

# Genetic Landscape of Sporadic Unilateral Adrenocortical Adenomas Without PRKACA p.Leu206Arg Mutation

European Network for the Study of Adrenocortical Tumors (ENSAT)

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1                   **Genetic landscape of sporadic unilateral adrenocortical adenomas without**  
2                   ***PRKACA* p.Leu206Arg mutation**

3  
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24  
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41 **Abstract**

42 *Context:* adrenocortical adenomas (ACAs) are among the most frequent human neoplasias. Genetic  
43 alterations affecting the cAMP/PKA signaling pathway are common in cortisol-producing ACAs,  
44 while activating mutations in the gene encoding  $\beta$ -catenin (CTNNB1) have been reported in a subset  
45 of both benign and malignant adrenocortical tumors. However, the molecular pathogenesis of most  
46 ACAs is still largely unclear.

47 *Objective:* aim of the study was to define the genetic landscape of sporadic unilateral ACAs.

48 *Design and setting:* next-generation whole-exome sequencing was performed on fresh-frozen tumor  
49 samples and corresponding normal tissue samples.

50 *Patients:* 99 patients with ACAs (74 cortisol-producing and 25 endocrine inactive) negative for  
51 p.Leu206Arg PRKACA mutation.

52 *Main outcome measures:* identification of known and/or new genetic alterations potentially involved  
53 in adrenocortical tumorigenesis and autonomous hormone secretion, genotype-phenotype correlation.

54 *Results:* 706 somatic protein-altering mutations were detected in 88/99 tumors (median: 6 per tumor).  
55 We identified several mutations in genes of the cAMP/PKA pathway, including three novel mutations  
56 in PRKACA, associated with female sex and Cushing's syndrome. We also found genetic alterations  
57 in different genes involved in the Wnt/ $\beta$ -catenin pathway, associated with larger tumors and endocrine  
58 inactivity, and, notably, in many genes of the Ca<sup>2+</sup>-signaling pathway. Finally, by comparison of our  
59 genetic data with those available in the literature, we describe a comprehensive genetic landscape of  
60 unilateral ACAs.

61 *Conclusions:* This study provides the largest sequencing effort on ACAs up to now. We thereby  
62 identified somatic alterations affecting known and novel pathways potentially involved in adrenal  
63 tumorigenesis.

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## 69 **Introduction**

70 Adrenocortical adenomas (ACAs) are among the most frequent human neoplasias with a prevalence of  
71 2-3% in the general population. They are endocrine inactive in 70% of cases, mostly incidentally-  
72 discovered, or associated with autonomous cortisol or aldosterone secretion. The genetic basis of  
73 several adrenal disorders has been elucidated over the last years following classical genetic approaches  
74 and utilizing next-generation sequencing techniques. In particular, the cAMP/protein kinase A (PKA)  
75 pathway plays a central role in adrenocortical growth and steroidogenesis. Specifically, genetic  
76 alterations affecting the cAMP/PKA pathway, such as germline or somatic mutations in genes  
77 encoding the regulatory subunit 1  $\alpha$  of PKA (*PRKARIA*), the protein Gsa (*GNAS*), and the  
78 phosphodiesterases 11A and 8B (*PDE11A* and *PDE8B*) have been reported in cortisol-producing  
79 ACAs (CPA) and bilateral micronodular adrenal hyperplasias (1-5).

80 Recently, we and others have found somatic mutations in the gene encoding the catalytic  
81 subunit  $\alpha$  of PKA (*PRKACA*) in 35-70% of unilateral ACAs associated with Cushing's syndrome (6-  
82 10). These mutations translate into a constitutive activation of PKA by interfering with binding  
83 between its regulatory and catalytic subunits (11). Activating mutations in the gene encoding  $\beta$ -catenin  
84 (*CTNNB1*) represent another important contributor of adrenocortical growth. At variance with  
85 mutations in *PRKACA*, *CTNNB1* mutations had been reported in both adrenocortical adenomas and  
86 carcinomas with similar prevalence (10-30%) (12-14), and had been most frequently observed in non-  
87 cortisol-secreting tumors (15). Moreover, by using SNP array profiling, we have identified the  
88 presence of several recurrent copy number alterations (CNA) in specific chromosomal regions that  
89 may also play a role in the pathogenesis of these tumors (16-17).

90 Despite these recent advances, the pathogenesis of a large proportion of ACAs has remained  
91 elusive. In particular, despite representing the most frequent subtype, endocrine inactive adenomas are  
92 the least thoroughly investigated, due to their infrequent surgical treatment and thus  
93 underrepresentation in tissue based studies. Therefore, the aim of the current study was to define the  
94 genetic landscape of sporadic unilateral ACAs by next-generation whole-exome sequencing (WES). In  
95 particular, we intended to clarify the molecular mechanisms involved in adrenocortical tumor  
96 development and provide genotype-phenotype correlation studies.

97 **Methods**

98 *Tissue samples, patients, and clinical annotations*

99 Fresh-frozen ACA tissues (n=99) and corresponding blood or normal adrenal tissues were included  
100 from 11 centers belonging to the European Network for the Study of Adrenocortical Tumors (ENSAT,  
101 [www.ensat.org](http://www.ensat.org)). Only histologically confirmed unilateral ACAs were included (18). We selected  
102 endocrine inactive ACAs (EIA) and CPA without known p.Leu206Arg *PRKACA* mutation (6-10). A  
103 subgroup of patients (n=42) had been included in an earlier report (8). All patients provided written  
104 informed consent and the study was approved by the ethics committee of each participating institution.

105 Clinical and hormonal data were collected through the ENSAT registry  
106 (<https://registry.ensat.org/>). Overt Cushing's syndrome (CS) and subclinical CS (SCS) were diagnosed  
107 according to current guidelines (19) and defined as previously reported (6). The final series consisted  
108 of 74 CPA (39 CS and 35 SCS) and 25 EIA (**Table 1**).

109 A comparative analysis was performed with data available from previous WES studies on  
110 CPAs (n=79) (6, 7, 12, 13) and ACC (n=176) (12, 20), and from "The Cancer Genome Atlas" project  
111 (21, <https://tcga.data.nci.nih.gov/tcga/tcgaCancerDetails.jsp>).

112

113 *WES and data analysis*

114 DNA was extracted from fresh-frozen tissues and checked for signs of degradation as previously  
115 described (6). Exomes were enriched in solution and indexed with the SureSelect XT Human All Exon  
116 (50Mb kit, version 5, Agilent Technologies, Santa Clara, CA, USA) for library preparation.  
117 Sequencing was performed as paired-end reads of 100 bp on HiSeq2500 systems (Illumina, San  
118 Diego, CA, USA). Pools of 12 indexed libraries were sequenced on four lanes to an average depth of  
119 coverage between 82x and 170x. Image analysis and base calling were performed with Real-Time  
120 Analysis software (Illumina). Reads were aligned against the human assembly hg19 (GRCh37) using  
121 the Burrows-Wheeler Aligner tool (BWA, v 0.7.5a). Moreover, we performed single-nucleotide  
122 variant and small insertion and deletion (indel) calling specifically for the regions targeted by the  
123 exome enrichment kit, using SAMtools (v 0.1.19). Subsequently the variants were filtered using the  
124 SAMtools varFilter script using default parameters, with the exception of the maximum read depth

125 parameter, which we set to 9999. Variant detection was done as described earlier (6). In brief, to  
126 reduce false positives we filtered out variants that were already present in our in-house database  
127 (currently 8,000 exomes) or had variant quality less than 40. Raw read data of the remaining variants  
128 are then manually investigated using the Integrative Genomics Viewer (IGV). The frequency of each  
129 mutated allele was then evaluated in large population genomics projects, such as the EXAC (Broad)  
130 and the “1000 Genomes AF (allele frequency)” data set (Supplementary Table 1).

131 The Gene Set Enrichment Analysis software (MSigDB database v5.0) (22) was used to  
132 identify enriched gene ontology (GO) terms in ranked lists of genes and to perform gene family and  
133 pathway analysis (1330 gene sets), including the KEGG (Kyoto Encyclopedia of Genes and Genomes)  
134 and the REACTOME pathway (v55) databases.

135

#### 136 *In silico analysis*

137 Somatic variants were evaluated by both Polymorphism Phenotyping v2 algorithm tool (PolyPhen-2)  
138 (<http://genetics.bwh.harvard.edu/pph2>) (23) and SIFT (Sorting Tolerant From Intolerant) algorithm  
139 (<http://sift.jcvi.org/index.html>) (24) to predict the possible impact of an amino acid substitution on the  
140 structure and function of a human protein. The variants were classified as possibly pathogenic according to  
141 the given thresholds (Supplementary Table 1). Most interesting recurrent genetic alterations were  
142 evaluated by *in silico* analysis to predict whether the variants may be damaging. Structural images  
143 were prepared with PyMOL software ([www.pymol.org](http://www.pymol.org)). The 3D structures of the mammalian PKA  
144 holoenzyme containing catalytic subunit  $\alpha$  and regulatory subunit 2  $\beta$  (PRKACA-PRKAR2B), the  
145 stimulatory G-protein  $\alpha$  subunit (GNAS, isophorm 15), and the ryanodine receptor RYR1 were  
146 acquired from Protein Data Bank (<http://www.rcsb.org/pdb/>, entries 3TNP, 1AZS, and 4UWA,  
147 respectively). Aminoacid changes induced by mutations were identified and displayed using the  
148 Chimera v1.10 Software.

149

#### 150 *Copy number alterations*

151 We compared the results of WES in the present study with previously published CNA data by SNP  
152 array profiling (17) available in 14/99 patients.

153 *Transcriptome analysis*

154 Transcriptome analysis was performed by Affymetrix HGU133Plus2, as previously described (25), on  
155 an independent cohort of 41 ACAs, including 11 EIAs and 30 CPAs (20 CS and 10 SCS). Targeted  
156 next-generation sequencing (AmpliSeq design, IonTorrent sequencing) for *CTNNB1* (Ser45 hotspot,  
157 exons 7 and 8), *PRKACA* (L206 hotspot), *GNAS* (R201 hotspot), *PRKACB* and *PRKARIA* was  
158 performed on 37/41 ACAs. Reads were aligned using the human genome assembly hg19 (GRCh37)  
159 and variant calling was performed using Torrent Suite Software (v. 4.2.1). Variants were annotated by  
160 ANNOVAR package (March, 22nd 2015 release). Variants were visually validated by IGV. Mutations  
161 were validated by Sanger sequencing. The mutation status for *CTNNB1* was not available for one  
162 ACA, whereas the one for *PRKARIA* and *PRKACB* was not available in four ACAs.

163 Transcriptome data were analyzed in R (<https://cran.r-project.org/>). Unsupervised hierarchical  
164 clustering was performed using hclust based on the top 1000 variable transcripts. Differential gene  
165 expression was generated with Limma (Linear Models for Microarray Data (26)) R package, using  
166 Benjamini-Hochberg correction to adjust p-values. An extensive list of calcium-signaling related  
167 genes was provided by the KEGG “Calcium Signaling Pathway” gene list. Enrichment in these  
168 calcium genes was sought among the differentially expressed genes, using the Fisher exact test.

169

170 *Statistical analysis*

171 Unsupervised complete linkage clustering was performed on the rows and columns using the  
172 Hamming distance as a similarity metric, to investigate interdependency among genetic alterations.  
173 The Fisher’s exact or Chi-square tests, and Mann-Whitney U test were used to investigate dichotomic  
174 and continuous variables, where appropriate. Kruskal-Wallis test, followed by Bonferroni *post-hoc*  
175 test, was performed for comparison among groups for non-normally distributed variables. Data are  
176 shown as median and ranges, if not otherwise specified. Statistical analyses were made using  
177 GraphPad Prism (version 5.0, La Jolla, CA, USA) and SPSS Software (version 21, SPSS Inc.,  
178 Chicago, IL, USA). P values  $<0.05$  were considered as statistically significant.

179

180

181 **Results**

182 *Overview of genetic findings*

183 Clinical and hormonal characteristics together with the genetic data of patients are provided in **Table**  
184 **I**. We identified 706 non-synonymous protein-altering somatic mutations in 88/99 samples. In 11  
185 tumors no mutations were detected. The somatic variants included 597 missense, 45 nonsense, 31  
186 frameshift, 24 direct splicing, and 9 indel alterations, resulting in a median of 6 somatic mutations in  
187 exonic regions per tumor (range: 0-55) (**Figure 1** and **Table I**). According to the PolyPhen-2  
188 algorithm, 203 mutations were classified as probably damaging, 116 as possibly damaging, 271 as  
189 benign, and 116 remained undefined. The most frequent substitutions were the C:G>T:A transition  
190 and the C:G>A:T transversion (29% and 28% of cases, respectively, **Supplementary Figure 1**). The  
191 complete list of somatic mutations **including all the information about the type and localization of**  
192 **genetic alterations, the frequency of the variants in different available databases and the pathogenic**  
193 **classification** is summarized in **Supplementary Table 1**.

194

195 *Specific genetic alterations*

196 Recurrent somatic mutations (n=56) are shown in **Table 2**. The most frequent alterations were  
197 missense mutations at *CTNNB1*, in a hot-spot region encoding serine in position 45 (n=39). *CTNNB1*  
198 mutations occurred in 7/39 patients (18%) with CS, 19/35 subjects (54%) with SCS, and 13/25  
199 patients (52%) with EIA. Moreover, alterations in genes encoding several members of the cadherin  
200 superfamily were identified, but only those occurring in *PCDHGA6* were found in at least two  
201 samples.

202 *GNAS* somatic mutations were identified in 8/74 patients with CPAs (11%), two of them with  
203 SCS and six with CS, but in none of the EIAs. In seven patients known activating mutations were  
204 found at codon 201, whereas in one patient with CS a novel probably damaging mutation was  
205 observed (76A>C, p.Lys58Gln). The 3D *in silico* analysis showed that lysine 58 is near the critical  
206 position 201, suggesting a functional significance for p.Lys58Gln substitution, similar to the known  
207 *GNAS* activating mutations (**Supplementary Figure 2**).



208            Interestingly, we found three novel somatic mutations in *PRKACA* in three patients with CS  
209 (p.Trp197Arg, p.245\_248.del and p.Glu32Val). Although those mutations occurred outside the known  
210 hot-spot region of *PRKACA* in exon 7, the 3D *in silico* analysis pointed towards a potential pathogenic  
211 role for two of them. p.Trp197Arg mutation is located at the interface between the catalytic and the  
212 regulatory subunit. The exchange of the hydrophobic tryptophan with the hydrophilic, positively  
213 charged arginine might lead to alteration in the interaction between the subunits. Moreover, the  
214 p.245\_248.del affects a region of the catalytic subunit of PKA at the interface with the regulatory  
215 subunit, likely inducing a modification that alters the binding of the regulatory to the catalytic subunit.  
216 In contrast, the mutation p.Glu32Val, with a hydrophilic, negatively charged glutamate replaced by a  
217 hydrophobic valine, is situated outside the interaction region (**Figure 2**).

218            Several alterations were found in different ryanodine receptors, and those occurring in RYR1  
219 and RYR3 were recurrent. The 3D *in silico* analysis revealed that mutations in *RYR1* (p.Arg1469Gly  
220 and p.Val3218Leu) and *RYR2* (p.Lys2264Asn) were located in the clamp regions of the cytoplasmic  
221 assembly, while the mutation in *RYR3* (del4516) was pinpointed in the sliding helix between  
222 transmembrane and cytoplasmic assemblies (**Supplementary Figure 3**).

223            Finally, different potentially relevant “private” mutations were detected, including alterations  
224 in genes encoding ionotropic (*GRIA1*, *GRIA2*, *GRID1*, *GRIK2*, *GRIN1*, *GRIN3B*, *GRIP1*) and  
225 metabotropic glutamate receptors (*GRM3*, *GRM4*, *GRM6*). Moreover, a missense mutation in *ARMC5*  
226 (p.Pro866Leu) was observed in a 22-mm unilateral left adenoma associated with CS. However, no  
227 second hit at the *ARMC5* gene was observed in this tumor. Finally, a probably damaging frameshift  
228 mutation (532\_533insG) at *TP53* was detected in a 40-mm, endocrine inactive, oncocytic adenoma.  
229 Unfortunately, no follow-up data were available to ascertain the clinical course of this patient during  
230 the post-operative period.

231

### 232 *Gene enrichment and pathway analysis*

233            The gene enrichment analysis identified 605/706 (86%) mutated genes associated with GO terms.  
234 Interestingly, Ca<sup>2+</sup>-signaling, collagen formation, and extracellular matrix organization were  
235 recognized as the most significantly represented pathways (**Supplementary Table 2**). The gene family

236 analysis further showed that eight cytokines and growth factors, 60 transcription factors, including  
237 *ATRX* and *MED12*, 16 protein kinases, including *PRKACA*, 14 oncogenes, including *CREB1*,  
238 *CREBBP*, *CTNNB1*, and *GNAS*, and four tumor suppressor genes, including *APC* and *TP53* were  
239 included among the mutated genes. None of them were mutated in more than one sample  
240 (*Supplementary Table 3*).

241

#### 242 *Genotype-phenotype correlation and transcriptome analysis*

243 No statistically significant relationship was found between the mutation frequency and clinical data  
244 (sex, age, tumor size, and cortisol secretion pattern). We classified patients into three groups according  
245 to the known or potential biological consequences of the most frequent mutations: subjects with  
246 mutations in genes encoding components of the classic Wnt/ $\beta$ -catenin pathway (*CTNNB1*, *APC*,  
247 *APC2*, *PCDH15*, *PCDHA8*, *PCDHB11*, *PCDHA10*, *PKP2*), those with alterations in genes encoding  
248 components of the cAMP/PKA pathway (*GNAS*, *PRKACA*, *PRKARIA*, *CREB1*, *CREBBP*, *ADCY3*,  
249 *GRM3*, *GRM4*, *GRM6*), and those with mutations in genes encoding components of Ca<sup>2+</sup>-dependent  
250 signaling (*CACNA1C*, *CACNA1E*, *CACNG8*, *RYR1*, *RYR2*, *RYR3*, *GRIA1*, *GRID1*, *GRIK2*, *GRIN1*,  
251 *GRIN3B*, *GRIP1*) (*Supplementary Table 4*). The results of the unsupervised binary clustering analysis  
252 and the relationship between the genetic landscape of tumors and the clinical phenotype of the three  
253 groups of patients are shown in *Figure 3A* and *Supplementary Table 5*. Patients with mutations in  
254 genes encoding components of the Wnt/ $\beta$ -catenin pathway were older, had larger tumors, and carried a  
255 higher total number of mutations than those without these aberrations ( $P < 0.05$ ). In contrast, patients  
256 with mutations in the genes encoding component of the cAMP/PKA pathway were more frequently  
257 female and younger, in comparison to subjects not carrying mutations ( $P < 0.01$ ). Mutations in genes  
258 encoding components of Ca<sup>2+</sup>-dependent signaling were associated with a higher number of mutations  
259 when compared to those without ( $P = 0.001$ ), whereas no difference in clinical and hormonal  
260 parameters was evident.

261 The results of the unsupervised clustering according to the results of the transcriptome  
262 analysis are shown in *Figure 3B* and *C*. After considering the expression level, transcriptome profile  
263 could clearly identify four groups and well separated patients with CS from those with EIA and SCS,

264 and tumors with mutations of the cAMP/PKA pathway from those with mutations in the Wnt/ $\beta$ -  
265 catenin or without mutations in one of those two pathways, showing significant enrichments in  
266 calcium-related genes (**Figure 3B**). Surprisingly, restricting the analysis only to genes of the Ca<sup>2+</sup>  
267 signaling pathway, the transcriptome profile was also able to clearly divide the patients in four groups.  
268 The four clusters showed a good separation in patients with CS vs those with EIA or SCS, as well as  
269 tumors with mutations in the cAMP/PKA vs Wnt/ $\beta$ -catenin pathway (**Figure 3C**).

270

### 271 *Combined genetic and genomic analysis*

272 We further analyzed current WES data in combination with those from SNP array profiling available  
273 for a subgroup of 14/99 ACAs (three with CS, seven with SCS, and seven with EIA) (17). As  
274 summarized in **Table 3**, some large chromosomal regions (16p13.3-13.2, 19p13.3-12, 7p22.3-22.1,  
275 11p15.5, 20q13.3) and several genes were affected by recurrent CN gains, including genes involved in  
276 Wnt/ $\beta$ -catenin (*APC2* in two samples), cAMP/PKA pathways (*PRKACA*, *PRKR1B*, *AKAP8* in two  
277 samples) or Ca<sup>2+</sup>-dependent signaling (*CACNA1H* in five samples, *CACNA1A* and *CACNA1B* in two  
278 samples). There was no significant difference in total number of CNA between tumors with or without  
279 somatic mutations.

280 In 4/14 tumors no somatic mutations were detected by WES. One of those (CS) showed a  
281 large amplification at 19p13.2-12 including the genes *AKAP8*, *CACNA1A*, *PDE4C* and *PRKACA*. The  
282 second tumor (SCS) had amplifications at 7p22.2, which included *PRKR1B* and 16p13.3. The third  
283 sample (EIA), presented a CN gain at chr11p15.5 and several micro-amplifications, whereas the last  
284 one (SCS), did not show any CNA in regions or genes with presumed functional relevance.

285

### 286 *Systematic review of genetic data available in unilateral adrenocortical tumors*

287 We compared the genetic findings of the present analysis with WES data available in the literature for  
288 ACA (n=69 CPA) and ACC (n=176) (**Supplementary Table 6**). The analysis of *PRKACA* wild-type  
289 benign tumors (n=94 CPA+25 EIAs) showed that mutations in genes involved in cAMP/PKA pathway  
290 were present only in CPA (20% of cases), whereas alterations of genes involved in Wnt/ $\beta$ -catenin

291 signaling were mutated in 49% of CPA and in 76% of EIAs. Alterations in genes involved in Ca<sup>2+</sup>-  
292 dependent signaling were found in 14% of CPA and in 16% of EIA.

293 We performed an unsupervised clustering with all WES data available for ACAs (n=168),  
294 subdividing the mutations according to the three groups defined above (*Supplementary Figure 4*). We  
295 also performed a canonical pathway analysis considering all the 168 ACA samples together and  
296 subdividing them into the three groups (49 CPA with *PRKACA* mutations, 94 CPA without *PRKACA*  
297 mutations and 25 EIA, *Supplementary Table 7*). In brief, genes involved in the “cancer pathways”  
298 were present in all groups, while genes of the “calcium signaling pathway”, “collagen formation” or  
299 “ECM organization” were not recorded among the *PRKACA* mutated CPAs.

300 Finally, we observed that 23% of somatic mutations observed in our cohort were previously  
301 reported in at least one of the 176 ACC samples and 6% in at least two ACCs (*Supplementary Table 6*  
302 and *Supplementary Figure 5*). As expected, mutations in *CTNNB1*, the most frequent alterations,  
303 were detected in 15% of ACC and in 25% of ACA (34% of *PRKACA* wild-type CPA and 52% of  
304 EIA). Interestingly, mutations in different members of proto-cadherin family were frequently observed  
305 in 13% of CPA negative for *PRKACA* mutations, 24% of EIA and 15% of ACC.

306

## 307 **Discussion**

308 The present study represents the most comprehensive genetic characterization of unilateral ACA. In  
309 this large European series we analyzed also for the first time endocrine inactive adenomas that  
310 represent the most frequent but less investigated type of ACAs. By restricting the investigation to  
311 patients without mutations in the predominant hot-spot region of *PRKACA* (p.Leu206Arg), WES  
312 analysis highlights substantial heterogeneity of the genetic background of cortisol-producing and  
313 endocrine inactive ACAs, and separates well those tumors from aldosterone-producing ACA (27, 28).  
314 Overall, we identified 706 somatic mutations with a median of 6 per tumor. Many of the 605 mutated  
315 genes encoded components of the cAMP/PKA, the Wnt/ $\beta$ -catenin or, more surprisingly, the Ca<sup>2+</sup>-  
316 dependent signaling pathway.

317 Among genetic alterations of the cAMP/PKA pathway, *GNAS* somatic mutations were the  
318 most frequent, being associated with cortisol production, accordingly with published data (7, 9-10). In

319 addition to the previously reported hot-spot mutations, a novel substitution p.Lys58Gln was found in a  
320 patient with CS with potential functional relevance in our *in silico* model. Likewise, three novel  
321 somatic mutations in *PRKACA* were detected in three CPA associated with CS. Interestingly, *in silico*  
322 data provide evidence that the p.Trp197Arg substitution and the p.245-248 deletion may be able to  
323 alter the interaction between the catalytic and the regulatory subunit of PKA, similarly to what  
324 described for the p.Leu206Arg mutation (11). Moreover, the essential role of the phosphorylation site  
325 Trp197 in the binding to PKA regulatory subunit was already described in 1997 (29). In contrast, the  
326 localization of the mutation p.Glu32Val outside known interacting regions of the catalytic subunit, do  
327 not allow any speculation on the biological relevance of this substitution. Other mutated components  
328 of the cAMP pathway included *PRKARIA*, *CREB1* (cAMP responsive element binding protein),  
329 *CREBBP* (CREB binding protein) and three genes encoding metabotropic glutamate receptors  
330 (mGluRs, *GRM3*, *GRM4*, *GRM6*). The mutated mGluRs in our cohort belong to the group II and III  
331 mGluRs, which are G-protein-coupled receptors involved in regulation of intracellular cAMP levels.  
332 Interestingly, mGluR3 has been previously suggested to be involved in the regulation of  
333 steroidogenesis in adrenocortical tissues (30). Considering the relationship with the clinical data,  
334 mutations in component of the cAMP/PKA pathway occur invariably in young patients with cortisol-  
335 secreting tumors. Those results are in line with the data previously published by our group (6, 8) and  
336 others (7, 9-10), confirming that additional alterations of the cAMP pathway, apart from the well-  
337 known *PRKACA* mutations, are associated with a severe hormonal phenotype and, likely, early  
338 diagnosis.

339 Among mutations affecting genes of the Wnt/ $\beta$ -catenin pathway, as expected, the most  
340 common were somatic mutations in *CTNNB1* (39% of cases). They occurred more frequently in  
341 patients with SCS and EIA (54% and 52% of cases, respectively) than in those with CS (18%), as  
342 previously reported (15). These findings may further confirm a predominant role of *CTNNB1*  
343 mutations in early adrenocortical tumorigenesis. Among the components of the Wnt/ $\beta$ -catenin  
344 pathway, genes encoding for the plakophilin (*PKP2*), member of the arm-repeat (armadillo) gene  
345 family, the adenomatosis polyposis coli (*APC*) and *APC2*, and four members of the protocadherin  
346 family (*PCDH15*, *PCDHA8*, *PCDHA10*, *PCDHB11*) were recognized. Protocadherins play a major

347 role in cell-cell adhesion and interfere with the  $\beta$  catenin signaling proliferation pathway (31). Some  
348 members of the protocadherin family have recently been recognized as candidate tumor suppressor  
349 genes (31), and somatic mutations have been reported in squamous cell carcinoma, colon  
350 adenocarcinoma and melanoma (see COSMIC, <http://cancer.sanger.ac.uk/cosmic/gene/analysis>).  
351 Moreover, protocadherins may play a role in cell-cell adhesion and interfere with the Wnt/ $\beta$ -catenin  
352 signaling pathway (32), supporting the hypothesis that alterations of this Wnt/ $\beta$ -catenin regulatory  
353 signal may be relevant for adrenocortical tumorigenesis. In this context, it is important to mention that  
354 the regulator of Wnt/ $\beta$ -catenin pathway *ZNRF3*, recently reported as one of the most frequently altered  
355 genes in ACC (15), was not identified among mutated genes in our ACA series. In general and  
356 similarly to what previously reported for *CTNNB1* mutations, the genetic alterations in components of  
357 the Wnt/ $\beta$ -catenin pathway were mostly found in older patients with larger and inactive tumors (19).

358 Among  $\text{Ca}^{2+}$ -dependent signaling pathways, genes encoding  $\text{Ca}^{2+}$  receptors (*CACNA1C* and  
359 *CACNA1E*), ryanodine receptors (RyRs), ionotropic glutamate receptors (iGluRs) and one glutamate  
360 receptor interacting protein (*GRIP1*) were included. The RyRs are intracellular  $\text{Ca}^{2+}$ -release channels  
361 found on the sarcoplasmic reticulum of myocytes and on the endoplasmic reticulum of several non-  
362 muscular organs (33). There is some evidence on the potential role of RyR alterations on adrenal  
363 function (34). According to our *in silico* analysis of *RYR1* and *RYR2* mutations and considering that  
364 the interaction between transmembrane and cytoplasmic domains of those receptors is an important  
365 mechanism in  $\text{Ca}^{2+}$  release modulation (35), it is well conceivable that the mutations found in our  
366 cohort may be biologically relevant. Several genes responsible for regulation of intracellular  $\text{Ca}^{2+}$   
367 levels are known or suspected to be involved in the pathogenesis of endocrine tumors, such as  
368 aldosterone-producing adenomas (*KCJN5*, *ATP1A1*, *ATP2B3*, and *CACNA1D*) (27, 28) and GH-  
369 secreting pituitary adenomas (36, 37). In contrast, the role of alterations of  $\text{Ca}^{2+}$  signaling in the  
370 pathogenesis of CPA is not well understood, even though it has been demonstrated that adrenal  
371 fasciculata cells express high levels of T-type and L-type  $\text{Ca}^{2+}$  channels that may regulate cortisol  
372 secretion (38). Additionally,  $\text{Ca}^{2+}$  channels could be involved in molecular mechanisms of apoptosis  
373 regulation and cancer transformation (39), leading us to speculate on the proliferative role of this  
374 pathway in adrenocortical cells. Interestingly, the transcriptome analysis performed on our

375 independent cohort clearly showed that the expression of  $\text{Ca}^{2+}$  signaling-related genes in ACAs not  
376 associated with primary hyperaldosteronism is able to classify patients into meaningful clusters. In  
377 fact, the unsupervised clustering restricted to the expression levels of those genes, provided a good  
378 separation of patients with CS from those with SCS and EIA, and tumors with mutations in the cAMP-  
379 PKA pathway from those with Wnt/ $\beta$  catenin alterations. This finding, together with the identification  
380 of somatic mutations in  $\text{Ca}^{2+}$  signaling genes in our study, provides indirect evidence for a role of  
381  $\text{Ca}^{2+}$ -related pathways in the tumorigenesis and steroidogenesis of non-aldosterone secreting ACAs.  
382 Further studies will be necessary to unravel the specific underlying mechanisms.

383 Additional insights come from the combined analysis with CNA available from a previous  
384 SNP array profiling (17) in a well representative subgroup of present ACAs (three CS, seven SCS, and  
385 seven EIA), including four samples without any somatic mutations, four with mutations in the Wnt/ $\beta$   
386 catenin pathway, four with mutations in the cAMP/PKA pathway and two samples without known  
387 driver mutations. Here, we observed amplifications in several components of the Wnt/ $\beta$ -catenin,  
388 cAMP/PKA or  $\text{Ca}^{2+}$ -dependent signaling pathways. While this provides additional evidence for a  
389 major role in the pathogenesis of ACA, no differences were observed between ACA with or without  
390 somatic mutations.

391 According to the results of the pathway analysis, components of  $\text{Ca}^{2+}$  signaling, collagen  
392 formation, and extracellular matrix organization were among the most significantly represented.  
393 Extracellular matrices (ECM) are secreted molecules composed of glycoproteins, collagens,  
394 glycosaminoglycans and proteoglycans that can regulate cell migration, differentiation, proliferation  
395 and survival by communicating with intracellular cytoskeleton and growth factor signals (40).  
396 Interestingly, a putative role for ECM expression has been hypothesized in the development of human  
397 adrenal cortex (41). Moreover, a previous transcriptome study on ACAs identified enrichment in  
398 genes related to ECM (42). However, we observed only “private” mutations in ECM and collagen  
399 formation pathways and it is unclear whether they derive from proliferative processes or might  
400 represent early events in adrenocortical tumorigenesis.

401 We also performed an unsupervised clustering considering the WES data available for all  
402 ACA together (n=168) and separated for CPAs with or without *PRKACA* mutations (n=49 and 94,

403 respectively), providing results similar to that obtained in our present series (*Supplementary Figure*  
404 *4*). In addition, in this very large series, we observed that most genetic alterations in the cAMP/PKA  
405 signaling pathway were not associated with alterations at the Wnt/ $\beta$ -catenin or  $\text{Ca}^{2+}$ -dependent  
406 signaling pathway, further confirming their major role in the pathogenesis of CPAs.

407 The analysis of the genetic landscape of ACAs and ACCs provides indirect evidence for the  
408 existence of an adenoma-carcinoma sequence in adrenocortical tumors. For instance, the frequent  
409 C:G>T:A transitions observed in our patients has been found to be a feature of most cancer types (43),  
410 including ACC (12). Moreover, 6% of somatic mutations identified in our series were previously  
411 observed in at least two ACC samples (12, 20-21), giving support to a potential role of early genetic  
412 alterations in a multistep malignant transformation process. In this context, recurrent mutations in the  
413 hot-spot region of *CTNNB1*, were among the most commonly observed alterations in ACA and ACC.  
414 Thus, it is tempting to speculate that an adenoma-to-carcinoma multistep progression might occur in a  
415 subset of adrenocortical tumors bearing *CTNNB1* mutations, with  $\beta$ -catenin activating mutations as an  
416 early step in adrenocortical tumorigenesis. In sharp contrast, 11/99 tumors did not show any detectable  
417 genetic alteration by exome-sequencing. This finding might be due to limitation of the WES technique  
418 or to the pathogenesis of some ACA, which should be further evaluated for different genetic  
419 aberrations (alterations in intronic regions, alternative splicing, or gene fusions).

420 **One limitation of the current study is the lack of functional data so that we can only speculate**  
421 **on the biological role of newly identified genetic variants. However, also due to the large number of**  
422 **“private” mutations, this was beyond the scope of this report that was focused on providing a**  
423 **comprehensive overview of acquired genetic findings and potential genotype/phenotype correlations.**  
424 **Thus, targeted functional experiments will be required to characterize mutations not described in the**  
425 **literature.** In contrast, the large samples size, including for the first time also endocrine inactive  
426 adenomas, with detailed clinical characterization and the integration of previous WES data available  
427 for cortisol-secreting adenomas and carcinomas are relevant strengths of this collaborative project.

428 In summary, our study represents the largest sequencing effort on sporadic unilateral  
429 adrenocortical adenomas and demonstrates the heterogeneity of the genetic background of ACAs  
430 without *PRKACA* p.Leu206Arg mutation. Apart from the known somatic mutations, no other recurrent



431 mutation can alone explain the processes that lead to tumor formation and hormone hypersecretion.  
432 However, the provided landscape and the genetic alterations in newly described pathways (i.e. Ca<sup>2+</sup>-  
433 dependent signaling) are shedding light on the pathogenesis of adrenocortical tumors and are  
434 providing a solid basis for future molecular analysis.

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438

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623 **Figure legends**

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625 **Figure 1.** Total number of somatic mutations in each adrenocortical adenoma (n=99) evaluated by  
626 next generation exome sequencing (median: 6 mutations per tumor).

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628 **Figure 2.** *In silico* analysis of the 3D structure changes of three novel somatic mutations in *PRKACA*  
629 gene (589A->G, p.Trp197Arg; 95T->A, p.Glu32Val; and deletion in position 731-745, p.245-248).

630 a) wild type; b) the p.Trp197Arg mutation is at the interface between the catalytic and regulatory  
631 subunit. The exchange of the hydrophobic tryptophan with the hydrophilic, positively charged arginine  
632 leads to changes in this interaction. The p.245\_248.del also affects a region of the catalytic subunit of  
633 PKA at the interface with the regulatory subunit. The deletion of this region probably leads to  
634 modification of the 3D structure and affects the binding of the regulatory to the catalytic subunit. The  
635 mutation p.Glu32Val is situated outside the interaction region between the catalytic and regulatory  
636 subunits of PKA or any other reported interaction region of catalytic subunit of PKA.

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638 **Figure 3. A.** Heat map of the most recurrent somatic mutations classified according to their known or  
639 potential biological consequences: mutations in genes encoding components of Wnt- $\beta$  catenin  
640 pathway, those in genes encoding members of the cAMP/PKA pathway, and mutations in genes  
641 involved in Ca<sup>2+</sup>-signaling (n=99 samples). The relationship with the total number of somatic  
642 mutations and clinical parameters is also shown. **B and C.** Transcriptome analysis of the cohort of  
643 additional 41 adenomas. The unsupervised clustering performed according to the expression level  
644 from whole transcriptome profiling is shown in **B**, whereas the clustering restricted to Ca<sup>2+</sup> signaling-  
645 related genes is shown in **C**. The relationship with somatic mutations and clinical parameters, as well  
646 as the heat map of under-/over-expressed genes in the two pathways is also shown.

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