303 in hepatocytes, mitotane increases the enzyme activity (**Fig. 1D**).

304 We also used H295R cells treated with mitotane for 24 hours to perform gene expression
305 microarray analysis. Using as cutoff of 1.5 in fold change and a p value ≤ 0.05 we identified 344
306 transcripts regulated by mitotane. Importantly, an enrichment analysis for the categories of Gene
307 Ontology (GO), indicated that the drug preferentially increases expression of genes involved in
308 metabolism, and more specifically we looked into cholesterol metabolism (**Fig. 1E**). Among the
309 genes present in this GO group we further investigated sterol regulatory element-binding protein 1
310 (SREBP1) and Insulin induced gene 1 (INSIG1) encoding for proteins working as cholesterol
311 sensors, and ATP-binding cassette sub-family G member 1 (ABCG1), encoding for a protein that
312 mediates cholesterol efflux from the cells to ApoA1 (apolipoprotein A1), a component of HDL.
313 Results from microarray were confirmed by real-time PCR using short-term (24h) and long-term (2
314 weeks and 3 weeks) mitotane-treated H295R cells. As observed in microarray data, SREBP1,
315 INSIG1, and ABCG1 expression was decreased after 24h treatment (**Fig. 1F, 1I, 1L**). On the long
316 term treatment, expression of SREBP1 was maintained low (**Fig. 1G**). Survival data for this factor
317 show that when its expression is low patients have a trend to a worse outcome, even if not
318 significant (**Fig. 1H**). For INSIG1 and ABCG1 we found that long term treatment with mitotane did
319 not produce a decrease in gene expression, but mRNA levels were kept similar to those seen in
320 untreated samples (**Fig. 1J, 1M**). The higher expression of these genes maintained in the presence
321 of increased cholesterol amounts (caused by mitotane) indicate loss of cell ability to sense
322 cholesterol levels. Importantly, survival data for INSIG indicate that higher expression is associated
323 with worse survival (**Fig. 1K**). A similar trend in the association was observed for ABCG1, even if
324 there was not a significant difference between the two groups (**Fig. 1N**).

325

326 A decrease in ACC intracellular cholesterol positively associates with decreased tumor growth *in*
327 *vitro* and *in vivo*.

328 The use of simvastatin was able to reduce H295R cell viability in a time and dose-dependent
329 manner (**Fig. 2A**). Importantly, the decreased cell viability was rescued by addition of mevalonate,
330 the product of HMGCoA reductase activity (**Fig. 2B**). These effects were reproduced by fluvastatin
331 and rosuvastatin, (**Fig. S1A**). Additionally, we used two additional cell lines, SW13 and Y1, and
332 found that all tested statins produced effects similar to those observed in H295R cells (**Fig. S1 B,**
333 **C**). In the clonogenic assay, simvastatin treated cells formed significantly less colonies when
334 compared to vehicle treated cells, illustrating the tumor suppressor function of this drug (**Fig. 2C**).
335 When H295R cells were grown as spheroids in the presence of simvastatin, we observed a
336 substantial dose-dependent decrease in sphere numbers (**Fig. 2D**). To evaluate if intratumor
337 cholesterol depletion could reduce ACC growth *in vivo*, xenografts were generated by implanting
338 H295R cells in the flank of athymic nude mice. When tumors reached an average of 200 mm³ mice
339 were administered vehicle versus simvastatin at 4 mg/kg/day for 24 days and tumors were measured
340 twice a week. Tumor growth of the statin treated group was significantly smaller than the vehicle
341 treated group (**Fig. 2E**). Tumor volume at the end of the experiment was 60% smaller in animals
342 receiving simvastatin (**Fig. 2E**), and tumor weight was decreased by 58% (**Fig. 2F**). In parallel with
343 the decline in tumor size with simvastatin, there was a decrease in Ki-67 staining (**Fig. 2G and Fig.**
344 **S2A**).

345
346 Decreased cholesterol availability in ACC impairs estradiol production

347 After 48h treatment simvastatin at the dose of 10 μM caused a 33% reduction in intracellular
348 cholesterol (**Fig. 3A**). We also evaluated cholesterol content in H295R xenografts. By adjusting
349 to tissue weight we found a concentration of 9 ng/mg of tissue, while statin decreased this amount
350 to 6.3 ng/mg of tissue (**Fig. 3B**). In addition, frozen sections from tumors were stained for lipids
351 using Oil Red O, less red stain is observed in treated tumors, indicative of a reduced amount of lipid
352 deposition (**Fig. 3B and Fig. S2B**). Thus, intratumor cholesterol has an important cell-autonomous
353 role in ACC growth and in parallel statins lessens ACC tumor development. Treatment of H295R
354 cells for 48h with increasing concentrations of simvastatin decreased E2 production in a dose
355 dependent manner, as demonstrated by ELISA of H295R culture media, with 10 μM producing a
356 50% decrease in E2 content (**Fig. 3C**). When we evaluated aromatase (CYP19) gene expression we
357 did not find any change in mRNA, neither *in vitro* (**Fig. 3D**) nor *in vivo* (**Fig. 3G**), indicating that
358 simvastatin does not affect transcriptional regulation of this gene. In fact, expression of
359 steroidogenic factor 1 (SF-1) did not change after simvastatin treatment (**Fig. 3E**). However, WB

360 analysis indicated a decrease in aromatase protein content following statin treatment of H295R (**Fig.**
361 **3F**), data that was confirmed by IHC on xenografts tumors (**Fig. 3H and Fig. S2C**). Additionally,
362 the presence of mevalonate was able to overcome the inhibition on aromatase expression seen in the
363 presence of simvastatin (**Fig. S3A**).

364

365 Decreased E2 availability in ACC impairs ER α function.

366 ER α has a role in regulating transcription of mitochondrial genes involved in cellular respiration
367 (24). We evaluated both nuclear and mitochondrial ER α activity after simvastatin treatment.
368 Expression of ER α was decreased by simvastatin *in vitro* (**Fig. 4A Fig. S4A and S4B**) and *in vivo*
369 (**Fig. 4B Fig. S2D**), a similar effect was observed on cyclin E, a known target of ER α , both *in vitro*
370 and *in vivo* (**Fig. 4C, D Fig. S2E**). Additionally, the presence of mevalonate was able to overcome
371 the inhibition on ER α expression seen in the presence of simvastatin (**Fig. S3A**).

372 These effects are opposite to those elicited by E2, which instead increased cyclin E expression (**Fig.**
373 **S3B**). Mitochondrial protein fraction was used for WB analysis of ER α , we observed that
374 simvastatin treated samples had a lower content of the nuclear receptor (**Fig. 4E**). WB analysis of
375 all the components of the respiratory chain (COX I to IV plus ATP synthase) can be performed
376 using a mix of 5 different antibodies (OX-PHOS). With this approach we identified a decreased
377 expression of COXIV (**Fig. 4F and S4A, S4B**), a known target of ER α . On the contrary, E2
378 treatment increased COXIV expression, and is able to prevent statin inhibitory effect (**Fig. S3C**).
379 Data were also confirmed on statin-treated xenografts where we observed a reduced COXIV
380 expression (**Fig. 4G and Fig. S2F**) and activity (**Fig. 4H and Fig. S2G**) compared to vehicle treated
381 xenografts. We monitored cellular oxygen consumption rates (OCR), and demonstrated that
382 simvastatin is able to reduce oxygen consumption in a dose-dependent manner (**Fig. 5A**). Statin
383 exposure profoundly affected the oxidative metabolism of H295R cells. Indeed, 16 h of treatment
384 induced a clear dose-dependent decrease of the basal (**Fig. 5B**) and maximal respiration (**Fig. 5C**)
385 as well as ATP turnover (**Fig. 5E**) and spare capacity (**Fig. 5F**). No effect was observed on proton
386 leak (**Fig. 5D**). We also used immunoblotting to monitor the abundance of a known reliable marker
387 of mitochondrial mass, TOM20, in response to simvastatin treatment. We found that treated H295R
388 cells displayed a reduced expression of TOM20, *in vitro* and *in vivo* (**Fig. 5G, 5H and Fig. S2H**).

389

390 Decreased cholesterol availability in ACC activates an apoptotic pathway.

391 BAK expression and PARP-1 cleavage, were increased in H295R cells treated for 48h with
392 simvastatin, indicating activation of apoptosis (**Fig. 6A and Fig. S4C, S4D**), further confirmed by
393 TUNEL assay (**Fig. 6B**). Similarly, evaluation of apoptosis on H295R xenografts sections revealed

394 an increase of TUNEL positive cells under simvastatin treatment (**Fig. 6C**). Since Bak gene is under
395 c-Jun transcriptional control (25), we evaluated c-Jun protein levels after simvastatin treatment.
396 After 48h we observed increased levels of c-Jun and its phosphorylation status, as well as increased
397 levels of pERK1/2, whose sustained activation is associated with apoptosis (26). Addition of
398 mevalonate prevented activation of these kinases in response to simvastatin (**Fig. 6D and Fig. S4C,**
399 **S4D**). Specific inhibitors for ERK1/2 and JNK abrogated Jun and ERK1/2
400 activation/phosphorylation preventing apoptosis, as indicated by the loss of PARP1 cleavage. These
401 data indicate ERK1/2 and JNK as part of simvastatin-induced apoptotic mechanism (**Fig. 6E**).
402 Similarly to simvastatin, fluvastatin and rosuvastatin inhibited estrogen signaling and activated
403 apoptosis in H295R cells (**Fig. S4E and S4F**).

404 Discussion

405 Mitotane represents the first-line therapy for patients with ACC. However, mitotane alone or
406 combined with chemotherapy shows limited efficacy on advanced disease. In addition, mitotane has
407 high toxicity and several side effects among which hypercholesterolemia (6,27). Data from almost
408 40 years ago report the ability of mitotane to increase liver HMGCR activity *in vitro* and *in vivo* (5).
409 Since the adrenal synthesizes cholesterol *de novo*, mitotane could have a direct effect on adrenal
410 cholesterol synthesis. Having higher cholesterol bioavailability, tumor cells can foster their own
411 growth. To support our hypothesis, we first demonstrate an increase in intratumor cholesterol in
412 ACC patients treated with mitotane compared to untreated patients. Using previously published
413 microarray data publically available (28) we demonstrated the presence of increased HMGCR
414 expression in ACC samples, which, however, was not associated with decreased survival rate.
415 Increased intratumor cholesterol following mitotane treatment could be due to an increased activity
416 of HMGCR rather than to an increased expression, with the former influencing survival more than
417 the latter. Microarray analysis of mitotane-treated H295R cells helped us in identifying genes
418 involved in cholesterol metabolism and modulated by the drug. Among them, we validated INSIG1,
419 SREBP1 and ABCG1. INSIG1 encodes for a protein that retains a chaperone protein (SCAP) in the
420 endoplasmic reticulum, SCAP is necessary for delivery of SREBP1 to the Golgi, where SREBP1
421 becomes active. SREBP1, increases transcription of cholesterol synthesizing genes among which
422 HMGCR (29).

423 Short-term mitotane decreases INSIG1 and SREBP1 expression, while long term-mitotane
424 maintains low SREBP1 but high INSIG1 expression. When looking at survival data of ACC
425 patients, high INSIG1 is associated with a shorter survival. ABCG1 belongs to the family of ATP
426 binding cassette and mediates cholesterol efflux (30). Its expression is decreased by short-term
427 mitotane treatment, but this effect is lost after prolonged treatment, favoring cholesterol
428 accumulation. Since, mitotane has a prevalent accumulation in adipose tissue, the circulating levels
429 are often reduced (13,31), and the therapeutic concentrations of mitotane (between 14–20 µg/mL,
430 40–60µM) are not always reached in patients. Importantly, lower doses (10 and 25 µM) produce
431 effects that are different from what seen using 40µM. This raises a question, could cells, in the
432 presence of lower doses of mitotane, escape the normal control of cholesterol homeostasis? Can the
433 increase in cholesterol be responsible for long-term adjustment to mitotane?

434 Several reports propose a promising role for statins in cancer treatment (32). Here we demonstrate
435 that simvastatin can reduce intratumor cholesterol synthesis. Based on MTT assay IC50 for
436 simvastatin was calculated to be 10 µM. Since 1 µM simvastatin in the media corresponds to the
437 dose of 0.4586 mg/kg of body weight, we decided to treat mice with 4 mg/Kg/day. This dose is

438 equivalent to a human dose of 20 mg/d based on body surface area equivalency. This dose was
439 effective in producing more than 60% decrease in tumor growth. Importantly, this dose decreased
440 intratumor cholesterol content, supporting our hypothesis that a reduction in intratumor cholesterol
441 can decrease ACC growth. Interest for statins is not new for the therapy of ACC, however, it has
442 been considered in association with mitotane to reduce hypercholesterolemia (6,33). Our data
443 instead suggest the possibility of using lipophilic statins without mitotane but eventually with
444 cytotoxic drugs. Simvastatin, which appears to be the most effective among the tested drugs,
445 shouldn't be combined with mitotane, which is a known inducer of CYP3A4, a member of the
446 cytochrome P450 family involved in simvastatin metabolism. A recent case-study evaluated
447 management of hypercholesterolemia induced by mitotane treatment. A patient was co-
448 administered with mitotane and statins, but despite cholesterol lowering drugs, it was observed a
449 rise for total cholesterol and LDL-c level. Importantly the patient had 2 local recurrences within a 7
450 year-period, however, the course of ACC in this patient's case has been better than average. This
451 data supports our hypothesis that cholesterol can be implicated in ACC progression.

452 Cholesterol in tumor adrenal cells is used for steroid synthesis, and a decrease in its availability
453 would affect estradiol production. Our previous study demonstrated that E2 increases tumor growth,
454 and Tamoxifen, which blocks ER α activity, prevents its effects (9). With this information as
455 background, we wanted to investigate if the reduced E2 production, seen after simvastatin
456 treatment, could interfere with ER α function. We first observed a reduction in CCNE, a known
457 nuclear target of ER α . Additionally, we investigated if the expression of mitochondrial targets of
458 ER α could be influenced by simvastatin. To support a role for ER α in the mitochondria of tumor
459 adrenal, we show that E2 treatment increases the amount of COXIV levels and prevents simvastatin
460 inhibitory effect.

461 Additionally, it was reported a direct effect of statins on mitochondrial function, consequent to a
462 deficiency of complex I (34). The novelty of our data is the involvement of ER α /complex IV in
463 statin-mediated apoptosis. The reduction in COXIV alters the functioning of mitochondrial
464 respiration, changing the mitochondrial potential ultimately leading to organelle damage. High
465 COXIV activity within the tumor occurs in a significant subset of patients with high grade gliomas
466 and is an independent predictor of poor outcome (35). Importantly, it has been postulated that
467 COXIV activity may be required for the anchorage-independent growth of lung cancer cells (36). In
468 general, mitochondria appear to be an appealing target for the treatment of cancer (37). Effects on
469 COXIV negatively influence mitochondrial function as demonstrated by reduced oxygen
470 consumption rate (OCR). We have previously demonstrated that a reduced cell growth is observed
471 in breast cancer cells treated with XCT790, a drug that targets ERR α , a master regulator of cell

472 metabolism. $ERR\alpha$ inhibition reduces OCR and prevents tumor growth (20). We have also used
473 XCT790 to block $ERR\alpha$ in ACC and demonstrated its efficacy in reducing tumor growth (38),
474 further establishing that impairing mitochondrial function, reduces ACC growth. It has been shown
475 that mitotane significantly impairs mitochondrial respiratory chain function by selectively inhibiting
476 enzymatic complex IV activity. However, as a consequence of respiratory chain inhibition, mitotane
477 causes a compensatory increase of mitochondrial biogenesis (39). Differently from mitotane,
478 simvastatin reduces TOM20, a marker of mitochondrial mass. Reduced mitochondrial function after
479 treatment with simvastatin causes cell death by apoptosis, the same type of cell death that is
480 observed in H295R and SW13 cells in response to mitotane. This apoptotic mechanism requires
481 activation of c-Jun and sustained ERK1/2 phosphorylation. Farnesyl pyrophosphate (FPP) or
482 geranylgeranyl pyrophosphate (GGPP) are products of mevalonate that can be anchored onto
483 intracellular proteins through prenylation, thereby ensuring the re-localization of the target proteins
484 in the cell membranes (40-42). Ras is a prenylated protein upstream of ERK1/2 activation. The
485 observation that ERK1/2 phosphorylation is maintained in the presence of simvastatin, evidences
486 that its phosphorylation is independent of Ras and potentially involves different pathways. We
487 found that ERK phosphorylation is prevented by addition of p38 and JNK inhibitors, implicating
488 these kinases in the observed sustained ERK activation. Jun expression and activation are increased
489 by treatment with simvastatin and are reversed by addition of mevalonate, which also prevents
490 PARP-1 cleavage, confirming that the apoptotic mechanism is dependent on cholesterol depletion.

491 Collectively our data support the hypothesis of using statins for the treatment of ACC. Further
492 preclinical studies are warranted to establish effects on tumor growth when used in combination
493 with chemotherapy. However, their use in therapy as cholesterol lowering drugs will easily translate
494 preclinical studies into a clinical trial.

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510

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649 **Figure legends**

650 **FIGURE 1. Mitotane changes the expression of genes involved in cholesterol homeostasis in**
651 **ACC and negatively affects patients' survival. A)** Cholesterol was extracted from human ACC
652 samples and its content (ng/mg tissue) measured by colorimetric assay (- Mitotane, n=5, +
653 Mitotane, n=7, *p<0.03). **B)** Box plot graph for HMGCR gene expression in ACA (adrenocortical
654 adenoma), ACC (adrenocortical carcinoma) and NC (normal adrenal) human samples. ACC-ACA:
655 p-value=0.08, ACA-NC: p-value=0.26, ACC-NC: p-value=0.26. Statistical significance was
656 calculated using *limma*. **C)** Survival time in ACC patients according to HMGCR gene expression.
657 **D)** HMGCR expression and activity was evaluated in H295R cells untreated (basal) or treated for 2
658 and 14 days with mitotane 10µM. **E)** RNA from H295R cells left untreated (basal) or treated for
659 24h with mitotane (25µM) was processed for microarray analysis. Enrichment analysis for the
660 categories GO and heat map from microarray data with the most highly up-regulated (red) and
661 down-regulated (blue) genes involved in the cholesterol biosynthesis pathway. **F-G, I-J, L-M)**
662 mRNA expression of SREBP1 (**F-G**), INSIG1 (**I-J**) and ABCG1 (**L-M**) in H295R cells. The
663 mRNA was extracted and analyzed by QPCR from cells left untreated (0) or treated for 24 h with
664 Mitotane (10-25-40 µM) (**F-I-L**) and from cells untreated (0) or treated for different weeks (2 or 3
665 weeks, w) with Mitotane (10µM) (**G-J-M**). Each sample was normalized to 18S rRNA content.
666 Final results are expressed as n-fold differences of gene expression relative to calibrator. Data
667 represent the mean ±SD of values from at least three separate RNA samples (*p < 0.05, ***p<
668 0.001 versus calibrator). (**H, K, N**) Survival time in ACC patients according to the expression of
669 SREBP1 (**H**), INSIG1 (**K**) and ABCG1 (**N**) genes. Statistical significance was calculated using t-
670 test (**A, B, C, H, K, N**) or one-way analysis of variance followed by a Tukey post-hoc multiple
671 comparison test (**D, F, G, I, J, L, M**), P < 0.05 was considered significant.

672

673 **FIGURE 2. Simvastatin reduces H295R cell growth, in vitro and in vivo. A.** H295R cells were
674 left untreated (0) or treated with increasing doses (2.5, 5, 10 µM) of simvastatin for 24 and 48 h. **B)**
675 H295R cells were left untreated (0) or treated with increasing doses (2.5; 5; 10 µM) of simvastatin
676 with or without mevalonate (200 µM) for 48h. **A and B)** Cell viability was evaluated by MTT assay
677 (*p< 0.05, ***p<0.001 vs 0). **C)** Representative image of colony formation assay performed on
678 H295R cells (1000 cells/well) plated for 2 weeks in the presence of simvastatin (2.5, 5, 10 µM). **D)**
679 H295R cells were plated on low-attachment plates and then left untreated (0) or treated with
680 Simvastatin (2,5, 5 and 10 µM), tumor-spheres formation efficiency (TSFE) was evaluated 5 days
681 later (*p<0.05 vs untreated cells). **E)** H295R cells were injected subcutaneously in the flank region
682 of nude mice and the resulting tumors were grown to an average of 200 mm³ 21 days after
683 inoculation and then treated with vehicle (n= 8) or simvastatin (n= 7) (4mg/kg/day) for 24 days.
684 Values represent the mean ±SE of measured tumor volume over time (*p<0.05 versus control). **F)**
685 Representative tumors and final tumor weights, values are mean ±SEM, (*p<0.05 vs vehicle). **G)**
686 Ki67 immunohistochemistry and H&E staining of H295R xenografts (Magnification X 20, scale bar
687 =25 µm). Statistical significance was calculated using t-test (**F**) or one-way analysis of variance
688 followed by a Tukey post-hoc multiple comparison test (**A, B, D, E**), P < 0.05 was considered
689 significant

690

691 **FIGURE 3. Simvastatin decreases cholesterol and aromatase content in ACC. A)** H295R cells
692 were left untreated (0) or treated for 48h with simvastatin (2.5, 5, 10 µM) in growth medium
693 containing 10% lipoprotein-free serum. Cholesterol was extracted and measured by colorimetric

24

694 assay (* $p < 0.05$ vs untreated cells). **B, bar graph**) Cholesterol content in H295R xenografts
695 samples (* $p < 0.05$ vs vehicle) (n=8 vehicle; n=7 Simvastatin). **B, photograph**) Frozen sections of
696 H295R xenografts from vehicle- or simvastatin-treated mice were used for lipids droplets staining
697 by Oil Red O (Magnification X40, scale bar 12,5 μ m). **C**) H295R cells were treated for 48h with the
698 indicated doses of simvastatin added to 5% DCC-FBS and estradiol (E2) release in the culture
699 medium was measured by ELISA. Values represent the mean \pm SE (* $p < 0.05$ vs untreated cells). **D-
700 F**) H295R cells untreated (0) or treated for 24h with simvastatin (2.5, 5, 10 μ M) were analyzed for
701 CYP19 gene expression normalized to 18S rRNA by real-time PCR (**D**), and for SF-1 (**E**) or
702 Aromatase (Arom) (**F**) protein content by WB. GAPDH was used as a loading control. Blots are
703 from 1 representative experiment out of at least 3 performed. **G**) CYP19 expression in H295R
704 xenografts samples from vehicle- or simvastatin-treated mice by real-time PCR (n=8 vehicle; n=7
705 Simvastatin). **H**) Immunohistochemical staining of Aromatase in untreated or simvastatin-treated
706 H295R xenograft samples (Magnification X 20, scale bar =25 μ M). Statistical significance was
707 calculated using t-test (**B, G**) or one-way analysis of variance followed by a Tukey post-hoc
708 multiple comparison test (**A, C, D**). $P < 0.05$ was considered significant.

709

710 **FIGURE 4. Simvastatin reduces nuclear and mitochondrial ER α activity.** **A** and **C**) WB
711 analysis of ER α (**A**) and Cyclin E (**C**) was performed on equal amounts of total protein extracts
712 from H295R cells left untreated (0) or treated with Simvastatin (2,5, 5 and 10 μ M) for 48h. GAPDH
713 was used as a loading control. Blots are representative of three independent experiments with
714 similar results. **B and D**) Immunofluorescence analysis of ER α expression (**B**) and
715 immunohistochemical staining of Cyclin E (**D**) on H295R xenograft tumor samples obtained from
716 vehicle- or simvastatin-treated mice (Magnification X 20, scale bar=25 μ M). **E and F**) H295R cells
717 untreated (0) or treated with simvastatin (5 μ M) were used for mitochondrial protein extraction.
718 ER α (**E**) and OXPHOS (**F**) protein expression was analyzed by WB. GAPDH was used as a loading
719 control. Blots are representative of three independent experiments with similar results. **G and H**)
720 Immunostaining (**G**) and activity (**H**) of COX IV was evaluated on H295R xenograft samples
721 obtained from vehicle- or simvastatin-treated mice (Magnification X 20, scale bar = 25 μ m).

722

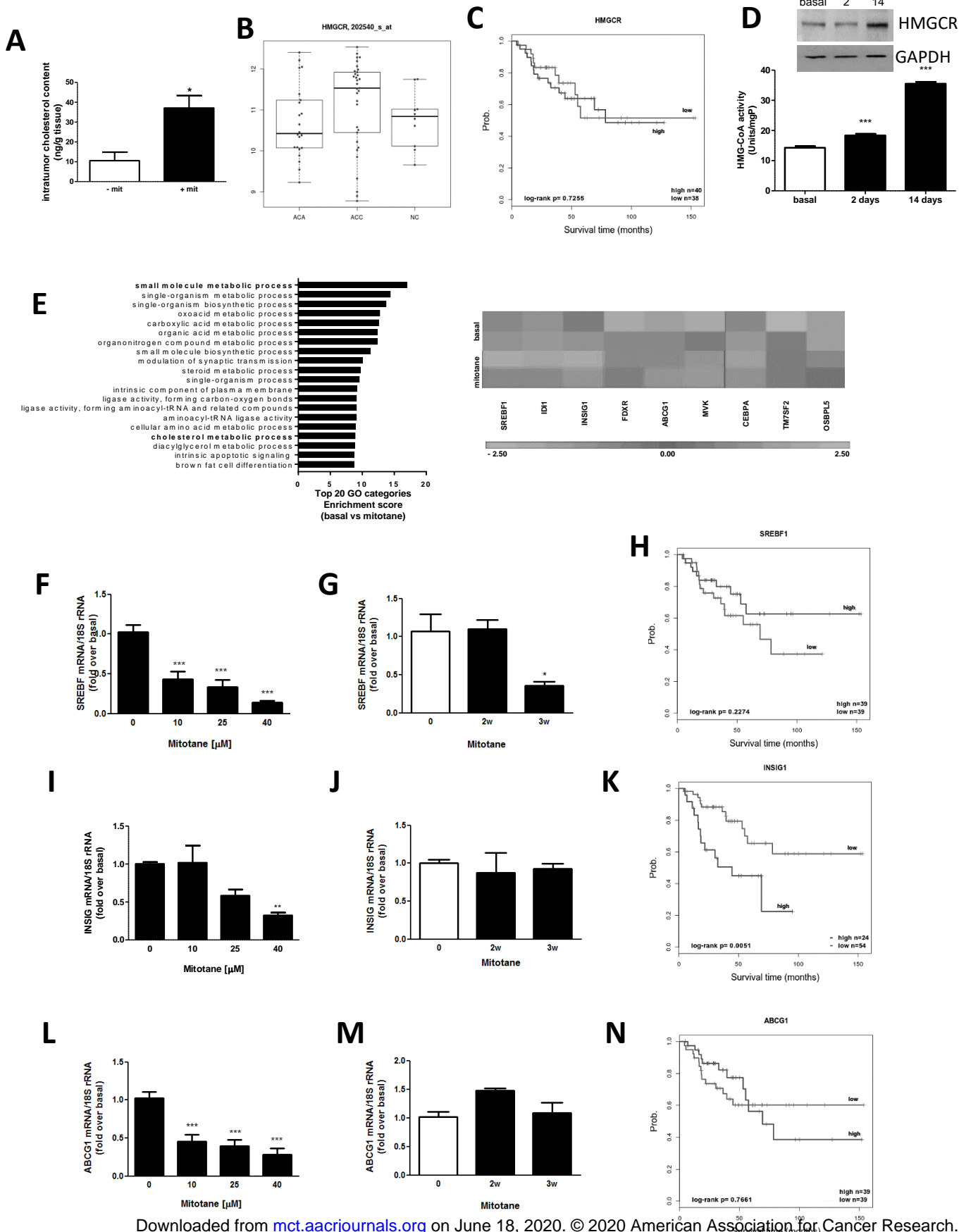
723 **FIGURE 5. Simvastatin reduces mitochondrial functions.** **A-F**) Mitochondrial respiration
724 described as OCR (oxygen consumption rate) levels was detected in H295R cells left untreated or
725 treated with Simvastatin (2.5, 5, 10 μ M) for 16h by Seahorse XFe96 analyzer. **A**) The linear graph
726 shows time course measurements but with three different injections to evaluate the OCR 1- after the
727 oligomycin injection, 2- after the injection of carbonyl cyanide-(trifluoromethoxy)phenylhydrazine
728 (FCCP), 3- after the injection of rotenone/antimycin. **B-F**) The histograms are derived from the
729 obtained measurements: (**B**) basal respiration, (**C**) maximal respiration, (**D**) proton leak, (**E**) ATP
730 turnover and (**F**) spare capacity (* $p < 0.05$, *** $p < 0.001$ simvastatin vs untreated cells). **G**)
731 Mitochondrial extracts from H295R cells treated for 48h were analyzed for TOM20 protein
732 expression by WB. **H**) TOM20 protein expression was evaluated by immunohistochemistry on
733 H295R xenograft samples obtained from vehicle- or simvastatin-treated mice (Magnification X 20,
734 scale bar = 25 μ m).

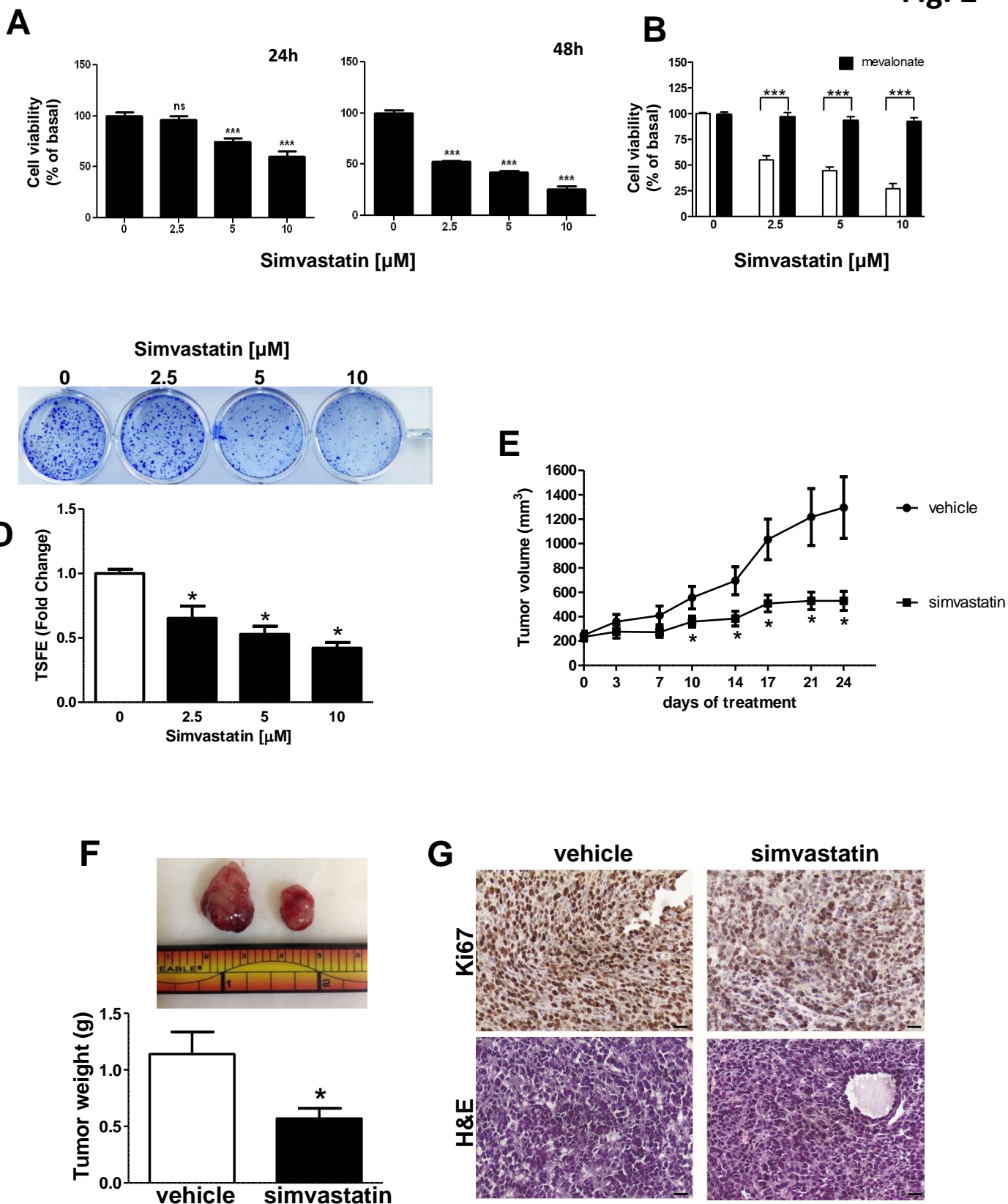
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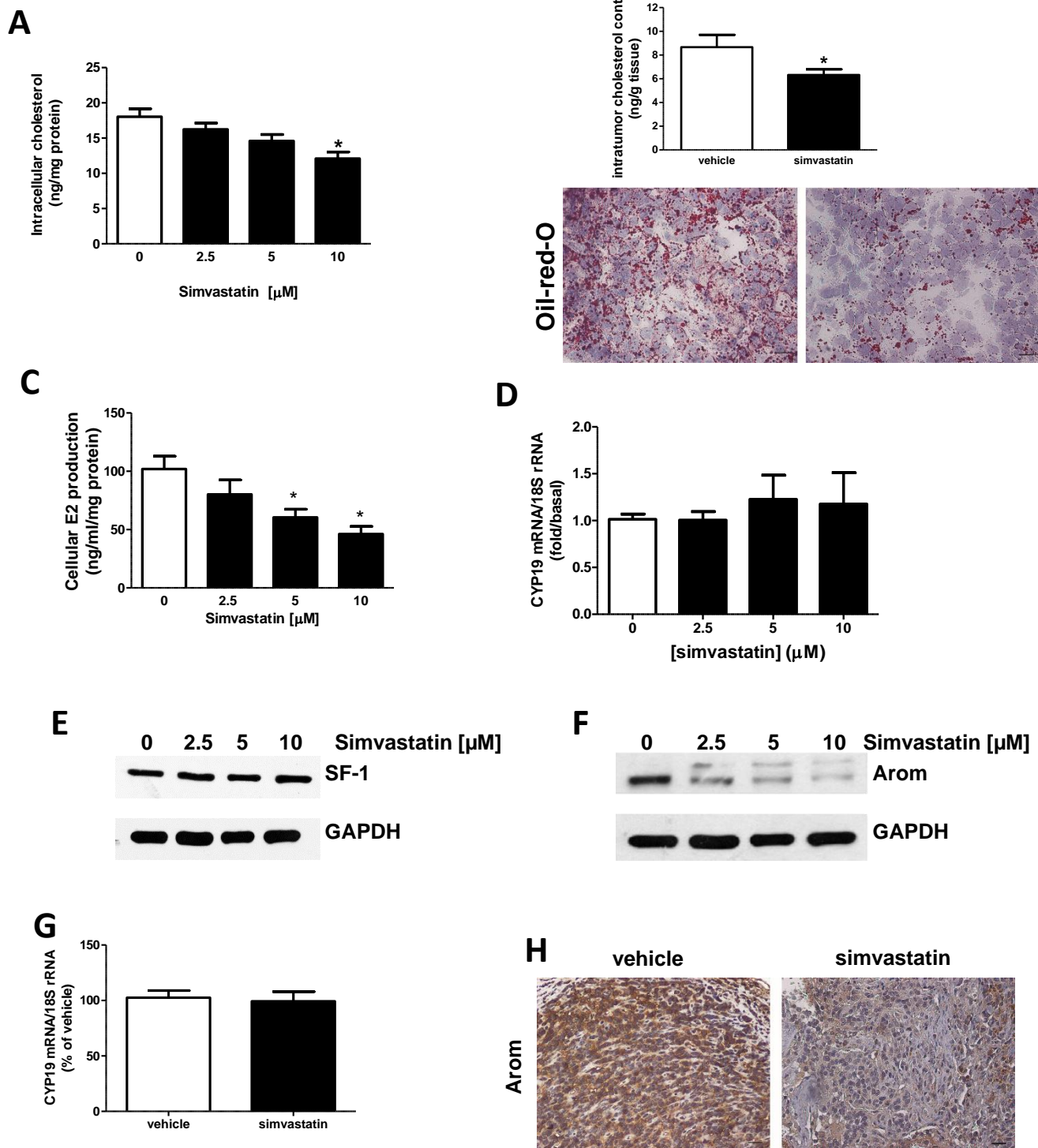
736 **FIGURE 6. In vitro and in vivo activation of apoptosis by simvastatin in ACC.** **A**) Cells were
737 left untreated (0) or treated with simvastatin (2.5, 5, 10 μ M) for 48h. WB analyses of Bak and
738 PARP-1 were performed on equal amounts of total protein extracts. GAPDH was used as a loading

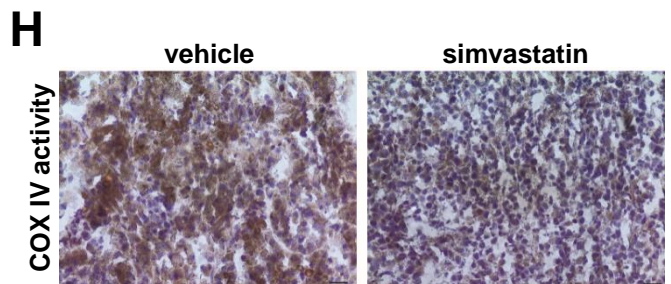
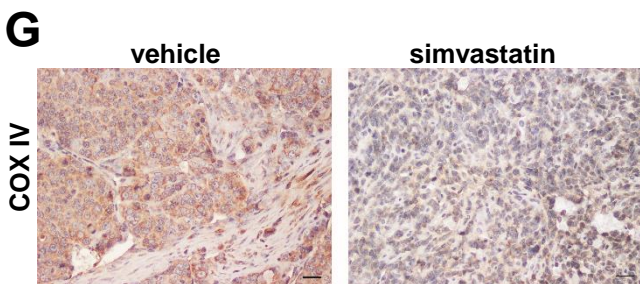
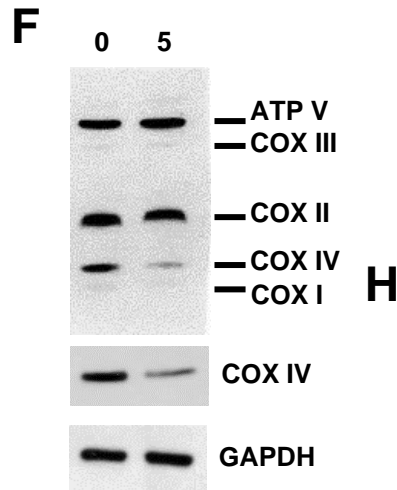
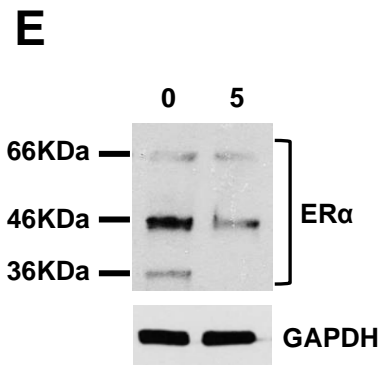
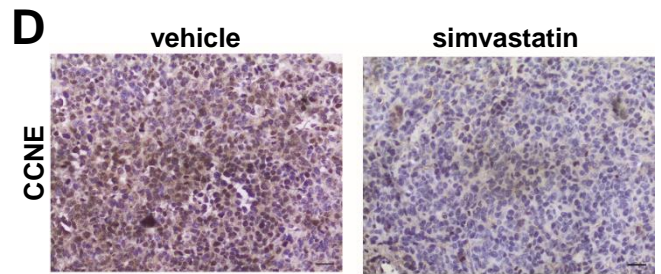
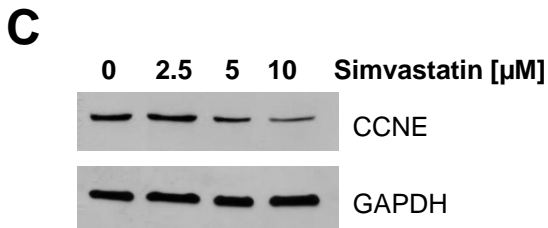
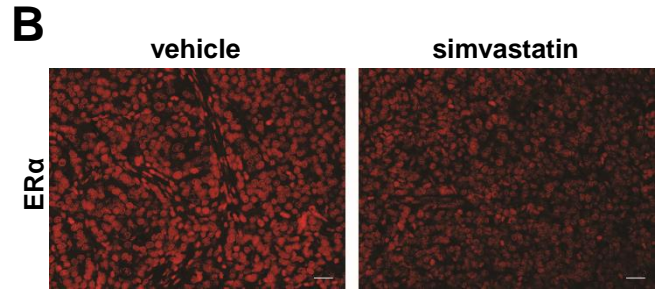
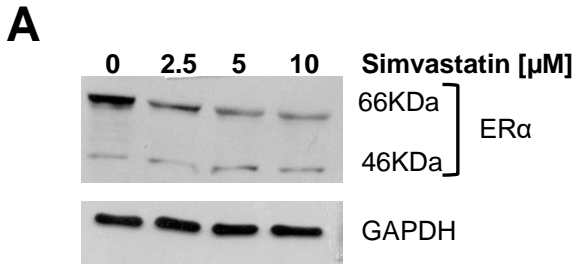
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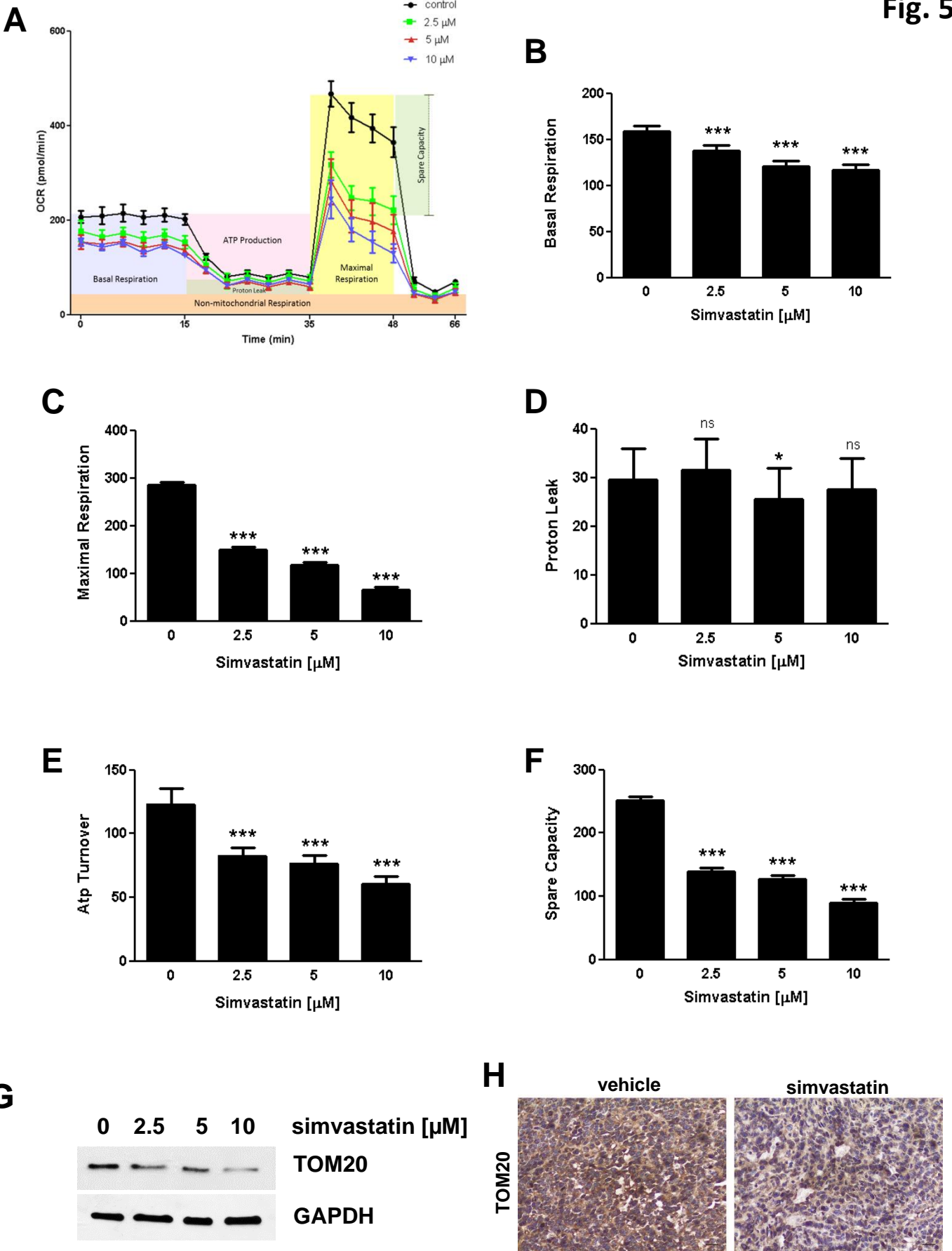
739 control. Blots are representative of three independent experiments with similar results. **B)** TUNEL
740 assay was performed on cells treated as described in **A)**. DAPI was used as nuclear counterstain.
741 Fluorescent signal was observed under a fluorescent microscope. Images are from a representative
742 experiment. **C)** TUNEL staining was performed on frozen sections of H295R xenograft samples
743 obtained from vehicle- or simvastatin-treated mice (scale bar 25 μm). **D)** WB analyses for p-cJun,
744 c-Jun, pERK1/2, ERK2, PARP-1, were performed on total protein extracted from cells treated for
745 48h with simvastatin (5 μM), mevalonate (100 μM), or their combination. **E)** WB analyses for p-
746 cJun, Jun, pERK1/2, ERK2, PARP-1, were performed on total protein extracted from cells treated
747 for 48 h with simvastatin (5 μM) alone or combined with PD98059 (10 μM), SP600125 (10 μM),
748 SB203580 (10 μM). GAPDH was used as a loading control.

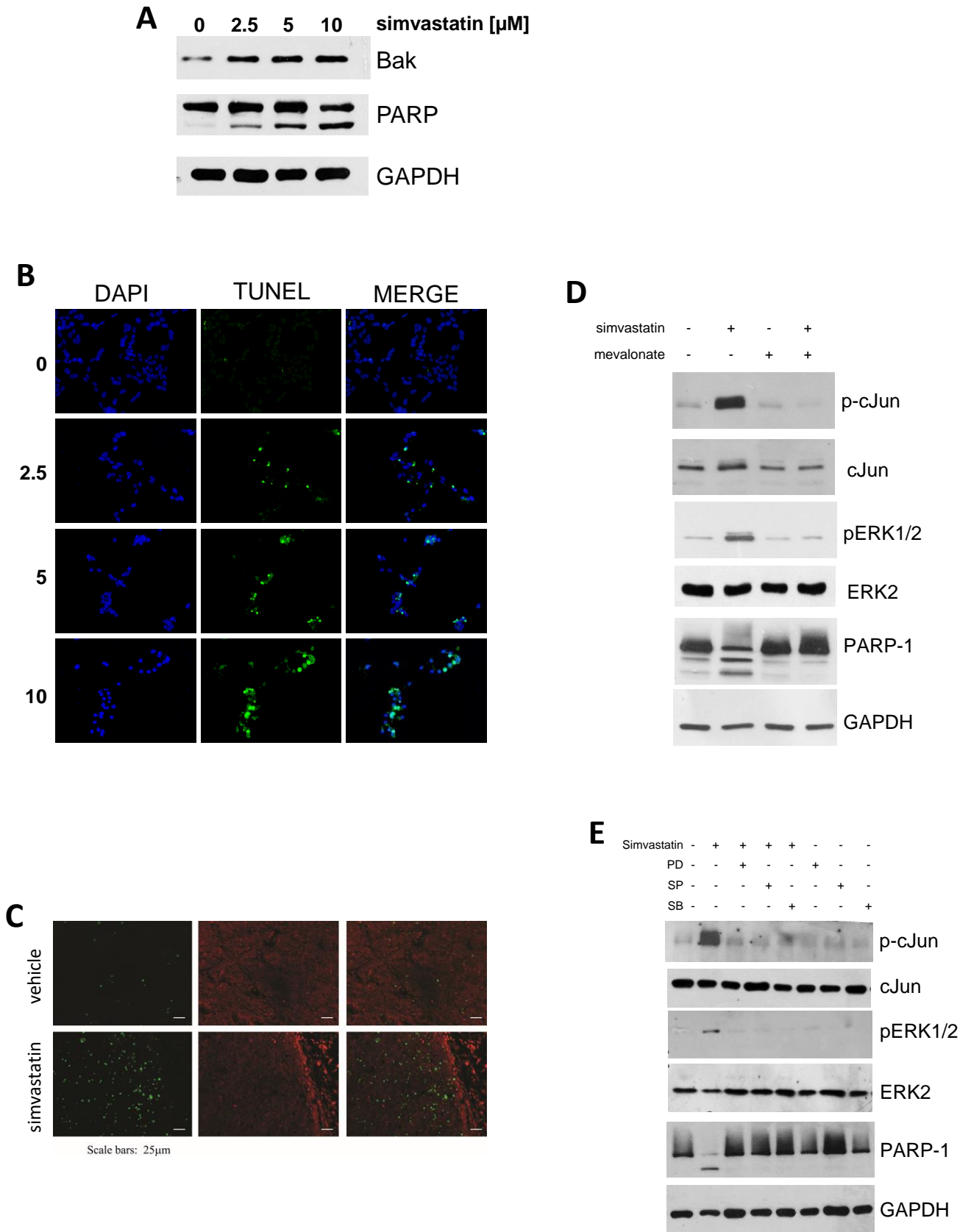












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Statins reduce intratumor cholesterol affecting adrenocortical cancer growth

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