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DNA METHYLATION OF STEROIDOGENIC ENZYMES IN BENIGN ADRENOCORTICAL TUMORS: NEW INSIGHTS IN ALDOSTERONE-PRODUCING ADENOMAS

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ABSTRACT

Context. DNA methylation has been identified among putative regulatory mechanisms for *CYP11B2* expression in primary aldosteronism.

Objective. To investigate DNA methylation and expression of genes encoding steroidogenic enzymes in benign adrenocortical tumors.

Design. Cross-sectional.

Setting. University Hospitals.

Patients. We collected fresh-frozen tissues from patients with benign adrenocortical adenomas (n=48) (non-functioning n=9, autonomous cortisol secretion n=9, Cushing syndrome n=17, aldosterone-producing [APA] n=13) and adrenal cortex adjacent to APA (n=12). We collected formalin-fixed paraffin-embedded (FFPE) specimens of paired APA and concurrent aldosterone-producing cell clusters (APCCs) (n=6).

Intervention. DNA methylation levels were evaluated by quantitative Bisulfite Next Generation Sequencing in fresh-frozen tissues (*CYP11A1*, *CYP11B1*, *CYP11B2*, *CYP17A1*, *CYP21A2*, *HSD3B1*, *HSD3B2*, *NR5A1*, *STAR*, and *TSPO*) and FFPE APA/APCC paired samples (*CYP11B2*). *CYP11B1*, *CYP11B2*, *CYP17*, *CYP21* and *STAR* gene expressions were examined by quantitative Real-Time PCR.

Main Outcome Measures. DNA methylation.

Results. *CYP11B2* methylation levels were significantly lower in APA than in other adrenal tissues (P<0.001). Methylation levels of remaining genes were comparable among groups. Overall, CYP11B2 expression and DNA methylation were negatively correlated (rho= -0.379; P=0.003). In FFPE paired APA/APCC samples, *CYP11B2* methylation level was significantly lower in APA than in concurrent APCCs (P=0.028).

Conclusions. DNA methylation plays a regulatory role for *CYP11B2* expression and may contribute to aldosterone hypersecretion in APA. Lower *CYP11B2* methylation levels in APA than in APCCs may suggest an APCC-to-APA switch via progressive *CYP11B2* demethylation. Conversely, DNA methylation seems not to be relevant in regulating the expression of genes encoding steroidogenic enzymes other than *CYP11B2*.

Keywords: methylation; steroidogenesis; CYP11B2; CYP11B1; APA; APCC.

Introduction

Epigenetic changes in DNA are well-known heritable mechanisms of regulation of gene expression, not caused by modification of DNA. Among them, one of the most studied regulatory mechanisms is DNA methylation of CpG islands, defined as DNA regions >500 bp with >55% of cytosine and guanine dinucleotides (1). Different levels of CpG methylation in promoter and non-promoter regions have been recognized in regulatory process of expression of genes involved in tumorigenesis, including apoptosis, DNA damage repair and cell cycle, among others (2).

The role of DNA methylation has been investigated in adrenocortical tumors. Several studies highlighted a specific methylation signature in adrenocortical carcinoma that could be used as a marker of malignancy (3, 4). Recently, genome-wide (5, 6) and targeted methylation analysis (7, 8) have investigated the methylation signature of benign adrenocortical tumors, showing that DNA methylation may be a putative regulatory mechanism of *CYP11B1* and *CYP11B2* expression in cortisol-producing and aldosterone-producing adenoma (APA), respectively. Interestingly, previous studies in H295R human adrenocortical cell lines showed that angiotensin II or potassium may change the DNA methylation status around transcription factor binding sites and transcription start site, activating *CYP11B2* expression and promoting aldosterone secretion (9).

Previous studies using antibodies against CYP11B2 has shown that APA may exhibit a uniform or heterogeneous expression of CYP11B2, while nests of CYP11B2-positive cells, called aldosterone-producing cell clusters (APCCs) beneath the adrenal capsule are frequently detected in normal adrenals and in the adrenal cortex adjacent to APA (10).

To date, no targeted analysis of methylation of steroidogenic enzymes has been performed in benign adrenocortical tumors according to their functional status. Additionally, the data on *CYP11B1* hypomethylation in cortisol-producing adenomas has not been confirmed in independent studies. Finally, no studies have investigated the methylation levels of *CYP11B2* in APCCs.

The first aim of our study was to investigate the DNA methylation of a selected panel of genes encoding key enzymes involved in steroidogenesis in benign adrenocortical tumors, including adenomas associated with autonomous cortisol secretion (ACS). The second aim was to assess the DNA methylation of *CYP11B2* in APA and concomitant APCCs.

Methods

Patients

We collected a total of 60 adrenocortical tissues from patients evaluated at two Italian centers, the Department of Medicine of the University of Padua and the Endocrinology Unit of the University Hospital of Bologna. Specifically, 9 tissues were non-functioning adrenal adenomas and 9 were adrenal adenomas associated with ACS from patients adrenalectomized for an incidentally-detected adrenal mass, 17 were cortisol-producing tumors from patients with Cushing syndrome, 13 were APA, and 12 were tissues derived from normal adrenal cortex adjacent to APA. All adrenal tumors were diagnosed as adrenocortical adenomas at histopathological examination according to the WHO Classification of tumors of endocrine organs (11).

Anthropometric, hormonal, and radiological characteristics of the patients were collected at the time of the diagnosis. Non-functioning adrenal tumors and tumors associated with ACS were defined according to the cortisol levels after 1 mg dexamethasone suppression test $\leq$ or $>$ 50 nmol/L, respectively (12). Cushing's syndrome and primary aldosteronism were defined according to the current guidelines (13, 14). In all patients with primary aldosteronism, adrenal vein sampling was performed under basal conditions and successful catheterization was defined with a selectivity index (adrenal vein to peripheral cortisol ratio) $\geq$2.0. Unilateral primary aldosteronism was defined by a lateralization ratio (dominant to contralateral aldosterone-to-cortisol concentration ratio) $\geq$4.0. According to PASO criteria, after unilateral adrenalectomy 9/13 patients with APA were judged as having complete clinical cure and 4/13 patients as having partial clinical cure. All patients showed biochemical and hormonal normalization (15).

In the absence of imaging criteria suggesting malignancy, patients with non-functioning adrenal tumors and with tumors presenting an ACS underwent surgery because of the relatively large size of the tumor at diagnosis and/or the progression of tumor size at radiological follow-up and/or the consideration of patient willing.

All patients gave written informed consent before participating in this study, which was approved by the local Ethics Committees.

Fresh-frozen tissues

All adrenocortical tissues were collected immediately after surgical resection of the adrenal gland and the central portion of each adrenocortical tumor was studied. The dissection of the adrenocortical tissue adjacent to APA was made by expert pathologists (V. M. and A.F). According to previously reported classification (18), subsequent histopathology showed that 4/12 cases had normal-appearing adrenal cortex adjacent to a single well-defined adenoma with positive CYP11B2 staining and 8/12 cases had hyperplasia (multiple CYP11B2-positive macronodules, focal thickening of the zona glomerulosa with CYP11B2-positive immunostaining or CYP11B2-positive diffuse hyperplasia with or without CYP11B2-positive micronodules). All tissues were snap frozen in liquid nitrogen and stored at -80°C until assayed. Total DNA was extracted from adrenal tissue using a Qiagen DNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) in accordance to the manufacturer's protocol. DNA concentration was measured on a NanoDrop ND-1000 UV–vispectrophotometerer (Isogen, Maarsen, The Netherlands) and DNA integrity was checked on an Agilent 2100 Bioanalyzer (Agilent Technologies, Amsterdam, The Netherlands) with 6000 Nano Chips according to the manufacturer's instructions. RNA was extracted from adrenal tissue using a Qiagen RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) in accordance to the manufacturer's protocol. RNA concentration was measured on a NanoDrop ND-1000 UV–vispectrophotometerer (Isogen, Maarsen, The Netherlands) and RNA integrity was checked on an Agilent 2100 Bioanalyzer (Agilent Technologies, Amsterdam, The Netherlands) with 6000 Nano Chips according to the manufacturer's instructions.

Formalin-fixed paraffin embedded (FFPE) tissues

FFPE tissues were available in 11/13 APA. All the hematoxylin and eosin (H&E) stained slides have been reviewed by the same two pathologists, who choose for each case the FFPE sample including the adenoma and the contiguous adrenal cortex, in the absence of necrosis. Consecutive 4 μm thick adrenal tissue sections were immunostained with the following monoclonal antibodies: CYP11B2

(clone 41-17B, dilution 1:120; Millipore, Burlington, MA), CYP11B1 (clone 80-7, dilution 1:500; Millipore), and CYP17A1 (clone D12, dilution 1:6000; Santa Cruz Biotechnology, Santa Cruz, CA). Immunostained sections were assessed with the correspondent H&E sections for each case. Each marker was assessed semiquantitatively by the McCarty H-score (ranging from 0 to 300), with all tissue examined under a 20x objective. In each field the percentage of immunopositive cells was registered and then multiplied by a factor (from 0 to 3) according to the intensity of the immunopositivity, scored as not present (0), weak but detectable above control (1 +), distinct (2 +), or very strong (3 +) for each marker (16, 17). APCCs were defined as clusters of CYP11B2-positive and CYP11B1- and CYP17A1-negative *zona glomerulosa*-like cells, localized in the subcapsular region extending into the *zona fasciculata* (18) (**Figure 1**). For total DNA extraction, the pathologists outlined the regions of interest (ROI) of APA and APCC, based on the CYP11B2 stained slides. One core (3 mm diameter and 6 mm in length maximum) per ROI was manually obtained using a manual tissue microarray punch (Beecher Instruments Inc., Sun Prairie, WI) (19).

Total DNA was extracted from FFPE adrenocortical tissues using a Qiagen DNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). DNA concentration and integrity were analyzed as described above.

Gene expression

Gene expression level of *CYP11B1*, *CYP11B2*, *CYP17A1*, *CYP21A2*, and *STAR*, with *β-Actin* as housekeeping gene, was evaluated by quantitative real-time (qRT)-PCR using a Sybr Green Assay kit (Thermo Fisher Scientific, Waltham, MA) in fresh-frozen samples with good-quality RNA: 7/9 non-functioning adrenal adenomas, 8/9 adenomas with ACS, 14/17 adenomas with Cushing syndrome, 12/13 APAs and 12/12 APA-adjacent adrenal cortex. The primer sequences are summarized in **Supplementary Table 1** (20). The specificity of the PCR for *CYP11B1* and *CYP11B2* was confirmed after mapping the primer sequences to the human genome using Primer-Blast (http://www.ncbi.nlm.nihgov/tools/primer-blast), to verify their uniqueness. Data were obtained as C_t values and used to determine ΔC_t values ($\Delta C_t = C_t$ of the target gene minus C_t of the housekeeping

gene). The equation (RQ) = 2 $(-\Delta C_t)/2(-\Delta C_t$ non-secreting adrenal tumors) $2^{-\Delta\Delta Ct}$ was applied to calculate the fold changes (Data Assist Software, Applied Biosystems, Foster City, CA).

Methylation analysis

The DNA methylation level of *CYP11A1*, *CYP11B1*, *CYP11B2*, *CYP17A1*, *CYP21A2*, *HSD3B1*, *HSD3B2*, *NR5A1*, *STAR*, and *TSPO* was analyzed from tissues extracted from fresh-frozen specimens. Additionally, we selected FFPE tissues extracted from the 11 cases of APA for DNA methylation analysis of *CYP11B2*. In three of those cases there was no evidence of concurrent APCCs, while concurrent APCCs were detected in the remaining 8 cases. In two cases of APA with concurrent APCCs, DNA extracted from APCCs was not enough for DNA methylation analysis. Therefore, we finally analyzed *CYP11B2* methylation levels in APA and concurrent APCCs in 6 paired samples. We did not investigate the *CYP11B2* methylation levels in APA-adjacent adrenal cortex. In fact, aldosterone-secreting cells were few and scattered to absent in APA-adjacent adrenal cortex, not allowing the isolation of a representative CYP11B2-immunostained area for molecular study (21).

DNA methylation level was evaluated by quantitative Bisulfite Next Generation Sequencing (bisulfite-NGS) as described previously (22). In brief, total DNA was treated with sodium bisulfite using the EZDNA Methylation-Lightning™ Kit (ZymoResearch, Irvine, CA, USA, cod. D5031) according to the manufacturer's instructions. Quantitative DNA methylation analysis was performed by next-generation sequencing using a targeted sequencing approach. In order to identify putative CpG island on promoter region of genes, genomic sequence stored on Ensembl genome browser (http://www.ensembl.org/index.html) were employed as query sequence. MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) designing was applied to identify CpGs and the best primers of choice (23). The list of genomic regions, primer sequences and mapping coordinates interrogated in this study are available on **Supplementary Table 2** (20). Libraries were prepared amplifying the regions of interest by a two PCR steps: target enrichment with tagged primers were generated using overhang adapters based on Nextera™ sequence at the 5′, to be recognized by a second round of short PCR step to add P5/P7 sequencing adapters (Illumina, San Diego, CA) and sample-specific indices to samples. Indexed DNA were quantified, pooled and loaded onto MiSEQ (Illumina, San Diego, CA, cod. 15027617). FASTQ output files were processed for quality control (>

Q30) in a Galaxy Project environment (24). The methylation ratio of each CpG was calculated in parallel by different tools: BWAmeth in a Galaxy Project environment (Europe) followed by the MethylDackel tool (24), and EPIC-TABSAT (25).

Mutational analysis

Genomic mutations of hot-spot regions of candidate driver genes (*KCNJ5*, *ATP2B3*, *ATP1A1*, and *CACNA1D*) in FFPE samples derived from APA and APCCs were analyzed by targeted sequencing after DNA amplification by polymerase chain reaction (PCR). The PCR products were purified with ExoSAP-IT (Affymetrix, Santa Clara, CA) and were sequenced using standard Sanger sequencing methodology and the same primers used for PCR (ABI 3100 automated DNA sequencing analyzer Applied Biosystems, Foster City, CA). The primers employed for DNA amplification are listed in **Supplementary Table 3** (20).

Statistical analysis

Data are expressed as median and range, if not otherwise specified. Comparison among groups was performed with Mann-Whitney U test or Kruskal-Wallis test for continuous variables, where applicable, and chi-square test for categorical variables, respectively. Significance values in multiple comparisons have been adjusted by the Bonferroni correction for multiple tests. The association between methylation and gene expression was calculated by using Spearman correlation coefficients. The comparison of related APA-APCC samples was performed by related-samples Wilcoxon signed rank test. Methylation plotter (26) was used to create **Figure 2A**. The methylation HeatMap (**Figure 2B**) and principal component analysis (PCA) analysis (**Figure 2C**) were created using ClustVis, a web tool for visualizing clustering multivariate data (27). Statistical analysis was performed by using IBM SPSS 21 (IBM, Armonk, NY). Statistical analysis for mRNA measurements was performed using the GraphPad Prism version 6.0 software (GraphPad Software). P value <0.05 was considered statistically significant.

Results

Anthropometric and hormonal characteristics of the patients and the radiological features of the adrenocortical tumors are reported in **Table 1**. As expected, APA size was smaller, when compared to the other groups (P=0.001).

The mean methylation level of the CpG islands considered for each gene among groups are reported in **Table 2**. The mean *CYP11B2* methylation level was significantly lower in APAs, when compared to other adrenal tumoral tissues (P<0.001), and the lower methylation level was detected in all CpG islands of *CYP11B2* (**Figure 2A**). No difference in methylation levels among groups was found for the remaining genes.

The heatmap showing the methylation level of the genes of interest among groups, the results of the unsupervised cluster analysis and principal component analysis are depicted in **Figure 2B and C**. According to the DNA methylation level, we could identify three groups: a cluster including mostly APA, clearly separated from the other groups, a second group including mostly cortisol-producing adenomas associated with Cushing syndrome and a third cluster with mainly non-functioning adenomas. Notably, adenomas associated with ACS were spread among the last two clusters. The PCA analysis confirmed that APA had a separate DNA methylation signature, whereas the other groups showed a large overlap, especially when considering adenomas with ACS.

As reported in **Figure 3**, analysis of gene expression showed that *CYP11B2* mRNA levels were significantly higher in APA than in the remaining adrenal tissues (P=0.001). *CYP21* mRNA was significantly higher in all but APAs, when compared to when compared to adrenal tissues adjacent to APA (P<0.001). Overall, we found a negative correlation between *CYP11B2* expression and respective DNA methylation (rho=-0.281; P=0.003), whereas no correlation was found between mRNA and DNA methylation levels for *CYP11B1*, *CYP17*, *CYP21A2*, and *STAR* (data not shown).

The analysis performed on FFPE tissues extracted from paired APA and APCC samples showed that the mean DNA methylation level of *CYP11B2* was significantly lower in APA than APCCs (P=0.028), with 4/7 CpGs showing significantly higher methylation levels in the latter (**Figure 4**). The H-score for CYP11B2 expression at immunohistochemistry was comparable between APA (280 [60-300]) and APCCs (300 [300-300]) (P=0.078). Unfortunately, it was not possible to extract high-

quality RNA from APCCs in FFPE sections, due to the tiny amount of available material. Therefore, a comparison of *CYP11B2* mRNA expression between APAs and corresponding APCCs could not be performed.

The analysis of somatic mutations in FFPE samples revealed that 5/11 APAs carried *KCNJ5* mutations (p.Leu168Arg in three and p.Gly151Arg in two tumors) and 2/11 APAs *ATP2B3* mutations (p.Leu425_Val426del and c.1227_1232delTGTGCT), whereas the remaining four APAs did not show mutations of genes involved in the analysis. No somatic mutations were detected in APCCs. No significant difference was found in methylation of *CYP11B2* (and other steroidogenic enzymes) according to specific somatic mutations (data not shown). Among APAs belonging to the 6 paired APA-APCC samples analyzed for DNA methylation of *CYP11B2*, 4 had *KCNJ5* mutation, 1 *ATP2B3* mutation and one had no mutations.

Discussion

The results of our study highlighted that *CYP11B2* methylation is an important regulatory mechanism for CYP11B2 expression in APAs. Additionally, we showed that APAs and concurrent APCCs have a different *CYP11B2* methylation level. At variance, our study did not highlight the role of DNA methylation as a regulatory mechanism of gene expression of several steroidogenic enzymes, including *CYP11B1*.

The PCA indicated *CYP11B2* hypomethylation as a common event in APA. In addition, we found a tendency to lower methylation level of CYP11B2 in APA than in paired adjacent adrenal frozen tissue. Moreover, the negative correlation between *CYP11B2* methylation and gene expression identified in the whole cohort of tumors is also consistent with previous publications. Since the first evidence of the pivotal mechanism of epigenetic regulation of *CYP11B2* expression in APA (5), subsequent studies confirmed that hypomethylation of the promoter region of *CYP11B2* was a common finding in APA, when compared to the adjacent adrenal gland in the same patients by methylome analysis (6), and compared to non-functioning adrenocortical tissues (7). These findings were consistent with other previous reports using similar methodologies comparing paired APA and

adjacent adrenal gland (6), and a recent report by Kometani et al. (28), who used pyrosequencing analysis. A limitation our study was that we dissected APA-adjacent adrenal gland macroscopically, and likely the samples contained not only zona glomerulosa but also other zones of adrenal cortex and medulla. Therefore, our analysis did not represent the pure comparison between APA and zona glomerulosa, the site of physiological aldosterone production. For strict comparison between APA and zona glomerulosa, in situ methodology, such as laser-capture microdissection, would be required.

The analysis performed in FFPE specimens of APAs allowed further insights into *CYP11B2* epigenetic regulatory mechanisms. In fact, we showed a significantly lower level of *CYP11B2* methylation in APA, when compared to concurrent APCCs, in most of the CpGs. Previous studies have proposed that APCCs may become a source of autonomous aldosterone production, when evolving into APA (29). In fact, some APCCs carry somatic mutations of genes driving aldosterone production in APA, like *CACNA1D*, *ATP1A1* and *ATP2B3* (30, 31). Moreover, recent reports have highlighted that some cases of APA may possibly arise by APCC-to-APA transitional lesions (pAATLs), with a subcapsular APCC-like portion and an inner APA-like structure (32). The different methylation levels of CYP11B2-positive APA and APCCs led us to speculate that a progressive demethylation process may occur at *CYP11B2* in APCCs, regulating CYP11B2 expression and aldosterone production in their concurrent APAs. However, CYB11B2 expression at immunohistochemistry was similar in APAs and APCC, suggesting that mechanisms which involve *CYP11B2* DNA hypomethylation would be only seen in APA. In consideration of genome wide methylation of APAs (5, 6, 33), some characteristics of APAs, such as tumorigenesis and aldosterone-driver gene mutation status, might also explain different methylation status between APA and APCC.

The data on the relationship between *CYP11B2* methylation levels and mutations in aldosterone-driver genes are contrasting. DNA methylation of *CYP11B2* was comparable in APA without known somatic mutations and those carrying *KCNJ5* and *ATP1A1* mutations in two studies (5, 7). Conversely, a combined methylome and transcriptome approach showed a different molecular signature between APA with *KCNJ5* somatic mutations and non-mutated tumors, with *CYP11B2*, *MC2R* and *ATP2B3* among the most hypomethylated and over-expressed genes (33). In the present study we could not identify differences in *CYP11B2* methylation levels between *KCNJ5*- or *ATB2B3*-

mutated and non-mutated samples. Nevertheless, the interpretation of these results may be limited by the low number of samples and the possible low sensitivity of the mutation detection technique. In fact, an important limitation arises from the mutation analysis of aldosterone-driver genes using Sanger sequencing methodology, particularly in APCCs. Due to the better gene coverage, NGS is more appropriate than Sanger sequencing in detecting somatic mutations, as highlighted in a very recent paper published by De Sousa *et al*. (34). Further targeted studies are needed to unravel the relationship between *KCNJ5* somatic mutations and *CYP11B2* methylation in APA in larger cohorts.

In contrast with a previous study, we did not show a different methylation pattern in *CYP11B1*, nor expression among different groups. Kometani et al. (8), by restricting the analysis to tumor sections with high CYP11B1 expression identified by immunohistochemistry, showed a lower DNA methylation level and a higher mRNA expression of *CYP11B1* in cortisol-producing adenomas, as compared with the adrenal tissue adjacent to the adenoma and non-functioning adenomas. The difference between our results and those obtained by Kometani et al. (8) may reside in the different tissue selected for analysis, which was irrespective of CYP11B1 expression at immunohistochemistry in our study, and in the different CpGs investigated. Nonetheless, the data on *CYP11B1* DNA methylation in our samples were consistent with those on gene expression, which did not differ among groups. A possible explanation may be the presence of APA with *KCNJ5* mutations, which generally express both CYP11B1 and CYP11B2.

According to previous studies, ACS in adrenal incidentalomas have not been related to specific molecular events or recurrent driver mutations in cAMP-PKA pathway (35), leading to the hypothesis that epigenetic regulatory mechanisms may have a role in cortisol overproduction. However, based on our results, DNA methylation of *CYP11B1* seems not to be relevant in determining cortisol overproduction in adrenal incidentalomas. The unsupervised clustering and the PCA performed in our study showed that adrenal incidentalomas and ACS were spread among two clusters, one with prevalence of adenomas associated with Cushing syndrome and the other with non-functioning adenomas. Thus, our data provide further support to the heterogeneity of the molecular background in adenomas associated with ACS. To date, a clear explanation of cortisol overproduction in adrenal incidentalomas and ACS is still lacking.

In conclusion, DNA methylation seems to be an important regulatory mechanism for CYP11B2 expression, and thereby epigenetic mechanisms may be responsible for aldosterone hypersecretion in APAs. Lower *CYP11B2* methylation levels in APAs than in concurrent APCCs may sustain the hypothesis of differential epigenetic mechanisms between APCC and APA, which requires further studies. At variance, we could not demonstrate a regulating role by DNA methylation for most of the remaining genes involved in steroidogenesis.

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Data availability statement: The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Declaration of interest: the authors declare no conflict of interest.

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FIGURES LEGEND

Figure 1. Upper box. A) H&E staining (case #15) with the corresponding immunostaining positive for CYP11B2 (B), and negative for CYP11B1 (C) and CYP17A1 (D) which demonstrate the typical expression pattern of the steroidogenic enzymes in the selected subcapsular area. This pattern defines an APCC (original magnification 10x). **Lower box.** A) H&E (case #11) which shows the adenoma and the remaining adrenal gland (original magnification 0.35x). B) Corresponding immunostaining for CYP11B2 (positive in the adenoma and APCC, negative in the remaining gland) (original magnification 0.35x), C) double staining for CYP11B2 (red) and CYP11B1 (brown) which demonstrate their complementary staining in the APCC/APA/remaining gland (original magnification 0.4x), and D) CYP17A1 (negative in the APCC and adrenal medulla, positive in the remaining adrenal cortex and partially positive in the adenoma) (original magnification 0.35x); (original magnification 5x in all the inserts). E) Higher magnification (20x) of a detail of the APA showed in (C) (double staining), with predominance of CYP11B2 (red) positive cells and a minority of CYP11B1 (brown), with few cells positive for both. F) Higher magnification (20x) of the same APA stained for CYP11B2 (B) which represents the predominant staining pattern, i.e. homogeneous positivity for CYP11B2.

Figure 2. A. Methylation of *CYP11B2* among different groups. The CpG position with respect to transcriptional start site were as follows: CpG Chr8:142917811: +33; CpG Chr8:142917803: +41; CpG Chr8: 142917777: +67; CpG Chr8: 142917757: +87; CpG Chr8: 142917753: +91; CpG Chr8: 142917740: +104; CpG Chr8: 142917731: +113. CPA: cortisol-producing adenoma. **B.** Heatmap showing the results of the unsupervised clustering analysis according to the methylation levels of the panel of genes. **C.** Principal component analysis showing clustering of patients according to methylation levels of genes of interest.

Figure 3. mRNA levels of CYP11B1, CYP11B2, CYP17A1, CYP21A2, and STAR among non-functioning adrenal adenomas (n=7), adenomas associated with autonomous cortisol-secretion (n=8) and Cushing syndrome (n=14), aldosterone-producing adenomas (APAs) (n=12), and APA-adjacent

adrenal gland (n=12). The equation (RQ) = 2 (−ΔCt)/2(−ΔCt non-secreting adrenal tumors) 2−ΔΔCt was applied to calculate the fold changes. Error bars indicate standard deviation. CYP11B2 mRNA levels were significantly higher in APA than in the remaining adrenal tissues (P=0.001). CYP21 mRNA was significantly higher in all but APAs, when compared to APA-adjacent adrenal gland (P<0.001).

Figure 4. A. Mean DNA methylation level of *CYP11B2* in paired samples (n=6) of aldosterone-producing cell clusters (APCCs) and aldosterone-producing adenomas (APAs) and B. Mean DNA methylation level of individual CpGs of *CYP11B2* in paired samples (APCCs and APAs) (n=6). *P<0.05.

TABLES

Table 1. Anthropometric, hormonal and radiological characteristics of the study cohort.

	Non-functioning adrenal tumors (n=9)	**Autonomous cortisol secretion (n=9)**	**Cushing syndrome (n=17)**	**Aldosterone-producing adenoma (n=13)**	**P value**
Age, years	49 (39-63)	57 (39-74)	46 (24-62)	42 (30-57)[a]	0.022
Females, n (%)	3 (33.3)	5 (55.5)	15 (88.2)	8 (61.5)	0.040
Tumor size, mm	40 (12-55)	38 (18-52)	32 (22-45)	17 (9-35)[b]	0.001
ACTH, pmol/L	8.0 (4.4-9.6)	2.6 (1.1-8.0)	1.1 (1.1-2.2)[b]	6.0 (5.3-7.1)	<0.001
Cortisol after DST, nmol/L	39 (30-44)	88 (58-248)	480 (348-775)[b]	n.a.[*]	<0.001
24h-urinary free cortisol, nmol/day	198 (110-298)	148 (97-345)	891 (283-2670)[b]	107 (44-180)	<0.001
24h-urinary free cortisol, ULN	0.6 (0.3-0.9)	0.4 (0.3-0.9)	2.7 (1.3-8.1)[b]	0.5 (0.3-1.0)	<0.001
Aldosterone, pmol/L	n.a.	n.a.	n.a.	714 (534-2256)[**]	n.a.
Serum K^+, mmol/L	n.a.	n.a.	n.a.	3.1 (1.4-3.8)	n.a.

ACTH, adrenocorticotrophic hormone. ULN, upper limit of normality. Data are expressed as median, with minimum and maximum in parenthesis. n.a.: not available.

[*]8/13 patients underwent dexamethasone suppression test, resulting in normal cortisol suppression.

[**]6/13 patients had suppressed plasma renin activity (<0.2 ng/mL/h); 3/13 patients had plasma renin activity between 0.21 and 0.5 ng/mL/h; the remaining 4/13 patients had suppressed direct renin concentrations (<2 mU/L).

Pairwise comparisons (significance values have been adjusted by the Bonferroni correction for multiple tests): [a]P<0.05 *vs* autonomous cortisol secretion; [b]P<0.05 *vs* all other groups.

Table 2. Mean methylation level of target genes in the selected CpG islands among adrenal gland tissue adjacent to aldosterone-producing adenomas and adrenocortical tumors.

	Aldosterone-producing adenoma (APA) (n=13)	**Non-functioning adrenal tumors (n=9)**	**Autonomous cortisol secretion (n=9)**	**Cushing syndrome (n=17)**	**Adrenal adjacent to APA (n=12)**	**P value**
CYP11A1	0.91 (0.86-0.95)	0.89 (0.87-0.96)	0.93 (0.89-0.98)	0.91 (0.86-0.95)	0.93 (0.88-0.97)	0.064
CYP11B1	0.33 (0.17-0.5)	0.32 (0.16-0.48)	0.3 (0.18-0.82)	0.35 (0.09-0.52)	0.42 (0.25-0.7)	0.257
CYP11B2	**0.31 (0.28-0.35)[a]**	**0.80 (0.56-0.91)**	**0.80 (0.66-0.91)**	**0.84 (0.60-0.98)[b]**	**0.49 (0.27-0.76)**	**<0.001**
CYP17A1	0.46 (0.23-0.73)	0.40 (0.15-0.72)	0.32 (0.23-0.81)	0.43 (0.15-0.67)	0.47 (0.31-0.71)	0.429
CYP21A2	0.33 (0.20-0.57)	0.45 (0.35-0.54)	0.39 (0.22-0.79)	0.44 (0.18-0.76)	0.45 (0.17-0.76)	0.172
HSD3B1	0.92 (0.85-0.94)	0.91 (0.88-1)	0.93 (0.88-1)	0.92 (0.85-0.95)	0.93 (0.61-0.95)	0.893
HSD3B2	0.37 (0.22-0.67)	0.55 (0.4-0.7)	0.41 (0.37-0.91)	0.46 (0.17-0.66)	0.47 (0.3-0.86)	0.081
STAR	0.0039 (0.0000-0.0078)	0.0047 (0.0004-0.0087)	0.005 (0.0012-0.014)	0.0031 (0.0001-0.0117)	0.0051 (0.0014-0.0117)	0.409
TSPO	0.95 (0.91-0.96)	0.94 (0.89-0.97)	0.94 (0.91-0.98)	0.96 (0.85-1)	0.95 (0.83-0.97)	0.768
NR5A1	0.05 (0-0.12)	0.09 (0.05-0.3)	0.14 (0-0.81)	0.07 (0-0.4)	0.08 (0.02-0.46)	0.109

APA: aldosterone-producing adenoma. Data are expressed as percentages. Pairwise comparisons (significance values have been adjusted by the Bonferroni correction for multiple tests): [a]P<0.05 *vs* non-functioning adrenal tumors, autonomous cortisol secretion, and Cushing syndrome; [b]P<0.05 *vs* adrenal tissue adjacent to APA.

Figure 1

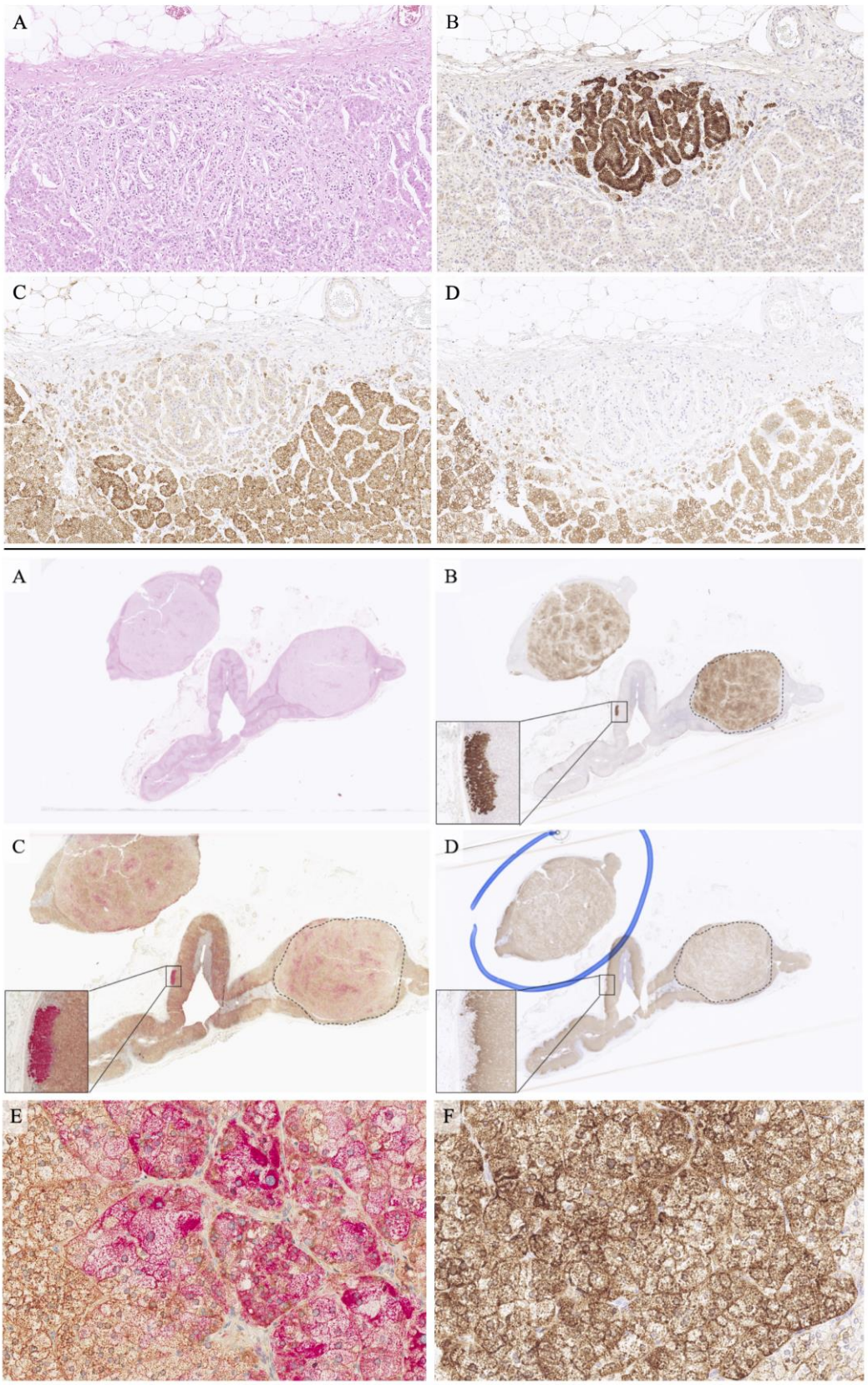


Figure 2

Fig.2A

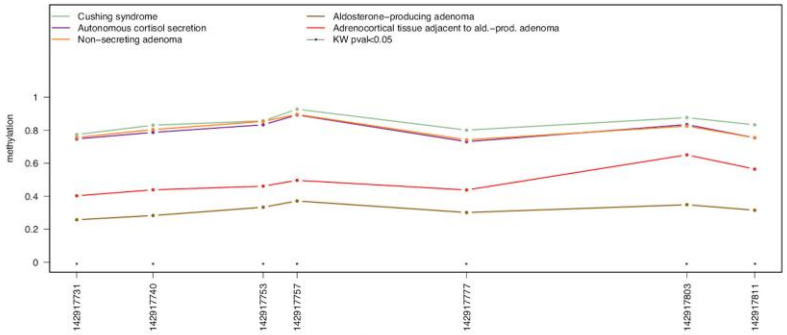


Fig.2B

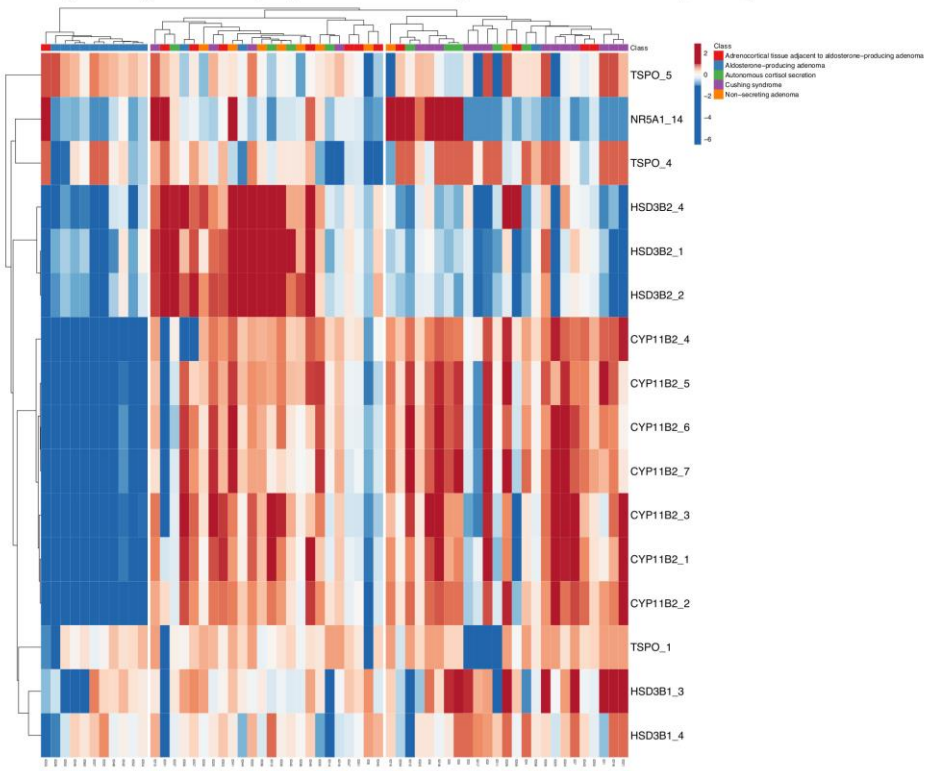


Fig.2C

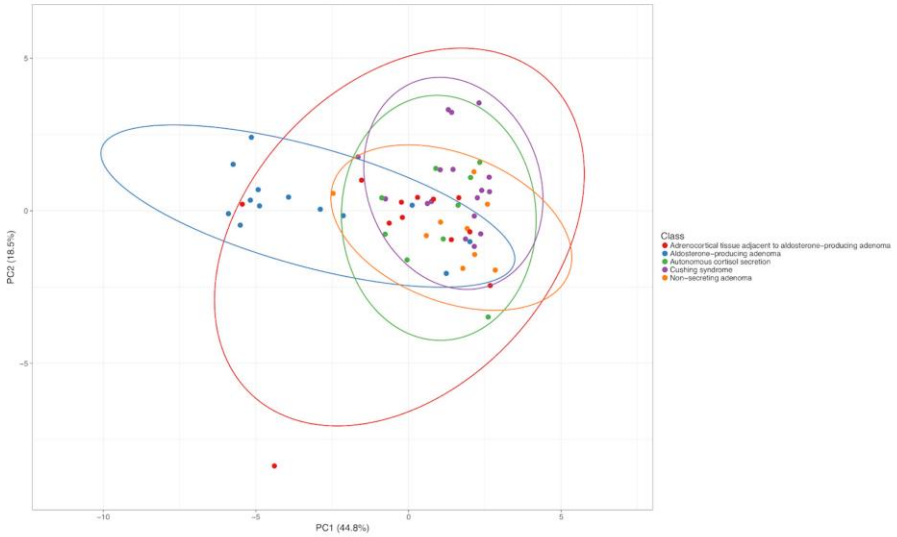


Figure 3

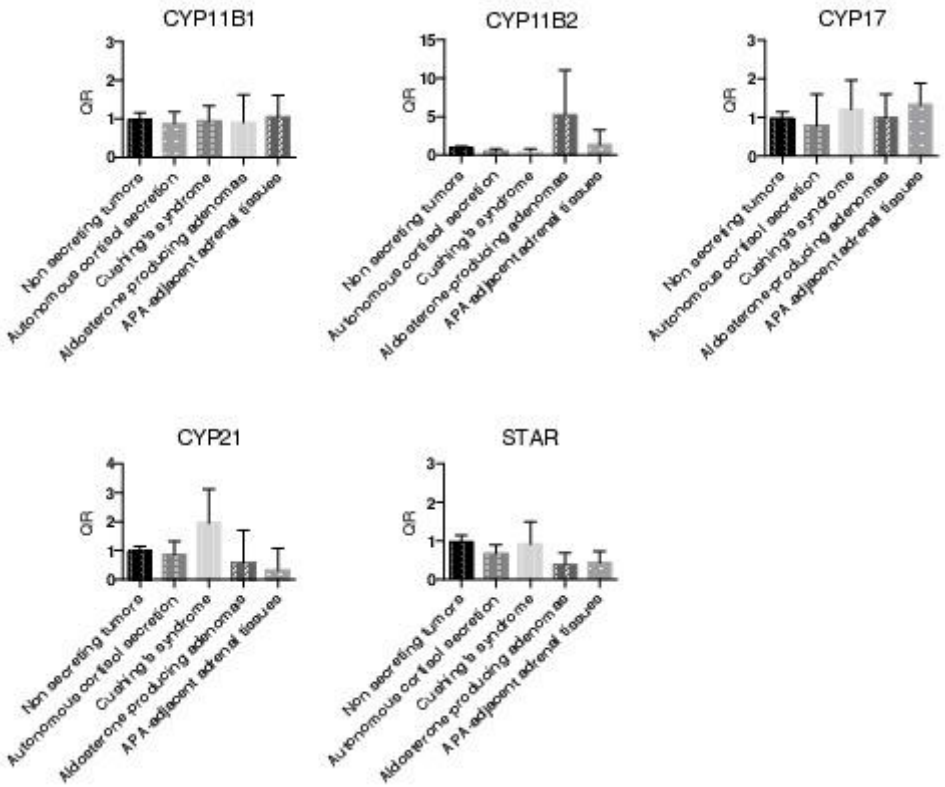
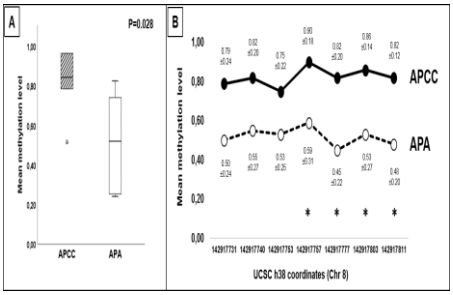

CYP11B1
CYP11B2
CYP17
CYP21
STAR
QR
Non secreting tumors
Autonomous cortisol secretion
Cushing's syndrome
Aldosterone-producing adenomas
APA-adjacent adrenal tissues

Figure 4


A
P=0.028
Mean methylation level
1,00
0,80
0,60
0,40
0,20
0,00
APCC
APA
B
Mean methylation level
1,00
0,80
0,60
0,40
0,20
0,00
0,79 ±0,24
0,82 ±0,28
0,75 ±0,22
0,86 ±0,18
0,82 ±0,20
0,86 ±0,14
0,82 ±0,12
0,60 ±0,24
0,56 ±0,27
0,53 ±0,25
0,59 ±0,31
0,45 ±0,22
0,53 ±0,27
0,43 ±0,20
APCC
APA
*
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143917731 143917740 143917753 143917767 143917777 143917803 143917811
UCSC h38 coordinates (Chr 8)