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RNA-Sequencing and Somatic Mutation Status of Adrenocortical Tumors: Novel Pathogenetic Insights.

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ABSTRACT

Context. Pathogenesis of autonomous steroid secretion and adrenocortical tumorigenesis remains partially obscure.

Objective. To investigate the relationship between transcriptome profile and genetic background in a large series of adrenocortical tumors and identify new potential pathogenetic mechanisms.

Design. Cross-sectional study.

Setting. University Hospitals of the European Network for the Study of Adrenal Tumors (ENSAT).

Patients. We collected snap-frozen tissue from patients with adrenocortical tumors (n=59) with known genetic background: 26 adenomas with Cushing syndrome (CS-CPA), 17 adenomas with mild autonomous cortisol secretion (MACS-CPA), 9 endocrine-inactive adenomas (EIA), and 7 adrenocortical carcinomas (ACC).

Intervention. RNA-sequencing.

Main Outcome Measures. Gene expression, long non-coding RNA (lncRNA) expression, and gene fusions. Correlation with genetic background defined by targeted Sanger sequencing or whole-exome sequencing.

Results. Transcriptome analysis identified two major clusters for adenomas: Cluster 1 (n=25) consisting of EIAs and MACS-CPAs with *CTNNB1* or no driver mutations, and Cluster 2 (n=25) consisting of CS-CPAs and MACS-CPAs with *GNAS* mutations and CS-CPAs with *PRKACA* or no drivers mutation. CS-CPA with *CTNNB1* mutations (n=2) clustered close to ACC. In Cluster 2, *FATE1* was among most common overexpressed genes. lncRNA analysis confirmed that patients clustered in four groups. Novel gene fusions were found, including *AKAP13-PDE8A* in one CS-CPA sample with no identified driver mutation.

Conclusions. MACS-CPAs and EIAs showed a similar transcriptome profile, independently of the genetic background, except for MACS-CPAs with *GNAS* mutations that clustered with most CS-CPAs. Still unrevealed molecular alterations in the cAMP/PKA or Wnt/beta catenin pathways might be involved in the pathogenesis of adrenocortical tumors.

Introduction

Over the last few years, the molecular and genetic events underlying the pathogenesis of adrenocortical tumors have been investigated by applying classical genetic approaches and next-generation sequencing techniques including whole-exome sequencing (WES). Alterations of several components of the cAMP/PKA pathway have been identified as a causative factor for tumorigenesis and cortisol hypersecretion in adrenocortical adenoma (ACA) [reviewed in (1,2)]. Independent studies have reported somatic mutations in the *PRKACA* gene as the pivotal pathogenetic event in approximately half of the ACAs associated with Cushing syndrome (CS), due to a constitutive activation of the catalytic subunit α of PKA (3-7). In a smaller proportion of patients with CS, additional mutations in members of the cAMP-PKA signaling pathway have newly been described, such as somatic mutations of the gene encoding the regulatory subunit II beta of PKA (*PRKACB,* very rare) (8), or confirmed, such as those occurring in the gene encoding the regulatory subunit 1 α of PKA (*PRKAR1A*) or the protein Gsα (*GNAS*) (4,5,7,9). Activating mutations in the gene encoding β-catenin (*CTNNB1*) have also been identified as an important contributor of adrenocortical growth. Interestingly, *CTNNB1* mutations had been reported in ACA and adrenocortical carcinoma (ACC) with a similar prevalence (10-30%) (9,10) with the highest frequency in endocrine inactive adenomas (EIA) (9,11). Despite these substantial advances, the molecular mechanisms underlying autonomous steroid secretion and adrenocortical tumorigenesis remain obscure for around 2/3 of cases that cannot be linked to known driver mutations.

Another important issue, is that, depending on the definition used, up to 40% of patients with apparently non-functioning ACA might present a mild autonomous cortisol secretion (MACS), a condition previously termed “subclinical CS”, since the classical clinical manifestations of overt hypercortisolism are absent (12,13). However, several evidences suggest that MACS could be associated with cortisol-dependent comorbidities, including higher risk of cardiovascular events, hypertension, dyslipidemia, diabetes mellitus type 2, obesity, osteoporosis and higher mortality rate (12,14-18). A previous study on transcriptome analysis on a small series of ACAs identified two different clusters according to hormone secretion: cluster 1, including only cortisol-producing adenomas (CPAs) associated to CS (CS-CPAs), and cluster 2 including non-functioning ACAs, as

well as MACS- and CS-CPAs, revealing an association between cortisol secretion and expression of a subset of genes implicated in steroid secretion (19). However, as to this point, the association between transcriptome and mutational status of benign adrenocortical tumors has not been investigated.

Our first aim was to analyze the association between the genetic background and the transcriptome profile assessed by RNA-sequencing (RNA-seq) in a large series of ACAs. Our second aim was to identify novel molecular events that may be involved in cortisol hypersecretion and adrenocortical tumorigenesis through the analysis of alternative gene spliced transcripts, long non-coding RNAs (lncRNAs), and gene fusions.

Materials and methods

Study protocol

This is a European multicentric retrospective study among centers belonging to the European Network for the Study of Adrenal Tumors (ENSAT), designed and conducted in accordance with the Declaration of Helsinki. The local ethic committees of each Institution approved the study protocol. Written informed consent was obtained from all subjects prior to study enrollment.

Selection of patient cohort

We selected snap-frozen samples from the previously published series of 99 non-aldosterone producing ACAs without exon 7 hot-spot *PRKACA* mutations, already characterized by WES (9). Among these, we took 54 samples with still available tumor material for RNA isolation. After excluding seven cases due to poor quality RNA (see paragraph "sample preparation"), we obtained a final series of 47 ACAs for RNA-seq analysis (**Figure 1**). According to the results of WES, we identified 22/47 (46.8%) ACAs with somatic mutations in known driver genes, such as *GNAS*, *CTNNB1*, and *PRKACA* (outside the hot-spot region). These included 7 CS-CPAs, 11 MACS-CPAs, and 4 endocrine inactive adenomas (EIAs). The remaining 25/47 (53.2%) tumors (n=14 CS-CPAs, 6 MACS-CPAs, and 5 EIAs) did not have somatic mutations in known driver genes at WES. In addition, we included 5 further CS-CPAs samples with known hot-spot L206R *PRKACA* mutations from the previously published cohort (6) and 7 early-stage/low aggressive ACCs (ENSAT tumor stage

1-3, Ki67 proliferation index <20%) that served as controls. The final cohort included 59 samples (**Figure 1**). Four normal adrenal glands (NAGs) obtained from surgery for renal carcinoma (n=1) or from tissue surrounding EIA (n=3) were used as controls for gene expression profiling. The dissection of the adrenal tissues from the snap-frozen specimen was made by expert pathologists at each Center participating in the study.

At variant calling performed on our RNA-seq data (see methods below), we could detect two additional somatic mutations in two CS-CPA cases classified as without driver mutations based on previous WES (see details shown in **Figure 1**). These mutations were in genes *PRKACA* (hot spot L206R found in CS-CPA2) and *GNAS* (hot spot R201S in CS-CPA6). In addition, two previously identified alterations in *CTNNB1* genes could not be verified, i.e. a 1602bp splice indel in CS-CPA22 and a missense mutation T41A in CS-CPA9. According to these findings, we classified our cohort as follows: 27 ACA with known driver mutations (7 CS-CPA_PRKACA, 4 CS-CPA_GNAS, 2 CS-CPA_CTNNB1, 9 MACS_CTNNB1, 1 MACS-CPA_GNAS, 4 EIA_CTNNB1) and 25 ACA without driver mutations (13 CS-CPA, 7 MACS-CPA and 5 EIA).

Clinical data collection

Clinical and hormonal data such as sex, age at diagnosis, initial tumor size, steroid secreting pattern, symptoms of overt CS were already available from previous studies (6,9).

Overt CS and MACS were defined according to the Endocrine Society guidelines (13), in order to maintain the same classification of previous studies (6,9). These guidelines recommend the use of the 1 mg overnight dexamethasone test (DST) with a cortisol cutoff of <1.8 µg/dL (50 nmol/L) as the primary test to exclude autonomous cortisol secretion. However, the newest European Society of Endocrinology (ESE)/ ENSAT guidelines from 2016 (12) recommend interpreting the 1 mg overnight DST as a continuous variable, considering post-dexamethasone serum cortisol levels between 1.9–5.0 µg/dL (51 and 138 nmol/L) as 'possible autonomous cortisol secretion' and levels >5.0 µg/dL (>138 nmol/L) as confirmed 'autonomous cortisol secretion'. Both guidelines recommend the use of additional biochemical tests to confirm cortisol secretory autonomy and assess the degree of cortisol

secretion, and MACS were defined in absence of clinical and catabolic signs of overt CS (12,13). To avoid bias in the interpretation of our results, we performed a second analysis classifying the patients according to the ESE/ENSAT guidelines. According to that, 2 MACS-CPAs (MACS-CPA2 and MACS-CPA11) without driver mutation were reclassified ad EIAs, and one EIA with *CTNNB1* mutation (EIA6) was reclassified as MACS-CPA. Differences between the results deriving from the two classifications were evaluated. In all our patients with MACS with values between 1.8 and 5 mcg/dL, ACTH was below 2.2 pmol/L (10 pg/mL) or midnight cortisol was above the reference range.

Clinical and histopathological data of ACC cases, including ENSAT tumor stage, Weiss score, and Ki67 index, were available from a previous publication (20) or were collected through the ENSAT registry (https://registry.ensat.org/).

Sample preparation and RNA-sequencing

RNA was isolated from snap-frozen tumor tissue by RNEasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) (n=23) or by Maxwell® 16 Total RNA Purification Kit used with the Maxwell® 16 Instrument (n=36), according to the manufacturers' instruction. Initial RNA quality control was performed by spectrophotometry at 260 nm and by running a denaturing agarose gel. Additional analysis was performed using an Agilent Technologies 2100 Bioanalyzer and an RNA Integrity Number (RIN) value ≥8 was required to ensure efficient mRNA sequencing.

TruSeq RNA Library Prep Kits was applied prior Illumina sequencing (NextSeq500) of pooled normalized libraries. Specifically, a paired-end 75nt mode (high-output flow-cells) was used for a minimum of 40-100 million reads per sample.

Data analysis

An initial quality assessment was performed using FastQC, v0.11.5 (Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Adapter- and quality trimming was done with Cutadapt, v1.1.5 (https://cutadapt.readthedocs.io/en/stable/). STAR v2.5.3a (21) was used to map the trimmed reads to the GENCODE human reference genome GRCh37 release 29. We used

Samtools v1.3 (22) utilizing htslib, v1.3 for sam-to-bam-conversions as well as sorting and indexing of the alignment files. For gene annotation, the GENCODE human reference genome GRCh37 release 29 was used. FPKM-values and differential gene expression as well as differential isoform expression were calculated with Cufflinks package, v2.2.1 (23). For principal component analysis (PCA) analysis, one was added to all FPKM values. The samples were normalized to the median of the four sequenced NAGs and log2-transformed. FPKM-values of all genes with a coefficient of variation ≥0.5. PCA was performed with the prcomp function from the R stats package, v3.4.4. Subsequently, 500 protein coding genes and 250 lncRNA genes with the highest PC loadings in one of the first three components were selected for unsupervised complete linkage clustering, respectively. The clustering was performed on the rows and columns using the Euclidian distance metric with the heatmap.2 function in the gplots R-package, v3.0.1. Arriba v1.1.0 was used for fusion detection with default settings (21-23). In the heatmap, we used log2(FC). The value 1 was added to each FPKM in order to make dividing by the median possible.

For pathway analysis, we used the Gene Set Enrichment Analysis (GSEA) software by Broad Institute v.MSigDB 7.0 (24,25) using gene sets from both Reactome and KEGGS pathways.

Variant calling using RNAseq data

Variant calling was performed following the GATK Best Practices workflow for SNP and Indel calling on RNAseq data (https://software.broadinstitute.org/gatk/documentation/article.php?id=3891). Trimmed reads TrimGalore, v06.6.1 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) utilizing Cutadapt, v2.3 (26) were mapped to the reference using STAR, v2.5.4b (21). The index and the dictionary of the reference sequence were created using Samtools v1.3 (22) and Picard, v2.18.11 (https://broadinstitute.github.io/picard/), respectively. Picard was also used to add read groups, to sort alignment files, to mark duplicates, and to create index files. The Genome Analysis Toolkit v3.8 (27) was used to reassign mapping qualities, indel realignment, base recalibration, variant calling, as well as variant filtering. Variants were annotated using ANNOVAR (v2019-10-24) (28). Somatic protein-altering variants and splice site variants were reported if they alter the protein sequence or affect a

splice site, are rare in the population (below a frequency of 2 % in 1000g2015aug_all, ExAC_nontcga_ALL, gnomAD_exome_ALL and gnomAD_genome_ALL) and the position is covered by at least 20 reads and the alternative allele is covered by at least 8 reads and comprised at least 10 %.

Validation of gene fusions

We selected a total of 7 high-confidence gene fusions to be validated by Polymerase Chain Reaction (PCR) and Sanger-Sequencing. Reverse transcription of RNA was performed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) as previously described (29). PCR amplification of 10 ng of cDNA was performed in a final volume of 25 µl using specific primer sets designed to span the fusion breakpoints, whereas GAPDH was used as internal control gene (Supplementary Table 2 (30)). Cycling conditions were: 96ºC for 2 min followed by 30 cycles of denaturing at 96°C (30 sec), annealing at 58°C (30 sec) and elongation at 72°C (1 min), and a final step of 72°C for 5 min. The PCR products were run on a 2.4% low-melt agarose gel with ethidium-bromide. PCR products were purified (ExoSAP-IT, Amersham Bioscences, Munich, Germany) and sequenced using the QuickStart Cycle Sequencing Kit (AB Sciex, Beckman Coulter, #608120, Krefeld, Germany) on a Genetic Analysis System (CEQ) 8000 (AB Sciex, #A16637-AA, Krefeld, Germany) capillary sequencer according to the manufacturer instructions. Data were analyzed using the CEQ8000 DNA Analyzer (AB Sciex).

Statistical analysis

To correlate the results of the unsupervised clustering analysis with the clinical features, the following parameters were evaluated: sex, age (cutoff 50 years old), tumor size (cutoff 35 mm), diagnosis (EIA, MACS-CPA, CS-CPA, and ACC), and mutational status (no driver mutations, *CTNNB1* mutation, mutations of the cAMP/PKA pathway, other driver mutations). A Fisher exact or Chi-square (χ^2) tests were used to investigate dichotomic variables, while a two-sided *t*-test or Mann-Whitney U-test was used to compare continuous variables, as appropriate. Kruskal-Wallis test, followed by Bonferroni

post-hoc test or Dunn's multiple comparison test, was used for comparison among several groups for non-normally distributed variables. Correlations and 95% confidence intervals (95% CI) between different parameters were evaluated by linear regression analysis. Statistical analyses were made using GraphPad Prism (v.6.0, La Jolla, CA, USA) and SPSS Software (v.23, SPSS Inc., Chicago, IL, USA). Data are presented as median with interquartile range in parentheses or mean ± standard deviation (SD). A p value <0.05 was considered statistically significant.

Results

Patient characteristics

Clinical parameters and hormonal levels at the time of diagnosis are reported in **Table 1**. Patients with CS-CPAs were mostly females and significantly younger than those of the other groups. As expected, they had significantly higher post-dexamethasone cortisol levels and 24-h urinary free cortisol, as well as lower ACTH levels in comparison to other groups. No other significant differences were observed in clinical parameters.

Among ACCs, 2 patients had non-secreting tumors and 5 had secreting ACC, including 2 overt-Cushing, 1 associated with mild autonomous cortisol secretion, and 2 associated with androgens excess.

Gene expression profile (overview)

The entire list of differentially expressed genes is reported in the **Supplementary Table 1**. Protein coding genes recognized at RNA-seq represented 86% of all detected genes, whereas the remaining gene types included processed and unprocessed pseudogenes, mitochondrial rRNA, and lncRNA (**Supplementary Figure 1** (30)). The number of significantly over- and under-expressed protein-coding genes in each group and the most frequently altered pathways are reported in **Table 2**. CS-CPAs showed 67 overexpressed genes, being mostly related to *GPCR ligand binding* and *G alpha (q) signaling*, with *NTS*, *TJP3, MMP7, ITIH1, GPRC6A, TNNT1, FATE1,* and *GIPR* among the top 40 genes. Conversely, MACS-CPAs and EIAs showed only few overexpressed genes (not enough for pathway analysis). ACCs samples presented 472 significantly overexpressed genes, mostly involved

in *Cell Cycle* and *Mitosis*. *DUSP9*, *PAX9*, *SULT1C2*, *PMAIP1*, *DHRS2*, *CPNE4*, *PBK*, and *DSG2* were among the top 40 overexpressed genes. Under-expressed genes in ACCs, like *GNG4*, *GNB3*, *CAMK2B*, *ADCY2*, and *SLC17A7,* were observed mostly in *Transport of small molecules*, *Neuronal System*, and *GPCR ligand binding*.

Relationship between gene expression profile and mutational status

At group 3D PCA (**Figure 2A**) and individual unsupervised clustering analysis (represented as heatmap in **Figure 2B**) for protein-coding genes we could classify tumor samples in four clusters: 1) "Cluster 1" (n=32), including MACS-CPAs and EIAs, either with *CTNNB1* mutations (MACS-CPA_CTNNB1, n=9, and EIA_CTNNB1, n=4) or without mutation in a known driver gene (MACS-CPA_none, n=6, and EIA_none, n=4), CS-CPA without known driver mutations (n=7) or with GNAS somatic mutations (n=2); 2) "Cluster 2", comprising mostly CS-CPAs with or without driver mutation (CS-CPA_PRKACA, n=7; CS-CPA_GNAS, n=2; and CS-CPA_none, n=6); 3) CS-CPAs with *CTNNB1* mutations (CS-CPA_CTNNB1, n=2), which show a gene expression pattern closer to ACC than most of the remaining benign tumors, and 4) "ACC", clustering separately from all other groups. The single MACS-CPA_GNAS case (MACS-CPA8) belonging to Cluster 2 was a 29-years old female patient, with a small ACA of 2 cm, with not suppressed post-dexamethasone serum cortisol levels (397.3 nmol/L), and 24-hour urine free cortisol (UFC) and ACTH levels within the normal range. The only MACS_no driver in Cluster 2 was a 34-years old male with 3.4 cm adrenal nodule, failed dexamethasone suppression test (100.2 nmol/L), and normal 24-hour urine free cortisol (UFC) and ACTH levels. Both had no clinical signs of CS.

Particularly, cases in Cluster 1 were mostly old male patients having large tumor, whereas those in Cluster 2 were predominantly young patients female with small tumor. The individual 3D PCA plots for protein-coding genes in the entire cohort (52 ACA, 7ACC, 4 NAG) is shown in the **Supplementary Figure 2A**.

Results from PCA and unsupervised clustering analysis did not change by classifying the adenomas according to the ESE/ENSAT guidelines (12), when considering all the differentially expressed genes. Therefore, further analyses were performed according the Endocrine Society guidelines.

Comment [GDD1]: Indeed, in the individual heatmap 1 of the 2 ctnnb1-cpas is included in cluster 2. Conversely, there is a gnas-cpa which cluster together with the other cpa-ctnnb1, which are both closer to ACC. Nonetheless, I would leave it as it is, since 3DPCA and individual heatmap are generated from different data (the whole expression data fro PCA and the top 500 genes for individual heatmap). Is it correct, Silke?

Comment [GDD2]: If we describe this case in detail, should we also describe the MACS-CPA13_none, which belongs to cluster 2? Otherwise, we can remove this detailed description. What do you think about it?

Comment [CR3]: I would add also the MACS-CPA13

Looking at specifically over-expressed genes in the different clusters (boxes in **Figure 2B**), we identified *IGFN1, CXCL2, DPEP1, PITX1, SHISA3* and *CXCL14* in Cluster 2. Among Cluster 1, no genes were homogeneously over-expressed. Even if not highlighted in the heatmap, among the most overexpressed genes in Cluster 2, we observed *FATE1* (Fetal and Adult Testis Expressed 1). Targeted data analysis of *FATE1* confirmed that CPAs with *GNAS* or *PRKACA* mutations had significantly higher *FATE1* mRNA expression than MACS-CPAs or EIAs with or without *CTNNB1* mutations ($p \leq 0.001$ and $p \leq 0.001$, respectively) (**Figure 3A**).

Moreover, genes like *PTTG1, TOP2A, CDK1, CCNB1, UBE2C,* and *PMAIP1* were highly expressed selectively in ACC (boxes in **Figure 2B**), most of them being involved in cell cycle regulation. Of note, the cluster with CS-CPAs_CTNNB1 showed some overexpressed genes in common with ACC (*SPINK13, GPX8, TMEM26, NPTX2,* and *HSPB8*). Targeted data analysis of *TOP2A* (Topoisomerase 2A) and *CCNB1* (cyclin B1) confirmed that ACC had significantly higher mRNA expression than other groups ($p \leq 0.001$ compared to MACS-CPAs or EIAs with *CTNNB1* mutation or without driver mutation and compared to MACS-CPAs and CS-CPAs with *GNAS* or *PRKACA* mutation, and $p \leq 0.05$ CS-CPAs_none, **Figure 3B**). Targeted analysis of *HSPB8* showed higher mRNA levels in both ACC and CS-CPA_CTNNB1 compared to other groups (**Figure 3B**). Moreover, as expected, ACC showed lower mRNA expression of *ZNFR3* than all other groups: -1.1±0.89 FKPM - P<0.05 only vs EIA/MACS_CTNNB1 (0.1±0.4) and EIA/MACS_no driver (-0.01±0.55) (**Figure 3A**). One CPA with *CTNNB1* mutation as well as few cases of EIA/MACS/CPA without driver mutations showed relatively low ZNFR3 levels, even if this was observed also in some CPA with known mutations in cAMP/PKA related genes. As shown in **Figure 3A**,

Considering other genes related to the steroidogenesis, *NR5A1* (Nuclear Receptor Subfamily 5 Group A Member 1, known also as steroidogenic factor 1, *SF1*) mRNA expression was significantly lower in ACC in comparison to MACS-CPAs or EIAs with CTNNB1 mutation or without driver mutations ($p \leq 0.001$ and $p \leq 0.05$, respectively), CS-CPA and MACS-CPA with *GNAS* or *PRKACA* mutation ($p \leq 0.001$) or CS-CPA_none ($p \leq 0.05$) (**Figure 3A**). Of note, CS-CPA with *GNAS* or *PRKACA* mutation showed significantly higher levels of *CYP11A1* and *CYP21A2* than EIA and MACS with *CTNNB1* mutations (**Figure 3A**).. On the other side, ACCs showed also significantly lower levels of

Comment [CR4]: I modified this

Comment [GDD5]: I cannot really say that by looking at the heatmap. Silke, could you please confirm these data?

Comment [CR6]: I modified this

Comment [CR7]: Silke/Guido please check P values for both genes.

Comment [CR8]: Add P value for HSPB8

Comment [CR9]: Add P values

several genes encoding for steroidogenic enzymes, including *CYP11B1* and *CYP11B2,* when compared to remaining tissues (data not shown). No other significant differences could be detected among other groups.

The analysis of clinical data among clusters identified by mRNA gene expression showed significant differences in sex ($\chi2$=9.90, p=0.02), age ($\chi2$=11.87, p=0.008), tumor size ($\chi2$=16.44, p=0.001), diagnosis ($\chi2$=113.74, p<0.001), and mutational status ($\chi2$=52.28, p<0.001) (**Figure 2A**). Particularly, patients in Cluster 1 were older (61% of the cases had ≥55 years) and had larger tumors (58% of the cases had tumors ≥35 mm) either inactive (all but one inactive tumors grouped in Cluster 1) or associated with MACS (47%). Except two CPAs, all remaining tumors with *CTNNB1* somatic mutations were also included in Cluster 1 (**Figure 2B**). On the contrary, in Cluster 2, all patients were female, except one, with diagnosis of overt Cushing syndrome in 15/18 cases, among which 77% of cases had <55 years. No driver mutation and mutation in the cAMP/PKA pathway were found in 44% of the cases. We could distinguish three sub-groups in Cluster 1 [1A (n=12), 1B (n=12), and 1C (n=8)] and two in Cluster 2 [2A (n=11) and 2B (n=7)] with slightly different clinical phenotype (**Supplementary Figure 2B** (30)). In particular, the homogeneous sub-cluster 2A includes all seven CS-CPAs with *PRKACA* mutations and three CS-CPAs without driver mutations, together with one MACS-CPA_none.

Differential exon usage

We investigated the alternative gene spliced transcripts in all our samples. Comparing the individual groups (ACCs, CS-CPAs, EIAs, MACS-CPAs each against NAGs) according both guidelines (12,13), no significant differential exon usage was detected (results not shown).

Long non-coding RNA

Several lncRNAs were differentially represented among the groups of tumors. As shown in **Supplementary Figure 4** (30), the PCA and the unsupervised clustering analysis confirmed that

Comment [GDD10]: 19/31 + 1 unknown

Comment [GDD11]: 18/31 + 1 unknown

Comment [GDD12]: From Barbara's email

Comment [GDD13]: This was the original sentence, before changing. Particularly, patients in Cluster 1 were older (68% of cases) and had larger tumors (64% of cases) either inactive (36%) or associated with MACS (64%) and with CTNNB1 mutations (52%) or unknown driver mutations (48%) (Figure 4 and Supplementary Figure 2B (30)).
By looking at the data, there are several differences between the one sent by Barbara and the one reported in new figure 2b (individual heatmap). Am I missing something? Barabara, could you please double check?

Comment [GDD14]: 14/18 + 1 unknown

patients clustered in four groups: ACCs, CS-CPAs_CTNNB1, lnc_Cluster 1 and lnc_Cluster 2 tumors, in analogy to the results of the coding gene expression profile.

IGFL2-AS1 was overrepresented in ACCs, whereas *LINC02539* and *LINC02104* were clearly overrepresented in CS-CPA_CTNNB1 tumors. Additionally, *LINC00400* and *DIO3OS* were overrepresented in lnc_Cluster 2. Both PCA and the heatmap for lnc showed similar results when we classified the tumors according to the ESE/ENSAT guidelines (12).

Gene fusions

All ACC samples provided evidence for gene fusions. The number of high- and medium-confidence fusions per sample was significantly higher in ACCs than ACAs (13.0±6.14 *vs* 1.67±1.95, $p<0.0001$). In contrast, no difference in the number of fusions was observed among CS-CPAs, MACS-CPAs and EIAs ($p=0.65$; **Supplementary Figure 3A** (30)). By considering only high-confidence gene fusions, ACCs had a significantly higher number of fusions per sample (8.14±5.43) compared to those found in Cluster 1 (0.76±1.13, $p=0.0005$) and Cluster 2 (0.72±0.89, $p=0.001$), but not to those observed in CS-CPA_CTNNB1 (2.00±2.82, $p=0.77$; **Figure 4A**). After restricting the analysis to CS-CPAs only, there was no significant difference in number of high-confidence fusions *per* samples considering the mutational status (**Figure 4B**). However, CS-CPA_CTNNB1 presented a trend towards higher number of high-confidence fusions compared to other subgroups (**Figure 4B**). We did not observe any significant difference in number of gene fusions when we classified the tumor according to the ESE/ENSAT guidelines.

The entire list of high confidence gene fusions detected in the present cohort is reported in the **Supplementary Table 3** (30). No recurrent fusions were detected in genes involved in cAMP/PKA nor Wnt/beta catenin pathways. However, we identified a "private" fusion between the gene coding for the A-kinase anchor protein 13 (*AKAP13*) and the gene coding the phosphodiesterase 8A (*PDE8A*) in one CS-CPA sample without known driver mutations (CS-CPA26; **Figure 4C-D**). This was validated by PCR. Regarding the other investigated fusions (**Supplementary Table 3** (30)), we were able to find 4 out of 6 gene fusions by PCR and Sanger-Sequencing, including *MAPKAPK5-ACAD10*,

379 *NUP155-WDR70*, *GTF2B-GBP5* and *SMC6-MSGN*, in the corresponding samples that were positive
380 at RNA-seq. *GADPH*, used as control gene, was found in all the evaluated samples.
381
382 **Discussion**
383 This is the first study employing RNA-seq to investigate the relationship between transcriptome and
384 the somatic genetic tumor background in a large, well-characterized cohort of benign adrenocortical
385 tumors. We found that transcriptome profiling was able to classify clearly distinct groups that also
386 differed in their hormonal profile and genetic background. Moreover, by applying deep RNA-seq, we
387 identified additional molecular alterations, including gene fusions and differentially expressed
388 lncRNAs associated with the genetic background of the tumors.
389 The transcriptome profile of benign adrenocortical tumors has been described in a previous
390 publication (19). By applying microarray analysis on a small number of ACAs (n=22), Wilmout
391 Roussel and colleagues identified a specific molecular signature of CS-CPAs, different from that of
392 EIAs or MACS-CPAs, which clustered together. The results of the unsupervised clustering analysis
393 performed in our study partly confirms those findings, showing that most benign adrenocortical
394 tumors could be divided in two groups, named Cluster 1, including EIAs and MACS-CPAs, and
395 Cluster 2, comprising CS-CPAs. The clinical features of patients in the two clusters revealed that
396 patients in Cluster 2 were mostly young females, with small hyperfunctioning tumors, whereas those
397 in Cluster 1 had mostly inactive or "low-functional" large tumors. Therefore, MACS-CPAs and EIAs
398 samples showed a similar transcriptome profile, which was clearly different from that of CS-CPAs
399 and ACCs. Those findings are consistent with previous clinical observations, showing that MACS-
400 CPA tumors seem to be a separate clinical entity from overt CS. Although patients with MACS could
401 present comorbidities associated to mild cortisol hypersecretion, such as risk of cardiovascular events,
402 hypertension, dyslipidemia, diabetes mellitus type 2, obesity, osteoporosis (12,14-18), the long-term
403 data did not provide evidence for an evolution from MACS to overt CS (31). Additionally, recent
404 analysis by liquid-chromatography tandem mass spectrometry in patients with adrenal incidentalomas

showed that the steroid profiling of subjects with EIA showed some similarities with those having MACS-CPA(32,33).

The combined analysis of transcriptome profiling with a tumor whole genome background performed in our cohort allowed us to pinpoint additional important findings. The similar transcriptome profile of MACS-CPA and EIA samples was independent of the presence of *CTNNB1* mutations, which were the most common alterations detected in tumors of this group. Additionally, the similar transcriptome profile among CS-CPAs is consistent with the activation of the cAMP/PKA pathway, independently of the underlying putative mutation. Conversely, the expression profile of the MACS-CPA associated with *GNAS* mutation was similar to that of CS-CPAs.

Among differentially expressed genes, it is worth mentioning *FATE1* as the most over-expressed gene in tumors belonging to Cluster 2, and specifically, in CS-CPAs samples carrying *PRKACA* and *GNAS* somatic mutations. *FATE1* encodes a known transcription factor involved in regulation of apoptosis and cell proliferation, regulated by steroidogenic factor 1 (SF1) at the promoter level (34). Considering the pivotal role of SF1 in steroid producing cells and the interplay between FATE1 and SF1 (35), our data provide additional evidence for the molecular events caused by cAMP/PKA pathway disruption due to *PRKACA* and *GNAS* somatic mutations at a transcriptional level.

Considering that the most evident difference between cluster 1 and cluster 2 is the steroid secreting pattern, we analyzed the expression of several genes involved in steroidogenesis. In particular, we could confirm that the expression of genes encoding for key enzymes such as *CYP11A1* (cytochrome P450 cholesterol monooxigenase) and *CYP21A2* (21-hydroxylase) was significantly higher in ~~*CYP11B2* was lower between~~ CPA carrying mutations in the cAMP-PKA genes compared to both ACC and EIA/MACS-CPA with CTNNB1 mutations.

Overall, the mRNA expression profile of the subgroup of CS-CPAs associated with *CTNNB1* mutations was more similar to that of ACCs than the remaining CS-CPAs and tumors included in Cluster 1, which were mostly carrying *CTNNB1* mutations. However, when analyzed at an individual level, CS-CPAs with *CTNNB1* mutations showed heterogeneity of the transcriptome profiling at unsupervised clustering. Notably, *TOP2A*, the gene encoding Topoisomerase II Alpha, was among the overexpressed genes in both CS-CPA samples with *CTNNB1* mutations and ACCs, different from the

Comment [CR15]: Guido please add one sentence about the relevance of this.

Comment [CR16]: I deleted this entire part and rewrote it. Those data are consistent with a suppression of the renin-angiotensin-system observed in severe hypercortisolism (as it is in tumors carrying cAMP-PKA pathway alterations), uncovered by lower levels of aldosterone, as shown in previous studies (Eisenhofer Clin Chem 2017). On the contrary, we could not demonstrate a difference in *CYP11B1* mRNA levels between CPA and EIAs. The expression level of *CYP11B1* in CPA has been investigated by different methods in several previous studies, with discordant results (Bassett, JCEM, 2005; Enberg, J Endocrinol Invest, 2009; Cao, Int J Urol, 2011; Kubota-Nakayama, Hum Pathol, 2016). In our study, the lack of difference of *CYP11B1* expression in CPA *vs* EIAs or MACS-CPA may be due by the lack of selective CYP11B1 immunohistochemistry-driven RNA tissue extraction.

remaining benign tumors. TOP2A is a cell cycle-related protein, involved in cell proliferation by controlling the topologic states of DNA during transcription in several types of cancer and is being studied as a potential druggable target for several anticancer agents (36). Several studies in ACC tissues have shown an overexpression of *TOP2A*, which was tightly correlated with Ki67, a marker of cell proliferation, and poor survival (37,38). Taken together, those observations reinforce the hypothesis proposed in several previous studies of a potential adenoma-carcinoma sequence in a sub-group of adrenocortical tumors. Indeed, studies on animal models and human tissues have shown that alterations of the Wnt-beta catenin pathway, IGF2 signaling and Notch signaling may boost the transformation of an ACA into ACC (39-41). Even though such an event is exceedingly rare (42), the alterations identified in benign adrenocortical tumors described in our study and in previous investigations may represent a preliminary phase in the multi-step potential evolution towards ACC.

The analysis of fusion genes, performed in benign adrenocortical tumors for the first time, showed that CS-CPAs samples carrying *CTNNB1* mutations had a number of high-confidence gene fusions that was set in between ACCs and remaining ACAs belonging to Cluster 1 and 2. Previous studies have identified fusion transcripts, a product of chromosomal rearrangements, as useful markers for several hematological malignancies and epithelial cancers, with important implications in treatment strategies (43). A recent pan-genomic characterization of ACCs has described a few private fusion transcripts, namely *EXOSC10-MTOR* and *MLL-ATP5L*, involving genes with well-known roles in cancer development (44), even though the impact of those events in the pathogenesis of ACC should be confirmed by further targeted studies. The results of the analysis of fusion transcripts in our cohort reinforce the hypothesis that CS-CPAs with *CTNNB1* mutations may have a different biological behavior than the remaining functioning and non-functioning benign adrenocortical tumors. As an additional interesting finding, we could identify the fusion transcript *AKAP13-PDE8A* in one case of CS-CPA without known driver somatic mutations. In a recent study, a similar fusion transcript has been frequently detected in colorectal cancer (45). The predicted PDE8B protein retained the conserved catalytic domain located at the C-terminal region, leading to the hypothesis that the promoter region of AKAP13, which is an A-kinase anchoring protein, might alter cAMP signaling through PDE8B dysregulation (45). Considering the involvement of phosphodiesterases in the

pathogenesis of Cushing syndrome (46), the involvement of *AKAP13-PDE8A* fusion transcript in the pathogenesis of a subset of CS-CPAs without driver mutations at WES is an intriguing mechanism that should be investigated in future functional studies.

Finally, the analysis of lncRNAs provided interesting insights into the molecular signature of adrenocortical tumors. LncRNAs are non-coding RNA transcripts >200 nucleotides in length, involved in epigenetic silencing, regulation of splicing and transcription (47). LncRNAs dysregulation has been described as a potential marker of ACC, with clinical implications. Indeed, recent studies have shown that the lncRNA molecular signature may discriminate ACCs from ACAs and NAGs and identify patients affected by ACC with poor prognosis (48,49). Our lncRNA signature analysis reinforced the results of the transcriptome analysis, showing that tumors could be divided in four clusters, including ACC, CS-CPA with *CTNNB1* mutations, lnc_Cluster 1 ("low-functional" tumors) and lnc_Cluster 2 ("functional tumors"). Indeed, ACC clustered separately from ACA and NAG tissue, confirming previous results (48,49). Notably, CS-CPAs with *CTNNB1* mutations clustered as a separate entity than CS-CPAs with known driver mutations affecting cAMP/PKA pathway and CS-CPAs with no driver mutations, giving further support to the different biological behavior of those tumors.

The main limitation of this study is represented by the rather small number of patients in selected groups of interest, which may limit the interpretation of the results and the possibility to perform correlations between transcriptome and clinical data. Nonetheless, this is the result of a strict selection process that relied on the genetic mutations identified in previous studies (6,9) and availability of high-quality RNA for RNA-seq. To avoid bias in the evaluation of our results, we performed the analysis following the definition of MACS-CPA and CS-CPA according to both the Endocrine Society and the ESE/ENSAT guidelines (12,13). The classification of three samples changes between the two guidelines: 2 MACS-CPAs without driver mutations, which are classified as EIAs according to the newest ESE/ENSAT guidelines, and 1 EIA with *CTNNB1* mutation MACS-CPS that was reclassified as EIA. By comparing the results, we found few differences only in the protein-coding heatmap, where CS-CPAs without known driver mutations do not cluster together with the CS-CPA with *GNAS* or *PRKACA* mutation as previously. In our opinion, this difference depends on the

relatively low number of cases in some diagnostic groups, leading to small changes in gene expression patterns and different clustering. Moreover, gene fusions, PCA, and heatmaps for lnc genes do not change between the tumor classification according the two guidelines. Therefore, we decide to shoe into details the results obtained by classifying the tumor according to the Endocrine Society guidelines, in order to keep the same tumor classification of the previous studies (6,9), allowing a better characterization of the tumor samples.

Moreover, another possible explanation for the mutation-negative cases might be the sensitivity of sequencing methods in detecting mutations with low allele frequencies, as shown in recent studies highlighting the possibility of increasing the detection of mutations by applying targeted immunohistochemistry (IHC)-driven deep-sequencing in primary aldosteronism (Nanba, JCEM, 2018; Nanba, Hypertension, 2019). Nonetheless, our retrospective cohort of fresh-frozen tumors was heterogeneous and an IHC-driven approach would not have been possible. Finally, another limitation of our study is that tumors were dissected macroscopically. Therefore, even though the proceudre was made by expert pathologists, our analysis may carry the chance of sampling error, in analogy to other studies dealing with snap-frozen tissues. More accurate data may derive by an *in situ* methodology, such as laser-capture microdissection. Of note, the strength of our study is that the characterization of transcriptome profile and additional RNA alterations (gene fusions and differential expression of lncRNA) is evaluated in a large cohort of adrenocortical tumors (n=59) and analyzed according to both the functional status and the presence of driver mutations.

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Conclusion

In conclusion, our findings suggest that still unrevealed molecular modifications (in cAMP/PKA pathway) might be involved in the pathogenesis of autonomous steroid secretion, some of them depending on the underlying genetic background. At variance, MACS-CPAs seem to be molecularly related to EIAs, independently from the genetic background. Finally, the similarity of molecular alterations between CS-CPAs carrying *CTNNB1* mutations and ACCs may pave the way for further targeted investigation aimed at defining the potential adenoma-carcinoma sequence in adrenocortical tumors.

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Data Availability

The raw data have been uploaded into the platform EGA (https://www.ebi.ac.uk/ega/home, accession number EGAS00001004533). Further datasets analyzed during the current study are available from the corresponding author on reasonable request.

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728 **Legend for Figures**

729 **Figure 1. Flow-chart of the selection process** of adrenocortical tumor samples, including diagnosis
730 and criteria for group classification. WES, whole-exome sequencing, ACA, adrenocortical adenomas.
731 NAG, normal adrenal glands. CS-CPA, cortisol-producing adenomas with overt Cushing Syndrome.
732 MACS-CPA, cortisol-producing adenomas with mild autonomous cortisol secretion. EIA, endocrine-
733 inactive adenoma. ACC, adrenocortical carcinoma. Old and new classification of samples according
734 to previous whole-exome sequencing data (Ronchi et al JCEM 2016) and to new variant calling from
735 RNA-seq data, respectively, is reported.

Comment [CR19]: Add reference

736

737 **Figure 2. Transcriptome profile analysis of adrenocortical tumor samples.**

Comment [CR20]: Silke check if everything correct

738 A) Three-dimensional principal component analysis for protein-coding genes in adrenocortical
739 carcinomas (ACCs) (n=7) and adenomas (ACAs, n=52) divided by functional status according the
740 Endocrine Society guidelines (CS-CPA, cortisol-producing adenoma; MACS-CPA, mild autonomous
741 cortisol secretion; EIA, endocrine-inactive adenoma) as well as mutational status (*PRKACA, GNAS* or
742 *CTNNB1* mutations or no known driver mutations).
743 B) Heatmap of the unsupervised clustering analysis of protein-coding genes in adrenocortical
744 carcinomas (ACCs) (n=7) and adenomas (ACAs, n=52) divided by functional status according the
745 Endocrine Society guidelines (CS-CPA, cortisol-producing adenoma; MACS-CPA, mild autonomous
746 cortisol secretion; EIA, endocrine-inactive adenoma) as well mutational status (*PRKACA, GNAS* or
747 *CTNNB1* mutations or no known driver mutations). Individual IDs can be seen at the bottom of the
748 Figure. Different clusters are recognized and highlighted as Cluster 1 (blue), divided in 1A (n=12), 1B
749 (n=12) and 1C (n=8), and Cluster 2 (pink), divided in 2A (n=11) and 2B (n=7). 2 CS-CPAs (one with
750 CTNNB1 and one without driver mutations) were not included in the previous clusters. Statistical
751 analysis to evaluate the differences on sex, age (cutoff 50-years old), tumor size (cutoff 3.5 cm),
752 diagnosis (EIA, MACS-CPA, CS-CPA, ACC) and mutation status (no driver *vs* mutation of
753 cAMP/PKA pathway *vs CTNNB1* mutation *vs* other driver mutation) among the clusters was
754 performed by Chi-square test. A p value<0.05 was considered statistically significant.

755

In box a) protein-coding genes which are upregulated in ACC are enlarged. In box b) and c) protein coding genes upregulated in Cluster2. The analysis was performed including the top 500 differentially expressed protein-coding genes using log_2 fold changes in principal component 1, 2, and 3. Color key bar on the top right corner shows log2(FC).

Figure 3. Targeted data analysis evaluating the mRNA expression (log_2FC) of A) steroidogenic genes: *FATE1*, NR5A1, CYP11A1, CYP21A2, and B) cell cycle-related genes: TOP2A, ZNRF3, HSPB8, CCNB1. The samples were grouped in adrenocortical carcinomas (ACC, n=7) and adrenocortical adenomas (ACAs, n=52) divided by functional and mutational status: cortisol-producing adenomas (CPAs) associated with Cushing syndrome (CS-CPAs) with *CTNNB1* mutation (n=2); mild autonomous cortisol secretion (MACS)-CPAs or endocrine-inactive adenomas (EIAs) with *CTNNB1* or without driver mutations (n=13 and n=12, respectively); CS-CPAs or MACS-CPAs with *PRKACA* or *GNAS* mutations (n=9); CS-CPA without driver mutations (n=13). Functional status was evaluated according the Endocrine Society guidelines. A non-parametric Kruskal-Wallis test was used significant to detect differences in the distributions of values among groups. The Dunn post-hoc analysis was performed using the dunnTest function of R's FSA package, v0.8.23.

Figure 4. Analysis of gene fusions by RNA-seq in adrenocortical tumours.

A) High confidence gene fusions recognized at transcriptome profile analysis in ACC compared to those found in cortisol-producing adenoma (CPA) associated to Cushing syndrome (CS-CPA) with *CTNNB1* mutation (CS-CPA_CTNNB1), and those in Cluster 2 and Cluster 1. ACCs had a significantly higher number of high-confidence gene fusions compared to the other groups. B) High confidence gene fusions among CS-CPAs according to the mutational status, showing that CS-CPA_CTNNB1 had a trend toward a higher number of high-confidence fusions (mean 2.00±2.82) than the other subgroups (mean 1.07±1.04 in CS-CPA_no driver, 0.43±0.53 in CS-CPA_PRKACA). To note, no one of the 4 CS-CPA_GNAS had highe-confidence gene fusions. In both A) and B) the

783 histograms represent the number of fusions ±SEM. Statistical analysis was performed by non-
784 parametric test with Dunn's multiple comparison test.
785 C) Specific breakpoint of the detected A-kinase anchor protein 13 (*AKAP13*)-phosphodiesterase 8A
786 (*PDE8A*) fusion verified by Sanger sequencing. D) Predicting fusion by Arriba software including the
787 coding sequence of the *AKAP13* gene and an intronic sequence of the *PDE8A* gene.
788

789 **Table 1. Clinical parameters, hormonal and genetic data of patients with adrenocortical tumors.**

	EIA (n=9)	MACS-CPA (n=17)	CS-CPA (n=26)	ACC (n=7)
Clinical and hormonal parameters				
Age, years	59.0 (55.0-71.5)	57 (47-62)	41.5 (35.0-47.3)**	51.0 (47.0-60.0)
Females, n (%)	4 (44%)	13 (76%)	25 (96%)**	4 (57%)
Tumor size, cm	3.3 (2.1-5.2)	4.0 (3.4-4.5)	3.3 (2.8-3.6)	11.0 (8.5-15.0)
Post-dexamethasone cortisol, nmol/L [n.a.]	35.9 (24.8-63.4)	84.1 (75.2-168.3)**	449.7 (373.8-551.8)**	59.3 (36.5-539.4) [3]
24-h UFC, nmol/day [n.a.]	n.a.	568 (174-1060) [4]	1327 (682-2118)* [12]	105.3 (74.2-248.8) [2]
ACTH, pmol/L [n.a.]	5.1 (1.9-7.3) [4]	0.8 (0.4-1.8)* [1]	0.7 (0.2-1.1)** [11]	14.1 (3.0-33.3) [1]
Ki67 index, % - median (range)	n.appl.	n.appl.	n.appl.	10 (2-15)
Genetic data				
Number of somatic mutations§ - median (range)	4 (1-15)	2 (0-17)	4 (0-40)	0 (0-1) [2]
Samples with known driver mutations§†, n (%)	4 (44%) *CTNNB1* n=4	11 (65%) *CTNNB1* n=10 *GNAS* n=1	12 (46%) *PRKACA* n=6 *GNAS* n=3 *CTNNB1* n=3	2 (40%) *TP53* n=1 *ZNRF3* n=1

Data are expressed as median and interquartile ranges in parentheses, if not otherwise specified. CPA-CS: cortisol-producing adenoma associated with overt Cushing syndrome, CPA-MACS: cortisol-producing adenoma associated with mild autonomous cortisol secretions, EIA: endocrine inactive adenoma, ACC: adrenocortical carcinoma, n.a.: number of patients with not available, n.appl., not applicable, UFC: urinary free cortisol.
Overt CS and MACS were defined according to the Endocrine Society guidelines (13).
§Number of somatic mutations were counted according to previously published data deriving from whole-exome sequencing for ACAs (9) and from targeted next generation sequencing of 160 genes for 5/7 ACCs (20), whereas for 2 ACCs this information was not available. Therefore, the number of mutations were not comparable between ACAs and ACCs. The use of a panel of 160 genes and the inclusion of only early-stage/low aggressive ACCs in this study, explains the very low number of found mutations.
†According to targeted sequencing of *PRKACA* exon 7 hot-spot mutations (6) or targeted next-generation sequencing for ACC (20).
*$P<0.05$ *vs* EIA (Kruskall-Wallis test followed by Bonferroni); for 24-h UFC MACS-CPA *vs* CS-CPA (Mann-Whitney test). **$P<0.005$ *vs* EIA (Kruskall-Wallis test followed by Bonferroni).

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791

792 **Table 2. Biological pathways in protein-coding genes differentially expressed in**
793 **adrenocortical tumors.**

	Under-expressed genes		Over-expressed genes	
	Total n genes	**Pathways**	**Total n genes**	**Pathways**
CS-CPA (n=26)	125	1) Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	67	1) GPCR ligand binding 2) G alpha (q) signalling 3) Peptide ligand-binding receptors 4) Class A/1 (Rhodopsin-like receptors) 5) Signalling by GPCR
MACS-CPA (n=17)	338	1) Neuronal system 2) Muscle contraction 3) Transmission across Chemical Synapses 4) Cardiac conduction 5) Potassium channel	7	No pathways significantly represented among the overexpressed genes
EIA (n=9)	246	1) Neuronal system 2)Transmission across chemical synapses 3) Neurotransmitter receptor binding	1	No pathways significantly represented among the overexpressed genes
ACC (n=7)	591	1) Transport of small molecules 2) Neuronal System 3) GPCR ligand binding 4) Signalling by GPCR 5) Class B/2 (Secretin family receptors)	471	1) Cell cycle 2) Mitosis 3) Rho GTPase 4) Glucoronidation 5) Kinesins

CPA-CS: cortisol-producing adenoma associated with overt Cushing syndrome, CPA-MACS: cortisol-producing adenoma associated with mild autonomous cortisol secretions, EIA: endocrine inactive adenoma, ACC: adrenocortical carcinoma. Under-expressed genes by $\log_2$ fold change values >2 and over-expressed genes by $\log_2$ fold change values <-2. Pathway analysis was performed by GSEA (Gene sets from Reactome and KEGGS).

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