Genetic background of adrenocortical adenomas associated with hypercortisolism

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an der Medizinische Fakultät
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vorgelegt von
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Table of content

Abbreviations

ACTH: adrenocorticotrophic hormone

PPNAD: primary pigmented nodular adrenocortical disease

BMAH: bilateral macronodular adrenal hyperplasia

cAMP: cyclic adenosine monophosphate

MC2R: melanocortin 2 receptor

PKA: protein kinase A

CREB: cAMP response element binding protein

PDE: phosphodiesterase

Publication list

First publication

Title: Novel somatic mutations in the catalytic subunit of the protein kinase A as a cause of adrenal Cushing's syndrome: a European multicentric study.

Authors: Guido Di Dalmazi, Caroline Kisker, Davide Calebiro, Massimo Mannelli, Letizia Canu, Giorgio Arnaldi, Marcus Quinkler, Nada Rayes, Antoine Tabarin, Marie Laure Jullié, Franco Mantero, Beatrice Rubin, Jens Waldmann, Detlef K Bartsch, Renato Pasquali, Martin Lohse, Bruno Allolio, Martin Fassnacht, Felix Beuschlein, Martin Reincke.

Journal/Year/Issue/Pages: The Journal of Clinical Endocrinology and Metabolism, 2014; 99(10): E2093–E2100. doi: 10.1210/jc.2014-2152.

Second publication

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

Authors: Ronchi CL*, Di Dalmazi G*, Faillot S*, Sbiera S, Assié G, Weigand I, Calebiro D, Schwarzmayr T, Appenzeller S, Rubin B, Waldmann J, Scaroni C, Bartsch DK, Mantero F, Mannelli M, Kastelan D, Chiodini I, Bertherat J, Reincke M, Strom TM, Fassnacht M, Beuschlein F; European Network for the Study of Adrenocortical Tumors (ENSAT).

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*Authors contributed equally to the work

Confirmation of co-authors

All authors listed in the publications gave their contribution to achieve the results of the two studies.

A confirmation with the signature of each co-author is submitted as a separate file.

Introduction

Cushing's syndrome is a pathological condition characterized by excessive cortisol production by the adrenals. The endogenous hypercortisolism can be caused by increased secretion of ACTH or can be the result of an autonomous activity of the adrenal glands. The ACTH-dependent form is the most frequent cause of Cushing´s syndrome, accounting for almost 70% of all cases of endogenous hypercortisolism. The corticotropin–dependent hypercortisolism is caused by excessive and autonomous secretion of ACTH by the pituitary gland (Cushing´s disease), mostly due to a pituitary adenoma, or by ectopic production of ACTH in up to 10% of all cases of endogenous hypercortisolism (ectopic Cushing´s syndrome). ACTH-independent Cushing's syndrome, the most frequent subtype, is due to unilateral adrenocortical adenomas and carcinomas (18-20%). Bilateral adrenal hyperplasia (primary pigmented nodular adrenocortical disease – PPNAD - and bilateral macronodular adrenal hyperplasia - BMAH) are rare causes (< 1%). The classification of endogenous hypercortisolism is summarized in **Figure 1A**. The clinical picture of Cushing´s syndrome is characterized by severe comorbidities and adverse events, which are related to the effects of the prolonged excessive cortisol production. Indeed, the deleterious consequences of cortisol hypersecretion involve many organ systems, considering that the glucocorticoid receptor is widespread expressed among almost all tissues. The classic clinical stigmata of a patient affected by Cushing´s syndrome are centripetal obesity, proximal muscle weakness, moon face, striae rubrae, and

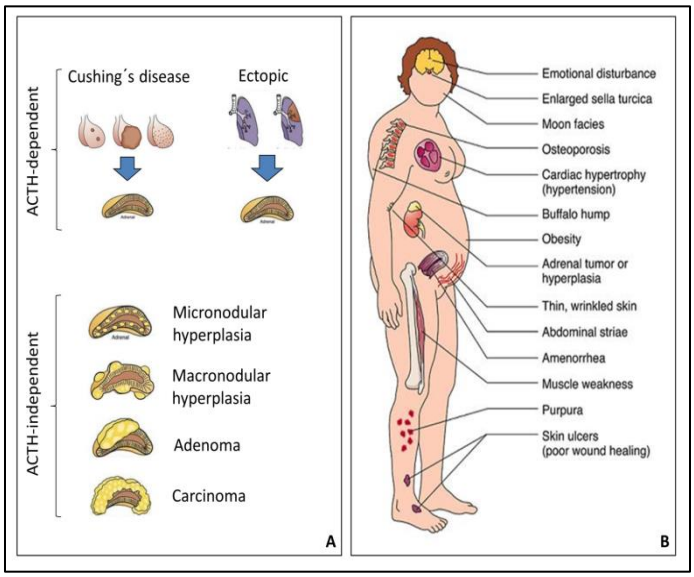

Figure 1. *Classification of endogenous hypercortisolism (A) and typical clinical features of Cushing´s syndrome (B).*

hirsutism (1), as depicted in **Figure 1B**. Moreover, the excessive cortisol secretion leads to resistant hypertension, impaired glucose metabolism, dyslipidemia, and hypercoagulable state due to alterations of clotting factors (2) that increase the incidence of cardiovascular events and the mortality of untreated patients (3, 4). The impairment of cardiovascular system and glucose metabolism is often associated with an increased rate of severe infectious complications (5), increased incidence of osteoporotic fractures, and psychiatric disorders (6). Therefore, Cushing´s syndrome is a severe condition that must be promptly recognized, to address rapidly the patient to the best therapeutic option.

Molecular basis of hypercortisolism and adrenocortical mass formation

Since the first discovery of somatic activating mutations of the Gs protein in growth hormone secreting adenomas (7), the role of alterations of the cAMP signaling pathway in endocrine tumorigenesis and hyperfunction has been extensively investigated. The cAMP pathway has been shown to be one of the major players in the regulation of steroidogenesis in adrenocortical cells (8). Therefore, it has been postulated that the impairment of one or more components of this intracellular pathway could lead to cortisol hypersecretion and adrenocortical growth. The cAMP pathway is a ubiquitous signaling pathway conserved in all eukaryotes (9). In the adrenal gland, this pathway is mainly involved in the production of cortisol. The melanocortin 2 receptor (MC2R) is a G protein-coupled receptor widely expressed in cells of *zona fasciculata* and, to a lesser extent, *zona glomerulosa*. It is activated by the pituitary hormone ACTH, and it is involved in activation of steroidogenic processes that lead to cortisol production. After the binding of ACTH to its receptor, the MC2R activates the intracellular adenylate cyclase through its stimulatory subunit α (Gsα), leading to the generation of cAMP, as shown in **Figure 2**. The increasing levels of this second messenger lead to activation of PKA, the main mediator of this

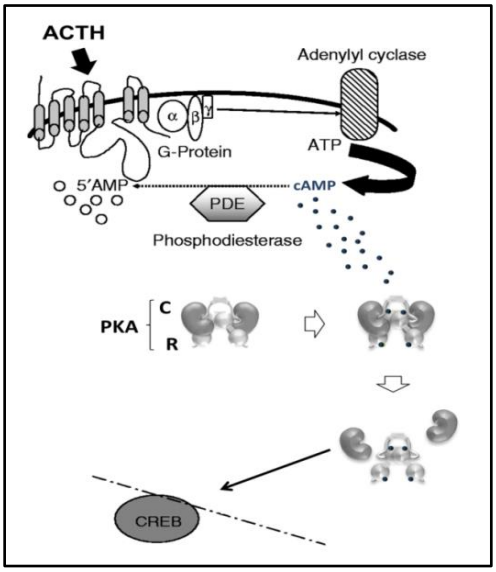

Figure 2. *Schematic representation of cAMP pathway. PKA: protein kinase A. C: catalytic subunit of PKA. R: regulatory subunit of PKA. CREB: cAMP response element binding protein. Adapted from ref. 11.*

intracellular signaling pathway, through the binding of cAMP with its ligand site. PKA is a holoenzyme composed of four different subunits, two catalytic and two regulatory. Up to now, four different catalytic (Cα, Cβ, Cγ, and Prkx) and four regulatory (RIα, RIβ, RIIα, and RIIβ) subunits have been identified. The catalytic subunits are under the inhibitory control of the regulatory subunits. When cAMP binds to its receptor, the regulatory subunit undergoes a conformational change that allows releasing the catalytic subunit, which in turn phosphorylate cytoplasmic targets and activate the cAMP response element binding protein (CREB). CREB binds to the cAMP response elements (regulatory sequences of DNA in specific target genes) leading to the activation of steroidogenic transcription factors. The cAMP signaling pathway is under the regulatory control of the phosphodiesterases (PDEs), a group of converting enzymes that degrade the cAMP by hydrolization into 5´-AMP. Among the 11 PDE gene families with 100 different isoforms of proteins described in humans, the PDEs 2A, 8A, 8B, and 11A have been found to be expressed in the adrenal cortex (10). Specifically, PDE2A, PDE8B, and PDE11A have shown a predominant role in cAMP signaling regulation through modulation of intracellular cAMP levels stimulated by ACTH (11).

Exome sequencing in sporadic adrenocortical tumors

In the last few years, the genetic basis of Cushing´s syndrome has become clearer by the analysis of familial disorders associated with bilateral adrenal diseases. For instance, mutations in *GNAS1* gene,

encoding for the Gsα subunit, in Mc-Cune Albright syndrome (12), genetic aberrations of *PRKAR1A* gene, coding for RIα subunit (13) in PPNAD in the context of Carney complex, and mutations in the *PDE11A* and *PDE8B* genes, encoding for PDEs, in micronodular adrenal hyperplasia (14) have highlighted a pivotal role of cAMP signaling pathway in the pathogenesis of hypercortisolism. Similarly, studies performed on sporadic adrenocortical tumors reported somatic mutations of *PDE8B* (15), *PRKAR1A* (16), and *GNAS* (17-19) in a small number of cases. The advent of next generation sequencing techniques has dramatically expanded our knowledge for genetic alterations in endocrine tumors. Examples include aldosterone producing adrenal adenomas with mutations in three groups of genes in approximately 50% of cases (20-22) and corticotroph pituitary adenomas with somatic *USP8* mutation in 35% of patients (23).

Recently, somatic mutations of the gene *PRKACA*, which encodes the Cα subunit of PKA, were found in a substantial proportion of patients with Cushing's syndrome due to sporadic adrenocortical adenomas (24). The most common alteration was a missense mutation (c.617A>C) leading to a single amino acid substitution (p.Leu206Arg). An insertion c.595_596insCAC (p.Leu199_Cys200insTrp) was also found in one patient. *In silico* and functional analysis demonstrated that those mutations generate an impairment of the interaction between regulatory and catalytic subunit, leading to a constitutive cAMP-independent activation of the latter. The functional autonomy of the Cα subunit resulted in in severely enhanced cortisol production, as shown by cortisol levels after dexamethasone suppression test, urinary free cortisol, and midnight cortisol values. The same mutation (p.Leu206Arg) has been also confirmed in three additional independent cohorts (17-19).

Research project and summary of results

The research subject of this dissertation was focused on two main aims. The first was a characterization of somatic *PRKACA* mutations in a large cohort of sporadic adrenocortical tumors, representing the first replication cohort following the discovery publication. The second aim was to complete and extend the current knowledge on the molecular pathogenesis of adrenocortical tumors associated with or without cortisol secretion, by performing next generation sequencing on a large cohort of patients with adrenocortical tumors, without *PRKACA* mutations.

To achieve these objectives, tissues were collected from several European centers in the context of the German Cushing Registry (CUSTODES) and the European Network for the Study of Adrenal Tumors (ENSAT – www.ensat.org). In 2010, a Network of Excellence for Neuroendocrine Tumors has been founded in Munich (NeoExNETM), with a clinical registry and associated biobank. The platform, which is funded as part of the m4 Cluster of Excellence (www.m4.de) has been set up to recruit patients with endocrine tumors, with a special focus on patients with Cushing's syndrome. To this end, a national registry for patients with Cushing´s syndrome has evolved from the NeoExNET consortium, with 7 centers being actively involved. The ENSAT has implemented a collection of adrenal tumor-related databases by defining a European biobank network devoted to research on adrenal tumors. Common standardized operational procedures (SOPs) have been developed for the collection of biological material from adrenal tumor patients (25) that can be subjected to integrated biomarker approaches. This infrastructure has been virtually linked via web-based Clinical Record Forms (eCRF) as part of the Research Networking Program 07-RNP-067 funded by the European Science Foundation (running period: July 2009 to June 2014, more information at: www.esf.org/esf-ensat). Common contentment forms for clinical and genetic testing have been implemented and validated in each participating country.

For the first part of the research project, 9 European centers were involved in the analysis of *PRKACA* mutations: Munich (Medizinische Klinik und Poliklinik IV, Klinikum der Universität München), Berlin (Bereich Klinische Endokrinologie, Charité Campus Mitte, Charité Universitätsmedizin), Marburg (Klinik für Visceral-, Thorax- und Gefäßchirurgie), Würzburg (Endocrine and Diabetes Unit, Department of Internal Medicine I, University Hospital, University of Würzburg), Florence (Department of Experimental and Clinical Biomedical Sciences), Ancona (Endocrinology Division, Department of Clinical and Molecular Sciences, University Hospital), Padua (Endocrinology Unit, Department of Medicine, University of Padua), Bologna (Endocrinology Unit, Department of Medical and Surgical Sciences, Alma Mater Studiorum University of Bologna), and Bordeaux (Department of Endocrinology, Centre Hospitalier Universitaire Bordeaux and University of Bordeaux).

Frozen tumoral samples (n=149) and corresponding frozen normal tissue or blood (n=68) were collected from patients with non-secreting and cortisol-secreting adrenocortical tumors. For each patient, genomic DNA was extracted from frozen tissues using the Maxwell® Blood DNA Kit (Promega Corp., Madison, WI), according to the manufacturer´s recommendation, and amplified by polymerase chain reaction (PCR), with focus on exon 7, the hotspot mutation site identified in the publications cited above (17-19, 24). PCR products were sequenced on automated sequencer in both directions, performed by the facility service of Eurofins MWG Operon (Ebersberg, Germany). Patients with evidence of *PRKACA* mutations underwent PCR amplification and screening of exon 7 also in corresponding normal tissue or blood. The results of the sequencing were evaluated using the Mutation Surveyor® software (SoftGenetics, State College, PA).

The identified somatic mutations of *PRKACA* were characterized by an in silico analysis, in order to predict the functional consequences of the genetic alterations on the respective proteins. Structural 3D images of wild-type and mutant proteins were prepared by using the software PyMOL

(www.pymol.org), in collaboration with the Rudolf Virchow Center for Experimental Biomedicine, University of Würzburg.

The results of this research project have confirmed and extended the important results already published in the first discovery study on the relevance of the somatic mutations of the Cα subunit in patients with Cushing´s syndrome. Indeed, we found somatic mutations of *PRKACA* in 34% of patients with Cushing´s syndrome (22/64 tumor samples). The mutations were highly specific for adrenocortical adenomas associated with overt cortisol hypersecretion, given that no patients with non-secreting tumors or subclinical hypercortisolism showed those alterations. The mutation c.617A>C (p.Leu206Arg) was the most frequent, occurring in 18/22 patients. However, we also identified two novel mutations: c.600_601insGTG - p.Cys200_Gly201insVal (three patients) and c.639C>G+c.638_640insATTATCCTGAGG - p.Ser213Arg+p.Leu212_Lys214insIle-Ile-Leu-Arg (one tumor). *In silico* analysis predicted a pathogenetic role also for those two novel mutations, which are able to alter the interaction between the regulatory and the catalytic subunits of PKA (**Figure 3**).

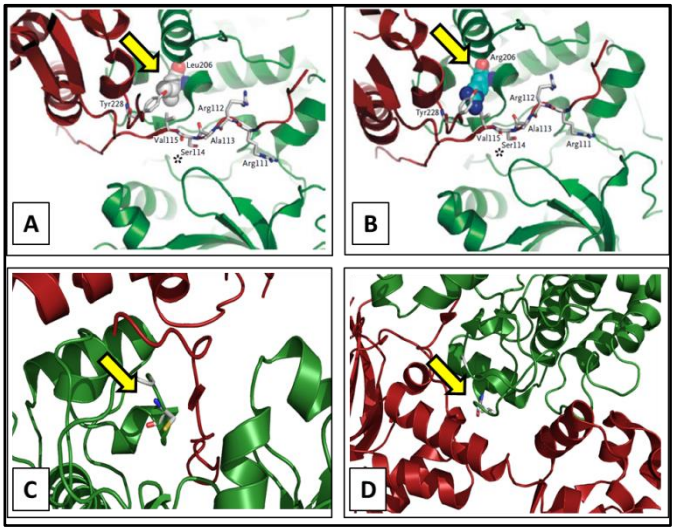

Figure 3. *In silico analyses of the mutations of PRKACA. Boxes A to D show the structure of the mouse full-length tetrameric RIIβ(2):Cα(2) holoenzyme prepared using the PyMOL software (www.pymol.org). The RIIβ subunit of PKA is shown in green and the Cα subunit in red. A; Wild-type situation with Leu206 (arrow). B; reconstruction of the mutation p.Leu206Arg (arrow). C; the arrow indicates the position of the Cys200, highlighting the site of p.Leu199_Cys200insTrp and p.Cys200_Gly201insVal mutations. D; the arrow indicates the site of the p.Leu212_Lys214insIle Ile Leu Arg mutation.*

Moreover, patients with somatic *PRKACA* mutations showed a more severe hypercortisolism, as shown by higher cortisol levels after dexamethasone test,

and smaller adenoma diameter. Those results are in line with the concept that mutations of PKA are able to induce severe functional consequences in terms of steroid production and a low proliferative rate.

Considering these important results, we performed a more comprehensive analysis of the genetic background of adrenocortical tumors by next generation sequencing, to accurately characterize the genetic alterations leading to tumor formation and cortisol secretion beyond *PRKACA*. For this second part of the research project, we selected tumor samples negative for mutations in exon 7 of *PRKACA* among the cohort of samples available from the aforementioned centers. In addition, samples from three additional centers were also included: Zagreb (Department of Endocrinology University Hospital Centre Zagreb, Croatia), Milan (Units of Endocrinology and Metabolic Diseases, Fondazione IRCCS Cá Granda-Ospedale Maggiore Policlinico, Italy), and Dresden (Departments of Clinical Chemistry and Laboratory Medicine, University of Dresden, Germany). A total of 99 unilateral non-secreting and cortisol-producing adrenocortical adenomas were enrolled. Corresponding normal tissue or blood was also obtained from all patients. DNA was extracted following the same procedures described above. After DNA quality check, exome sequencing was performed in all paired samples by the Institute of Human Genetics, Technische Universität München. Exomes were enriched in solution and indexed with the use of the SureSelect XT Human All Exon 50Mb kit, version 4 (Agilent Technologies). Sequencing was performed as paired-end reads of 100 bp on HiSeq2000 systems (Illumina Inc., San Diego, CA). Pools of 12 indexed libraries were sequenced on four lanes to an average depth of coverage between 88x and 160x. Image analysis and base calling was performed using Illumina Real Time Analysis. Reads were aligned against the human assembly hg19 (GRCh37) using Burrows-Wheeler Aligner (BWA v 0.5.9). By using SAMtools (v 0.1.18) and custom scripts, we analyzed single-nucleotide variants (SNVs) and small insertion/deletion (indel) for regions targeted by the exome enrichment kit. The varFilter script was applied to determine the variant quality. We set maximum read depth (-D) to

9999 and the minimum P-value for base quality bias (-2) to 1e-400. We used a custom script for marking of variants with adjacent bases of low-median base quality. Custom Perl scripts was used for annotating variants, including information on transcripts retrieved by UCSC Known Genes and RefSeq genes, variants recorded in dbSNP v.135, type of mutations, and corresponding protein amino acid change. We recorded all annotated variants in our in-house database. Therefore, we queried our database to identify variants in tumor tissue that were not present in the corresponding control tissue, in order to discover putative somatic variants. We filtered out variants that did not met the following criteria, in order to reduce false positives: variants already recorded in our database, those with quality <40, or variants that failed to pass one of the filters from filter scripts. Finally, we analyzed manually the raw data of the remaining variants, by using the Integrative Genomics Viewer (IGV) software. The Gene Ontology enRIchment anaLysis and visuaLizAtion tool (Gorilla) was used for identifying and visualizing enriched gene ontology (GO) terms in ranked lists of genes (biological process, function, and cell components) (26). The Gene Set Enrichment Analysis (GSEA) software was used to perform pathway analysis and gene family analysis. The protein-protein interactions were investigated by the STRING database (http://string-db.org) (27).

The results of this second research project gave in-depth clues on the pathogenesis of adrenocortical tumors and steroid secretion. We identified 706 candidate somatic mutations in 88/99 patients, resulting in a median of 6 somatic mutations per sample (range 0-55). The predominant substitutions were the C:G>T:A transition (29%) and the C:G>A:T transversion (28%), consistent with most cancer types. A total of 23 single genes were found to be recurrent. Among them, the most frequently mutated genes were *CTNNB1* and *GNAS* (n=39 and n=8, respectively). The most common alterations were missense mutations at *CTNNB1* in a hot spot region, encoding a serine in position 45, with p.Ser45Pro and p.Ser45Phe occurring in 22 and 10 patients, respectively. *CTNNB1* mutations occurred mostly in patients with non-functioning adenomas and in those associated with subclinical

hypercortisolism. *GNAS* somatic mutations were identified only in cortisol-producing adenomas, as expected, mutation prevalence was 8/74 (11%). Seven patients showed the known activating mutations in the region encoding the arginine in position 201, leading to the following aminoacid substitutions: p.Arg201His (n=3), p.Arg201Ser (n=2), and p.Arg201Cys (n=2). In one case of Cushing´s syndrome the novel mutation c.76A>C (p.Lys58Gln) was observed. The 3D structural in silico analysis showed that this aminoacid substitution is near the critical position 201, suggesting that it may alter its biological function. Moreover, mutations in *PRKACA*, occurring in regions outside the known hot spot site in exon 7 were detected in three cortisol-producing tumors. According to the 3D structural analysis, also those mutations were predicted to be damaging and potentially affecting the binding of the catalytic subunit to the regulatory subunit. Remarkably, mutations in genes encoding Ca^{++} channels were also found in a substantial proportion of patients. Among them, somatic mutations in genes encoding ryanodine receptors occurred in more than one patient. The *in silico* 3D structural analysis highlighted that mutations in *RYR1* (p.Arg1469Gly and p.Val3218Leu) and *RYR2* (p.Lys2264Asn) are located in the clamp regions of the cytoplasmic assembly, while the mutation in *RYR3* (del4516) was pinpointed in the sliding helix region between transmembrane and cytoplasmic assemblies.

Finally, we classified patients into three groups, according to the potential functional consequences of the most recurrent somatic mutations: subjects with mutations in genes encoding components of the classic Wnt-β catenin pathway (*CTNNB1*, *APC*, *APC2*, *PCDH15*, *PCDHA8*, *PCDHB11*, *PCDHA10*, *PKP2*), those with alterations in genes encoding components of the cAMP-PKA pathway (*GNAS, PRKACA, PRKAR1A, CREB1, CREBBP*, *ADCY3*, *GRM3, GRM4, GRM6*), and those with mutations in genes encoding Ca^{++} channels (*CACNA1C*, *CACNA1E*, *CACNG8*, *RYR1*, *RYR2*, *RYR3*, *GRIA1*, *GRID1*, *GRIK2*, *GRIN1*, *GRIN3B*, *GRIP1*). Mutations in components of the Wnt-β catenin pathway were mostly found in older patients, with endocrine inactive and larger tumors, whereas mutations in component of the cAMP/PKA pathway occur invariably in young patients with high endocrine activity. Those results are in line with

the data previously published by our group (24) and others (17-19), confirming that additional alterations of the PKA pathway, apart from the well-known mutations in the catalytic subunit of PKA, result indeed in a severe hormonal profile and, likely, in an early diagnosis. On the other side, alterations of components of Ca^{++} channels seem not to be associated with peculiar clinical or hormonal characteristics. Despite the well-defined role of genes involved in intracellular calcium homeostasis in the pathophysiology of aldosterone-producing adenomas (i.e. *KCJN5, ATP1A1, ATP2B3,* and *CACNA1D*) (20-22) and GH-secreting pituitary adenomas (28), the role of Ca^{++} channel mutations in non-aldosterone-secreting adenomas is not completely clear up to now. Recent data show that Ca^{++} channels could be involved in molecular mechanisms of apoptosis regulation and cancer transformation (29, 30), and, therefore, a role of those mutations in the proliferation of adrenocortical cells is conceivable.

Significance and future directions

The results of these two research projects are timely and provide novel information on the pathogenesis of adrenocortical adenomas. The strength of these studies relies on the high number of participants and on the rigorous methodology in the selection of the patients. Since the first discovery of alterations of *GNAS* mutations, the involvement of the cAMP/PKA pathway has been intensively studied. However, conclusions have been based on small number of patients. By the advent of highly accurate techniques like next generation sequencing, the power of the analysis of the genetic background of adrenocortical tumors has increased substantially. Here we show for the first time data on a follow-up study focused on *PRKACA* mutations (the most frequent alteration in cortisol-secreting adrenocortical tumors) and results of the analysis of exome sequencing in a large cohort of selected adrenal tissues, with precise and reliable clinical information. The future goal in the research field of

adrenocortical tumors is to study in detail the effects of the most relevant mutations, to identify novel mechanisms of tumor formation and steroid secretion, by adding the important information obtained by transcriptome analysis (RNA-sequencing) and metabolomic profile in tissue and blood. A second target for the next future is to better characterize the clinical phenotype of those patients, by integrating the genetic data with comprehensive steroid profiling performed by mass spectrometry, to be able to accurately select patients for genetic screening and targeted therapies.

Notes

In the first publications, I contributed to the research plan by collecting sample and clinical data, performing the genetic analysis of the whole cohort of samples and the statistical analysis, interpreting the results, doing literature research and writing the paper.

In the second publication (shared first authorship), I contributed to the research project by collecting patient's material (n=53/99 subjects), performing DNA extraction and quality controls, analyzing the results of the exome sequencing, performing statistical analysis and literature research, and writing the paper.

Summary

Cushing's syndrome is a severe pathological condition characterized by excessive cortisol production. Recently, our group and others identified somatic mutations in *PRKACA* gene, encoding the catalytic subunit of protein kinase A (PKA), in a high proportion of sporadic adrenocortical adenomas associated with Cushing´s syndrome. The first aim of this study was to identify the prevalence of *PRKACA* somatic mutations in a comprehensive replication cohort of different adrenocortical tumors. The second aim was to characterize the genetic landscape underlying adrenocortical tumor formation and cortisol production in those cases without PRKACA recurrent mutations.

In the first part of the study, we analyzed 149 tumor samples from several European centers by targeted sequencing. In the second part, 99 paired tumor/blood samples without *PRKACA* hot-spot mutations were analyzed by whole exome sequencing. Clinical and hormonal data were collected from the German Cushing Registry and from the databank of the European Network for the Study of Adrenal Tumors (ENSAT).

Targeted sequencing identified *PRKACA* somatic mutations in 34% of tumors of patients with Cushing's syndrome, whereas no genetic alterations were found in the remaining cases. We identified the *PRKACA* hot-spot mutation c.617A>C (p.Leu206Arg) in 18/22 patients and two novel variants of this gene: three tumors harbored the c.600_601insGTG variant (p.Cys200_Gly201insVal), whereas one patient showed the c.639C>G+c.638_640insATTATCCTGAGG variant (p.Ser213Arg+p.Leu212_Lys214insIle-Ile-Leu-Arg). All *PRKACA* mutations led to alterations of a region of the catalytic subunit of PKA implicated in the interaction with the regulatory subunit. Tumors with somatic *PRKACA* mutations were smaller and associated with higher levels of cortisol following dexamethasone suppression, compared to non-mutated tumors.

The exome sequencing of *PRKACA* wild-type tumors showed a median of 6 somatic protein-altering mutations per tumor (total n= 706), in 89% of the cases. We identified somatic mutations in genes encoding proteins involved in the cAMP/PKA pathway (associated with female sex and overt Cushing's syndrome) and alterations in different genes involved in Ca2+-signaling and Wnt/β-catenin pathway, in larger endocrine inactive tumors.

The first part of this study represents the very first follow-up analysis on genetic alterations in adrenocortical tumors, confirming and extending the findings of the exploratory study showing the prevalent role of *PRKACA* somatic mutations in cortisol-producing adrenocortical tumors. The second part of this study, which is the largest sequencing effort on adrenocortical tumors performed up to now, clearly shows that somatic alterations in genes of cAMP/PKA, Wnt/β-catenin, and Ca2+-signaling pathways are the main alterations underlying *PRKACA*-negative tumors, providing valuable information on the pathogenesis of adrenocortical tumorigenesis and cortisol-production.

Zusammenfassung

Das Cushing-Syndrom ist eine schwere Erkrankung, welche durch eine übermäßige Cortisol-Produktion gekennzeichnet ist. Vor kurzem identifizierte unsere Gruppe somatische Mutationen im *PRKACA* Gen, welches für die katalytische Untereinheit der Proteinkinase A (PKA) codiert. Diese Mutationen fanden vor allem in sporadischen Cortisol-produzierenden Nebennierenrindenadenomen mit floridem Cushing. Das erste Ziel dieser Studie war es, die Prävalenz von somatischen *PRKACA*-Mutationen in einer großen Serie von unterschiedlichen Nebennierenrindentumoren zu identifizieren. Das zweite Ziel war es, die genetischen Ereignisse, die der Nebennierenrindentumorbildung und der Produktion des Cortisols zugrunde liegen, in solchen Adenomen zu identifizieren, welche keine rekurrierte Hotspot-*PRKACA*-Mutation aufweisen.

Im ersten Teil der Studie wurden 149 Tumorproben aus mehreren europäischen Zentren durch gezielte Sequenzierung analysiert. Im zweiten Teil wurden 99 Tumorproben mit korrespondierender normaler DNA nach Ausschluß von *PRKACA*-Hot-Spot-Mutationen mittels Exome-Sequenzierung analysiert. Klinische und hormonelle Daten wurden aus dem deutschen Cushing-Register und aus dem European Network for the Study of Adrenal Tumors (ENSAT) extrahiert und analysiert.

Die gezielte Sequenzierung identifizierte somatische *PRKACA*-Mutationen bei 34% der Tumoren von Patienten mit floridem Cushing-Syndrom. In den verbleibenden Fällen wurden keine genetischen Veränderungen gefunden wurden. Wir identifizierten die PRKACA-Hot-Spot-Mutation c.617A> C (p.Leu206Arg) bei 18/22 Patienten und zwei neue Varianten dieses Gens: c.600_601insGTG (p.Cys200_Gly201insVal) bei drei Patienten und c.639C> G + c .638_640insATTATCCTGAGG (p.Ser213Arg + p.Leu212_Lys214insIle-Ile-Leu-Arg) bei einem Patienten. Eine *In-silico*-Analyse dieser *PRKACA*-Mutationen sagte Strukturveränderungen in Regionen der katalytischen Untereinheit von PKA vorher, die in Protein-Protein-Interaktionen mit der regulatorischen Untereinheit involviert sind. Patienten mit somatischen PRKACA-Mutationen zeigten höhere Cortisolwerte im Dexamethason-Hemmtest und hatten kleinere Adenome im Vergleich zu nicht-mutierten Probanden.

Die Exom-Sequenzierung von PRKACA-Wildtyp-Tumoren zeigten insgesamt 706 somatische Protein-verändernde Mutationen bei 88/99 Tumoren (Median: 6 pro Tumor). Wir identifizierten mehrere Mutationen in den Genen des cAMP/PKA-Signalweges, die mit weiblichem Geschlecht und einem floriden Cushing-Syndrom assoziiert waren. Größere endokrin inaktive Tumore wiesen Mutationen in Genen, die an Ca2+- Signalwegen und Wnt/β-Catenin-Signalwegen beteiligt sind, auf.

Der erste Teil dieser Studie bestätigt und erweitert die Ergebnisse der initialen Publikation, die die vorherrschende Rolle der somatischen *PRKACA*-Mutationen bei Cortisol-produzierenden Nebennierenrindentumoren zeigte. Der zweite Teil dieser Studie, die bislang größte Exome-

Sequenzierungsbemühung bei Nebennierenrindentumoren, zeigte eindeutig, dass somatische Veränderungen der Gene von cAMP/PKA, Wnt/β-Catenin und Ca2+-Signalwege die wichtigsten Veränderungen in PRKACA-negativen Tumoren sind. Diese Studie liefert wichtige Informationen über die Pathogenese der Nebennierenrindentumoren und Cortisol-Produktion.

First publication

Title: Novel somatic mutations in the catalytic subunit of the protein kinase A as a cause of adrenal Cushing's syndrome: a European multicentric study.

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Novel Somatic Mutations in the Catalytic Subunit of the Protein Kinase A as a Cause of Adrenal Cushing's Syndrome: A European Multicentric Study

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Context: Somatic mutations in *PRKACA* gene, encoding the catalytic subunit of protein kinase A (PKA), have been recently found in a high proportion of sporadic adenomas associated with Cushing's syndrome. The aim was to analyze the *PRKACA* mutation in a large cohort of patients with adrenocortical masses.

Methods: Samples from nine European centers were included (Germany, n = 4; Italy, n = 4; France, n = 1). Samples were drawn from 149 patients with nonsecreting adenomas (n = 32 + 2 peritumoral), subclinical hypercortisolism (n = 36), Cushing's syndrome (n = 64 + 2 peritumoral), androgen-producing tumors (n = 4), adrenocortical carcinomas (n = 5 + 2 peritumoral), and primary bilateral macronodular adrenal hyperplasias (n = 8). Blood samples were available from patients with nonsecreting adenomas (n = 15), subclinical hypercortisolism (n = 10), and Cushing's syndrome (n = 35). Clinical and hormonal data were collected. DNA amplification by PCR of exons 6 and 7 of the *PRKACA* gene and direct sequencing were performed.

Results: *PRKACA* heterozygous mutations were found in 22/64 samples of Cushing's syndrome patients (34%). No mutations were found in peritumoral tissue and blood samples or in other tumors examined. The c.617A>C (p.Leu206Arg) occurred in 18/22 patients. Furthermore, two novel mutations were identified: c.600_601insGTG/p.Cys200_Gly201insVal in three patients and c.639C>G+c.638_640insATTATCCTGAGG/p.Ser213Arg+p.Leu212_Lys214insIle-Ile-Leu-Arg) in

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Abbreviations: Cα, catalytic subunit α; DST, dexamethasone suppression test; PKA, protein kinase A.

one. All the mutations involved a region implicated in interaction between PKA regulatory and catalytic subunits. Patients with somatic *PRKACA* mutations showed higher levels of cortisol after dexamethasone test and a smaller adenoma size, compared with nonmutated subjects.

Conclusions: These data confirm and extend previous observations that somatic *PRKACA* mutations are specific for adrenocortical adenomas causing Cushing's syndrome. **(*J Clin Endocrinol Metab* 99: E2093–E2100, 2014)**

Endogenous hypercortisolism due to a unilateral adrenocortical mass is by far the most common cause of ACTH-independent Cushing's syndrome and this subtype of hypercortisolism accounts for almost 30% of patients with overt Cushing's syndrome (1). The clinical phenotype of patients with Cushing's syndrome is characterized by severe comorbidities, due to the excessive cortisol production. The presence of resistant hypertension, type 2 diabetes, and vertebral osteoporotic fractures are common features of the syndrome that are frequently found in association with typical catabolic signs such as easy bruising, purple striae, and proximal muscle weakness (2). If not adequately treated, severe and prolonged hypercortisolism could lead to an increased morbidity and mortality, mainly due to cardiovascular diseases (3, 4) and infectious complications (5).

In the last few years, the molecular basis of ACTH-independent Cushing's syndrome has been elucidated in bilateral adrenal disease in the context of familial disorders. The impairment of the various components of the cAMP pathway has been claimed to be related to adrenocortical growth and cortisol hypersecretion. For instance, mutations in the *GNAS1* gene, encoding for the subunit α of the stimulatory G protein, has been shown to be the causative factor of adrenal hyperplasia associated with Cushing's syndrome in Mc-Cune Albright syndrome (6). Hypercortisolism in primary pigmented adrenocortical disease in the context of Carney complex has been linked to genetic aberrations of the *PRKAR1A* gene that lead to an impairment of the regulatory subunit type 1A (R1α) of protein kinase A (PKA), one of the major effectors of the cAMP signaling pathway (7). Mutations in the *PDE11A* and *PDE8B* genes coding phosphodiesterases regulating the degradation of intracellular cAMP, have been associated with micronodular adrenal hyperplasia and hypercortisolism (8).

The analysis of molecular causes of ACTH-independent Cushing's syndrome has also been extended to sporadic adrenocortical tumors. Somatic mutations of the *PDE8B* gene (9) and *PRKAR1A* gene (10) have been found in patients with cortisol-producing adenomas. Activating *GNAS* mutations have also been lately reported in patients with adrenocortical adenomas associated with Cushing's syndrome (11, 12, 13). However, these mutations were able to explain only a small number of cases of adrenal hypercortisolism. Recently, somatic mutations of the *PRKACA* gene, encoding for the catalytic subunit α (Cα) of the PKA, have been identified in more than one third of patients with Cushing's syndrome due to sporadic adrenocortical adenomas (14). The most common genetic alteration found in these 22 patients was a missense mutation that leads to constitutive activation of the Cα subunit, resulting in cAMP-independent activity of the PKA and enhanced cortisol production. Moreover, patients with the mutation in the *PRKACA* gene had a more pronounced cortisol hypersecretion than nonmutated subjects. The same genetic alterations have been also confirmed in three very recent studies in a high proportion of patients with Cushing's syndrome associated with adrenocortical tumors (11, 12, 13).

The first aim of our study was to analyze somatic mutations of the *PRKACA* gene in a large cohort of patients with sporadic adrenocortical masses associated with Cushing's syndrome and different patterns of hormonal secretion. The second aim of the study was to investigate the hormonal and clinical phenotype of these patients.

Materials and Methods

Patient samples and clinical data

Nine European centers were involved in this project, four from Germany, four from Italy and one from France. A total of 149 frozen tumoral samples were collected from patients with nonsecreting adenomas (n = 32), subclinical hypercortisolism (n = 36), Cushing's syndrome (n = 64), androgen-producing tumors (n = 4), adrenocortical carcinomas (n = 5), and primary bilateral macronodular adrenal hyperplasias (n = 8). Normal adrenal specimens were also obtained from peritumoral samples of four adenomas (two nonsecreting and two associated with Cushing's syndrome), two adrenocortical carcinomas, and two macronodular adrenal hyperplasias. A total of 60 blood samples were also collected from patients with nonsecreting adenomas (n = 15), subclinical hypercortisolism (n = 10), and overt Cushing's syndrome (n = 35). The diagnosis was histologically confirmed after surgery in all cases. None of the patients in this cohort were reported in our previous study (14).

Clinical and hormonal data were recorded for each patient, if available. The presence of hypertension, type 2 diabetes, dyslipidemia, and osteoporosis was assessed. The history of major cardiovascular events was also recorded. The presenting symptoms at the time of diagnosis were investigated. The main hor-

monal parameters analyzed were cortisol levels after dexamethasone suppression test (DST), midnight serum and/or salivary cortisol, basal plasma ACTH levels, and 24-hour urinary free cortisol. If available, normal reference ranges were recorded to calculate the upper limit of normality. The size of the adrenal mass was finally collected of each patient.

All patients gave written informed consent for the genetic analysis. The study was approved by the ethics committee of each individual institution.

Diagnostic criteria of nonsecreting adenomas, subclinical hypercortisolism, and Cushing's syndrome

The diagnosis of ACTH-independent Cushing's syndrome was assessed in the presence of at least three biochemical hallmarks of hypercortisolism, such as cortisol levels after DST greater than 138 nmol/l (5 μg/dl), basal plasma ACTH less than 2.2 pmol/l (10 pg/ml), elevated 24-hour urinary free cortisol, and increased late-night serum and/or salivary cortisol. The diagnosis of Cushing's syndrome was also made in case of typical catabolic signs specific to overt hypercortisolism, such as proximal muscle weakness, skin fragility, easy bruising, in association with at least two impaired hormonal tests. Patients were defined as having nonsecreting adenomas if they lacked typical clinical signs of hypercortisolism and if they had normal hormonal tests. In these patients, the response to DST was considered normal if the cortisol levels decreased to less than 50 nmol/l (1.8 μg/dl). All the patients who did not fit in the previous two categories were considered as having subclinical hypercortisolism.

DNA extraction and *PRKACA* sequencing

Genomic DNA was extracted from frozen tissues using the Maxwell Blood DNA Kit (Promega Corp), according to the manufacturer's recommendation. The qualitative and quantitative evaluation of the DNA was assessed by spectrophotometry at 260 nm. The PCR was performed for amplification of exons 6 and 7, using the following primers: 5′-GTTTCTGACGGCTGGACTG and 3′-AGTCCACGGCCTTGTTGTAG. The PCR program was as follows: 2-minutes denaturation at 95°C, eight amplification cycles (15 s at 95°C, 15 s at 65°C, −1 C at each cycle, and 30 s at 72°C) followed by 30 amplification cycles (15 s at 95 C, 15 s at 58°C, and 30 s at 72°C), and 5 minutes at 72°C. PCR products were visualized on 1% agarose gel containing ethidium bromide. The amplicons were sequenced on automated sequencer in both directions. The results of the sequencing analysis were evaluated using the Mutation Surveyor software (SoftGenetics).

In silico analysis of *PRKACA* mutations

Structural images were prepared using the PyMOL software (www.pymol.org). The structure of the mouse full-length tetrameric RIIβ(2):Cα (2) holoenzyme (15) (PDB entry 3TNP) was used to display the catalytic (Cα) and the regulatory (RIIβ) subunits of PKA.

Statistical analysis

Mean, SD, and frequencies were used as descriptive statistics. Comparison between groups was performed using one-way ANOVA and χ^2 tests, where appropriate. Intergroup differences were assessed by simple contrasts applied to one-way ANOVA and logistic regression. Data were analyzed by SPSS software version 21.0.0.0 (IBM). Two-tailed *P* values less than .05 were considered significant.

Results

General characteristics

The general characteristics of patients with nonsecreting adenomas, subclinical hypercortisolism, and overt Cushing's syndrome are shown in Table 1. Patients with Cushing's syndrome were younger and the prevalence of female sex was higher when compared with subjects with nonsecreting adenomas and subclinical hypercortisolism. The prevalence of type 2 diabetes at the time of diagnosis was higher in patients with Cushing's syndrome than in those with nonsecreting adenomas, whereas no significant difference was found between Cushing's syndrome and subclinical hypercortisolism groups. The serum cortisol levels post-DST and at midnight, and the urinary free cortisol were higher in patients with Cushing's syndrome compared with those with subclinical hypercortisolism and nonsecreting adenomas.

Prevalence of the *PRKACA* mutations

Sequencing analysis showed mutations of exon 7 of the *PRKACA* gene in 22 of 64 adenomas with Cushing's syndrome (34%). No mutations were found in nonsecreting adenomas and in samples from patients with subclinical hypercortisolism, nor in the remaining 24 tumoral samples of androgen-producing tumors, adrenocortical carcinomas, and primary bilateral macronodular adrenal hyperplasias. In addition, no mutations were found in normal adrenal tissue taken from peritumoral specimens. Eighteen of 22 adenomas associated with Cushing's syndrome showed the missense mutation c.617A>C (p.Leu206Arg). In addition, two novel mutations were observed in four patients. The insertion c.600_601insGTG (p.Cys200_Gly201insVal) was found in three patients, and the missense mutation c.639C>G (p.Ser213Arg) associated with a 12 bp duplication (c.638_640insATTATCCTGAGG, p.Leu212_Lys214insIle Ile Leu Arg) was found in one patient. All the mutations reported were heterozygous. No mutations were observed in the peripheral DNA, available for 11 of the 22 patients with *PRKACA* mutation. Specifically, no mutations were found in blood samples of the three patients with the p.Cys200_Gly201insVal mutation, whereas the blood sample was not available for the patient with the p.Leu212_Lys214insIle Ile Leu Arg mutation.

Location of the mutations in the PKA catalytic subunit

All identified mutations involve regions that are located at the interface between the catalytic and regulatory sub-

Table 1. General Characteristics of Patients With Nonsecreting Adenomas, Subclinical Hypercortisolism, and Cushing's Syndrome

	Nonsecreting (N = 32)	Subclinical Hypercortisolism (N = 36)	Cushing's Syndrome (N = 64)	*P* value[a]
General Characteristics				
Age, y	56.1 ± 12.9 (n = 31)	60.8 ± 11.5 (n = 36)	42.0 ± 12.0[b,c] (n = 60)	<.001
Female, n (%)	14/32 (43.8)	22/36 (61.1)	60 (93.8)[b,c]	<.001
Adenoma size, mm	3.6 ± 1.5 (n = 32)	4.0 ± 1.2 (n = 36)	3.6 ± 1.4 (n = 54)	.330
Co-morbidities				
Hypertension, n (%)	13/27 (48.1)	23/31 (74.2)	35/54 (64.8)	.116
Type 2 diabetes, n (%)	1/28 (3.6)	6/31 (19.4)	16/54 (29.6)[d]	.021
Dyslipidemia, n (%)	7/28 (25.0)	10/30 (33.3)	19/54 (35.2)	.636
Osteoporosis, n (%)	2/25 (8.0)	7/26 (26.9)	9/50 (18.0)	.210
Cardiovascular diseases, n (%)	2/27 (7.4)	4/31 (12.9)	5/53 (9.4)	.773
Hormonal parameters				
Cortisol post-DST, nmol/l	30.1 ± 7.8 (n = 9)	165.6 ± 178.6[b] (n = 29)	523.9 ± 238.7[b,c] (n = 44)	<.001
ACTH, pmol/l	5.1 ± 5.6 (n = 21)	2.6 ± 1.5 (n = 33)	1.0 ± 0.6[c,e] (n = 50)	<.001
Urinary free cortisol, nmol/d	262.4 ± 164.7 (n = 11)	217.1 ± 265.4 (n = 29)	1045.5 ± 837.7[b,c] (n = 38)	<.001
Urinary free cortisol, ULN	0.6 ± 0.2 (n = 9)	0.7 ± 0.7 (n = 26)	3.3 ± 3.8[f,g] (n = 29)	.001
Midnight serum cortisol, nmol/l	141.5 ± 62.6 (n = 8)	198.5 ± 63.2 (n = 12)	495.9 ± 136.2[b,c] (n = 16)	<.001
Midnight serum cortisol, ULN	0.5 ± 0.1 (n = 8)	0.9 ± 0.3[b] (n = 12)	2.4 ± 1.2[b,f] (n = 14)	<.001

DST, 1 mg dexamethasone suppression test; ULN, upper limit of normal.

Plus-minus values are means ± SD Hormonal data are expressed in SI Units.

[a] One-way ANOVA (continuous variables), χ^2 test (categorical variables).

Pairwise comparison between groups (simple contrasts applied to one-way ANOVA and logistic regression):

[b] $P < .001$, reference category nonsecreting.

[c] $P < .001$, reference category subclinical hypercortisolism.

[d] $P = .020$, reference category nonsecreting.

[e] $P = .003$, reference category nonsecreting.

[f] $P = .001$, reference category nonsecreting.

[g] $P = .001$, reference category subclinical hypercortisolism.

units (Figure 1). We had previously performed an in silico analysis of the most frequent mutation (p.Leu206Arg), which suggested that the exchange of Leucine by a bulky and positively charged amino acid such as Arginine would lead to a steric hindrance with Val115 and Tyr228 in the regulatory subunit, likely impairing holoenzyme formation and, hence, leading to constitutive PKA activation (14). This hypothesis was supported by functional studies of the Leu206Arg variant showing high basal PKA activity, lack of suppression by the regulatory subunit, and absence of regulation by cAMP (14). Cys200 and Gly201, although not in direct contact to the regulatory subunit, are located in a region of the catalytic subunit that is oriented parallel to the inhibitory sequence (Figure 1). An insertion between these two amino acids could lead to "bulging" out of this region toward the inhibitory sequence and thereby also interfere with binding of the inhibitory loop. Similarly, Leu212 and Lys214 are located on the surface of the protein and are positioned in a region that adopts a "tip-like" structure, which is inserted into a complementary cavity of the regulatory subunit (Figure 1). Insertion of several amino acids at this position, likely leads to an increase of the size of the "tip" and its repositioning, which again could interfere with the interaction between the regulatory and the catalytic subunit.

Clinical and hormonal phenotype in mutated vs nonmutated patients

The clinical and hormonal phenotypes of the patients with Cushing's syndrome are shown in Table 2. The mean size of the adrenal mass was smaller in patients with *PRKACA* mutations, with respect to those without (2.8 ±

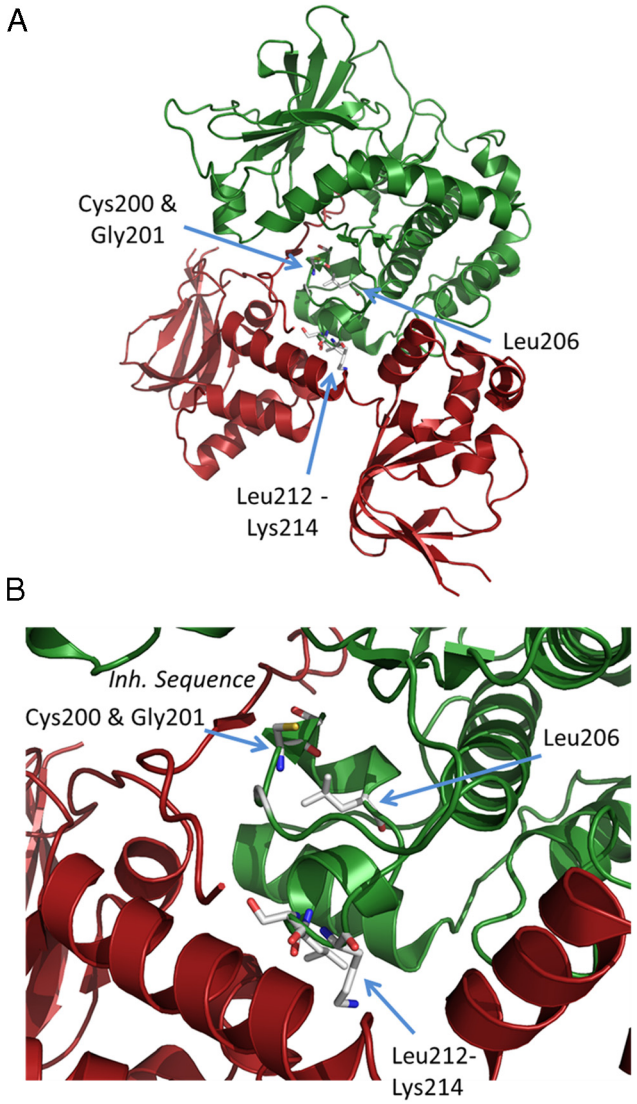

Figure 1. Position of PKA Variants. A and B, Structure derived from the PKA tetramer (PDB entry 3TNP) with the catalytic subunit depicted in green and the regulatory subunit in red. Cys200 and Gly201, Leu206, as well as Leu212 to Lys214 are shown in stick representation. A magnified view into the region of the three variants is shown in panel B.

0.5 cm vs 4.0 ± 1.5 cm, $P = .003$). No difference in other general characteristics and in the prevalence of preoperative comorbidities was found between the two groups of patients. The cortisol levels after DST were significantly higher in subjects with *PRKACA* mutations than in those without (615.1 ± 167.2 nmol/l vs 460.8 ± 262.4 nmol/l, $P = .033$), whereas the other hormonal parameters did not differ between groups. The prevalence of typical signs specific to Cushing's syndrome was not different within the two groups (data not shown).

Discussion

In this study we show that somatic mutations of the *PRKACA* gene, resulting in a constitutive activation of the Cα subunit of the PKA, occurred in more than one third of patients with adrenocortical adenomas associated with Cushing's syndrome. All these mutations were somatic and specific of patients with ACTH-independent Cushing's syndrome. Of note, the sequencing analysis revealed two novel mutations in exon 7 of the *PRKACA* gene never described before.

As the first discovery of somatic activating mutations of the Gs protein in somatotrophic-secreting adenomas (16), the role of alterations of the cAMP signaling pathway in endocrine tumorigenesis and hyperfunction has been extensively investigated. PKA is a pivotal component of the cAMP signaling cascade (17) and is involved in the regulation of steroidogenesis in adrenocortical cells (18). The constitutive activation of components of the cAMP pathway and, in particular, of one of its major effectors, PKA, has been related to the development of hypercortisolism associated with bilateral hyperplasias, mainly in case of germline mutations, and with sporadic unilateral adrenocortical adenomas, in case of somatic aberrations. Considering that PKA assumes a pivotal role in the regulation of steroidogenesis, it is not surprising that adrenal tumors carrying alterations in one of the subunits of PKA are mostly cortisol secreting. The functional characterization of the altered catalytic subunit α of PKA has been clearly highlighted in our recent article (14), which showed that somatic mutations of the Cα subunit occurred in more than one third of patients with unilateral adenomas associated with ACTH-independent Cushing's syndrome, who presented a more severe phenotype than patients without the mutation. Our results are consistent with these findings and with the report published by Goh et al (12), given that somatic mutations in the *PRKACA* gene have been found in 34% of patients with Cushing's syndrome. In the series described by Cao et al (11) and Sato et al (13), the prevalence of *PRKACA* gene mutations in patients with Cushing's syndrome was higher (65% and 52%, respectively). A feasible explanation could be that stricter diagnostic criteria could have been applied for the diagnosis of hypercortisolism. Nonetheless, the ethnic background must be considered in interpreting these results, because it is possible that Asian patients could have a higher frequency of *PRKACA* gene mutation. In addition, the data reported in this article confirm that the same patients had higher cortisol levels after the DST. The absence of mutations in the peripheral DNA suggests that these alterations occur only at a somatic level. The most frequent genomic alteration was a missense mutation that led to the previously described substitution of a Leucine with an Arginine in the active-site cleft of Cα. In our series of patients, we have confirmed that the Leu206Arg sub-

Table 2. Clinical and Hormonal Phenotype in Patients With Cushing's Syndrome With *PRKACA* Mutations Versus Nonmutated Subjects

	Cushing's Syndrome		
	No *PRKACA* Mutations (*N* = 42)	***PRKACA* Mutations (*N* = 22)**	***P* value[a]**
General Characteristics			
Age, y	43.3 ± 12.8 (n = 39)	39.5 ± 10.1 (n = 21)	.242
Age at onset of symptoms, y	41.6 ± 13.4 (n = 30)	37.1 ± 8.8 (n = 16)	.234
Females, n (%)	39/42 (92.9)	21/22 (95.5)	.683
Adenoma size, mm	4.0 ± 1.5 (n = 35)	2.8 ± 0.5 (n = 19)	.003
Co-morbidities			
Hypertension, n (%)	21/34 (61.8)	14/20 (70.0)	.541
Type 2 diabetes, n (%)	12/34 (35.3)	4/20 (20.0)	.235
Dyslipidemia, n (%)	13/34 (38.2)	6/20 (30.0)	.541
Osteoporosis, n (%)	6/31 (19.4)	3/19 (15.8)	.750
Cardiovascular diseases, n (%)	4/33 (12.1)	1/20 (5.0)	.390
Hormonal parameters			
Cortisol post-DST, nmol/l	460.8 ± 262.4 (n = 26)	615.1 ± 167.2 (n = 18)	.033
ACTH, pmol/l	1.1 ± 0.7 (n = 32)	1.0 ± 0.4 (n = 18)	.683
Urinary free cortisol, nmol/d	1012.6 ± 893.5 (n = 26)	1116.9 ± 733.6 (n = 12)	.727
Urinary free cortisol, ULN	3.5 ± 4.3 (n = 20)	2.8 ± 2.3 (n = 9)	.650
Midnight serum cortisol, nmol/l	510.9 ± 143.0 (n = 12)	451.0 ± 119.3 (n = 4)	.465
Midnight serum cortisol, ULN	2.6 ± 1.4 (n = 10)	1.7 ± 0.4 (n = 4)	.221

DST, 1 mg dexamethasone suppression test; ULN, upper limit of normal.

Plus-minus values are means ± SD.

[a] Oneway ANOVA (continuous variables), χ^2 test (categorical variables).

stitution is indeed a frequent finding in patients with adrenal Cushing's syndrome due to unilateral adenomas.

In our cohort, we were also able to identify two mutations in the exon 7 of the *PRKACA* gene not described before. All three mutations identified in this study are located at the interface between the catalytic and the regulatory subunit. Two of them (p.Leu206Arg, p.Cys200_Gly201insVal) affect regions that are near the inhibitory sequence of the regulatory subunit. The inhibitory sequence binds as tethered pseudosubstrate into the active site cleft of the catalytic subunit, thus impeding the access of other substrates and keeping PKA inactive in the absence of cAMP. Similarly, the third mutation (p.Leu212_Lys214insIle Ile Leu Arg) affects another region that extends as a "tip" into the regulatory subunit. Thus, we hypothesize that all three mutations interfere with the association between the regulatory and the catalytic subunit. This hypothesis is consistent with the results obtained with the functional analysis of the p.Leu206Arg and another mutation that we previously identified in the same region (Leu199_Cys200insTrp). These mutations have shown to lead to constitutive PKA activation and to loss of the normal response to cAMP (14).

The mutations in the *PRKACA* gene were found only in patients with Cushing's syndrome, whereas no genetic alterations were found in samples of patients with nonsecreting adenomas and subclinical hypercortisolism, or in other benign and malignant tumor entities. The absence of genetic aberrations of the *PRKACA* gene in nonsecreting adenomas and androgen-producing tumors is not surprising, considering the selectivity of hormone hyperproduction caused by the activation of the PKA, as described above. The absence of genetic aberrations of the *PRKACA* gene in samples taken from adrenocortical cancer is intriguing. The role of the impairment of PKA in malignant adrenocortical masses has not been completely elucidated. According to previously published studies, it has been postulated that PKA hyperactivation could be characteristic for benign rather than malignant adrenocortical tumors,

as hypothesized in several reports that showed a reduced expression of the transcription factor cAMP response element–binding protein in adrenocortical cancer (19, 20). The development of well-differentiated adrenocortical masses with a limited proliferative capacity caused by PKA alterations was also highlighted in the study by Bertherat et al (10), who proposed a differential tumorigenetic model involving the cAMP signaling cascade, which seems to be PKA dependent in benign adrenocortical masses and PKA independent in adrenocortical cancer (10). It is plausible that the mechanisms underlying the development of benign and hyperfunctioning tumors observed in patients with somatic mutations of the *PRKAR1A* gene described by Bertherat et al (10) could be similar to those occurring in patients with somatic mutations of the *PRKACA* gene, although this hypothesis has yet to be demonstrated.

The absence of mutations in patients with subclinical hypercortisolism is consistent with the previously reported finding that constitutive activation of PKA leads to profound hypersecretion of cortisol (14). Moreover, we used strict criteria for the classification of the patients who allowed us to create a well-defined group of subjects with true "overt" hypercortisolism, and a group of patients with the so-called subclinical hypercortisolism. It is tempting to speculate that the molecular mechanisms underlying the subclinical hypercortisolism is different from those of patients with ACTH-independent Cushing's syndrome, given that recent reports on the natural history of this condition have shown that patients with subclinical hypercortisolism do not develop the phenotype of full-blown Cushing's syndrome during long-term followup, even in case of increased cortisol production over time (21, 22). The absence of mutations in patients with subclinical Cushing's syndrome has been confirmed also in the study published by Cao et al (11), whereas in the series described by Goh et al (12) the p.Leu206Arg mutation was found in three patients with this condition. This difference could be most likely explained by the different diagnostic criteria used to diagnose the subclinical hypercortisolism.

The analysis of the hormonal data of patients with Cushing's syndrome have shown increased levels of cortisol after DST in mutated patients vs nonmutated ones. This result confirms previous findings (14). However, we did not find additional differences in the hormonal parameters analyzed. The lack of difference in basal plasma ACTH levels is understandable, given that ACTH is suppressed by definition in patients with ACTH-independent Cushing's syndrome and only small differences could exist, limited by the sensitivity of the different assay methods. The absence of differences in urinary free cortisol and midnight cortisol is not fully consistent with the values of cortisol after DST, but may be explained mainly by the lack of complete hormonal data for all patients, and partly by the sensitivity and the specificity of these tests that could be affected, especially for urinary free cortisol, by the different cutoffs, the methods of cortisol assays, and the accuracy of urine collection (2, 23).

The finding of a reduced adenoma size in patients with alterations of the *PRKACA* gene deserves particular consideration. It has been previously demonstrated that a correlation between adrenal mass size and increased number of genetic aberrations exists (24, 25). Recently, a higher total kinase activity due to high expression of the catalytic subunit of PKA has been shown in smaller rather than larger nodules of macronodular adrenal hyperplasias of patients with ACTH-independent Cushing's syndrome (26). Moreover, a reduced weight of adrenal masses has been clearly demonstrated in patients with sporadic adrenocortical tumors carrying somatic mutations in the *PRKAR1A* gene (10). It is plausible that in our series of patients the severity of the cortisol hypersecretion in mutated patients could have led to an early diagnosis, preventing the onset of additional mutations with higher proliferative potential. This hypothesis is also supported by studies performed in *Pde8b* KO mice. In this animal model, the increased PKA activity has been associated with an increased steroid production without an obvious increase in the size of the adrenal glands (27), confirming that PKA hyperactivation could lead to hypercortisolism and low proliferative rate in adrenocortical cells.

The main limitation of the study is the lack of complete clinical and hormonal data in a subset of patients, which has hampered a precise characterization of the patients' phenotype. On the other hand, this is the largest cohort of patients with adrenocortical adenomas who have been analyzed for mutations in the catalytic subunit of PKA until now.

In conclusion, we have confirmed that somatic mutations in the *PRKACA* gene (one previously known and two newly described) occur in more than one third of adrenocortical adenomas associated with Cushing's syndrome. This study has strongly reinforced the hypothesis that an aberrant cAMP pathway could indeed lead to tumoral mass formation and increase steroid production. Although these mutations are associated with increased hormonal activity and smaller size of the adrenal mass, the clinical correlates are yet to be fully clarified. Additional studies are also needed to complete the molecular landscape of unilateral adrenocortical adenomas associated with ACTH-independent Cushing's syndrome.

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Second publication

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

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

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

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

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

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

Results: 706 somatic protein-altering mutations were detected in 88/99 tumors (median: 6 per tumor). We identified several mutations in genes of the cAMP/PKA pathway, including three novel mutations in PRKACA, associated with female sex and Cushing's syndrome. We also found genetic alterations in different genes involved in the Wnt/β-catenin pathway, associated with larger tu-

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Abbreviations:

mors and endocrine inactivity, and, notably, in many genes of the Ca2+-signaling pathway. Finally, by comparison of our genetic data with those available in the literature, we describe a comprehensive genetic landscape of unilateral ACAs.

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

Adrenocortical adenomas (ACAs) are among the most frequent human neoplasias with a prevalence of 2%–3% in the general population. They are endocrine inactive in 70% of cases, mostly incidentally-discovered, or associated with autonomous cortisol or aldosterone secretion. The genetic basis of several adrenal disorders has been elucidated over the last years following classical genetic approaches and utilizing next-generation sequencing techniques. In particular, the cAMP/protein kinase A (PKA) pathway plays a central role in adrenocortical growth and steroidogenesis. Specifically, genetic alterations affecting the cAMP/PKA pathway, such as germline or somatic mutations in genes encoding the regulatory subunit 1 α of PKA (*PRKAR1A*), the protein Gsα (*GNAS*), and the phosphodiesterases 11A and 8B (*PDE11A* and *PDE8B*) have been reported in cortisol-producing ACAs (CPA) and bilateral micronodular adrenal hyperplasias (1–5).

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

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

Materials and Methods

Tissue samples, patients, and clinical annotations

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

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

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

WES and data analysis

DNA was extracted from fresh-frozen tissues and checked for signs of degradation as previously described (6). Exomes were enriched in solution and indexed with the SureSelect XT Human All Exon (50Mb kit, version 5, Agilent Technologies, Santa Clara, CA, USA) for library preparation. Sequencing was performed as paired-end reads of 100 bp on HiSeq2500 systems (Illumina, San Diego, CA, USA). Pools of 12 indexed libraries were sequenced on four lanes to an average depth of coverage between 82x and 170x. Image analysis and base calling were performed with Real-Time Analysis software (Illumina). Reads were aligned against the human assembly hg19 (GRCh37) using the Burrows-Wheeler Aligner tool (BWA, v 0.7.5a). Moreover, we performed single-nucleotide variant and small insertion and deletion (indel) calling specifically for the regions targeted by the exome enrichment kit, using SAMtools (v 0.1.19). Subsequently the variants were filtered using the SAMtools varFilter script using default parameters, with the exception of the maximum read depth parameter, which we set to 9999. Variant detection

Table 1. Overview of general characteristics, clinical data, and number of somatic mutations in patients with sporadic unilateral adrenocortical adenomas

	All	CS	SCS	EIA	P value
General characteristics					
Number of patients	99	39	35	25	
Age (yrs)	52.0 (40.5–61.5)	42.0 (35.0–50.5)	57.0 (49.0–67.0)	58.0 (51.0–66.0)	<0.001
Sex (M/F)	29/70	4/35	12/23	13/12	0.001
Clinical data					
BMI (kg/m^2)	28.6 (24.5–32.8)	25.5 (22.8–32.2)	28.2 (25.1–31.9)	32.2 (28.1–36.5)	0.04
Tumor diameter (mm)	38.0 (30.0–48.0)	35.0 (30.0–45.0)	40.0 (30.0–47.0)	35.0 (24.0–52.0)	0.86
Cortisol after DST (μg/dL)	4.2 (2.3–15.1)	15.7 (12.4–21.2)	3.3 (2.8–7.0)	1.8 (1.3–2.3)	<0.001
ACTH (pg/mL)	5.0 (2.0–9.0)	3.9 (1.9–5.0)	5.0 (1.9–9.7)	15.2 (6.9–24.5)	<0.001
UFC ULN>2 (n)	32	21	10	1	<0.001
Midnight cortisol ULN>2 (n)	13	9	4	0	0.015
Genetic data					
Total n° of somatic mutations	706	353	204	149	0.54
Median (range)	6 (0–55)	6 (0–55)	6 (0–16)	6 (0–19)	
Mean±SD	7.1 ± 7.9	9.0 ± 10.9	5.7 ± 4.9	6.0 ± 4.6	

Data are expressed as median with interquartile range in parenthesis or frequencies (if not otherwise specified). P value was evaluated by Kruskal-Wallis test and χ-square test, where appropriate. CS: Cushing´s syndrome, SCS: subclinical Cushing´s syndrome, EIA: endocrine inactive adenoma, M: male, F: female, BMI: body mass index, DST: 1-mg dexamethasone suppression test, ULN: upper limit of normal, UFC: urinary free cortisol, SD: standard deviation.

was done as described earlier (6). In brief, to reduce false positives we filtered out variants that were already present in our in-house database (currently 8000 exomes) or had variant quality less than 40. Raw read data of the remaining variants are then manually investigated using the Integrative Genomics Viewer (IGV). The frequency of each mutated allele was then evaluated in large population genomics projects, such as the EXAC (Broad) and the "1000 Genomes AF (allele frequency)" data set (Supplemental Table 1).

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

In silico analysis

Somatic variants were evaluated by both Polymorphism Phenotyping v2 algorithm tool (PolyPhen-2) (http://genetics.bwh-.harvard.edu/pph2) (23) and SIFT (Sorting Tolerant From Intolerant) algorithm (http://sift.jcvi.org/index.html) (24) to predict the possible impact of an amino acid substitution on the structure and function of a human protein. The variants were classified as possibly pathogenic according to the given thresholds (Supplemental Table 1). Most interesting recurrent genetic alterations were evaluated by *in silico* analysis to predict whether the variants may be damaging. Structural images were prepared with PyMOL software (www.pymol.org). The 3D structures of the mammalian PKA holoenzyme containing catalytic subunit α and regulatory subunit 2 β (PRKACA-PRKAR2B), the stimulatory G-protein α subunit (GNAS, isophorm 15), and the ryanodine receptor RYR1 were acquired from Protein Data Bank

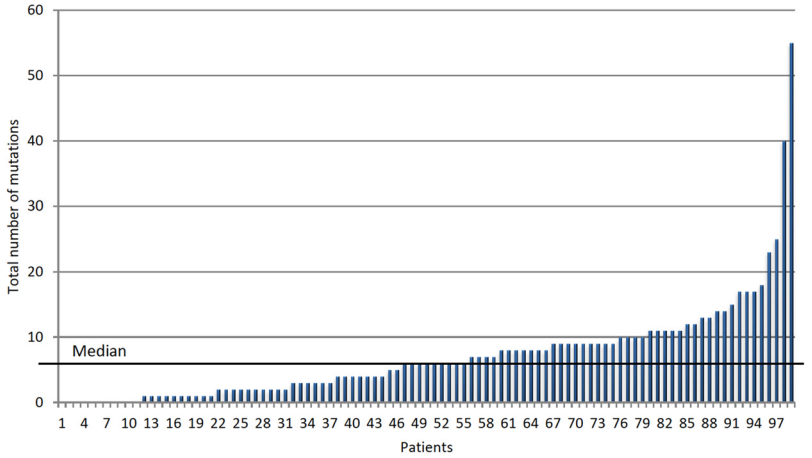

Figure 1. Total number of somatic mutations in each adrenocortical adenoma (n = 99) evaluated by next generation exome sequencing (median: 6 mutations per tumor).

(http://www.rcsb.org/pdb/, entries 3TNP, 1AZS, and 4UWA, respectively). Aminoacid changes induced by mutations were identified and displayed using the Chimera v1.10 Software.

Copy number alterations

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

Transcriptome analysis

Transcriptome analysis was performed by Affymetrix HGU133Plus2, as previously described (25), on an independent cohort of 41 ACAs, including 11 EIAs and 30 CPAs (20 CS and 10 SCS). Targeted next-generation sequencing (AmpliSeq design, IonTorrent sequencing) for *CTNNB1* (Ser45 hotspot, exons 7 and 8), *PRKACA* (L206 hotspot), *GNAS* (R201 hotspot), *PRKACB* and *PRKAR1A* was performed on 37/41 ACAs. Reads were aligned using the human genome assembly hg19 (GRCh37) and variant calling was performed using Torrent Suite Software (v. 4.2.1). Variants were annotated by ANNOVAR package (March, 22nd 2015 release). Variants were visually validated by IGV. Mutations were validated by Sanger sequencing. The mutation status for *CTNNB1* was not available for one ACA, whereas the one for *PRKAR1A* and *PRKACB* was not available in four ACAs.

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

Statistical analysis

Unsupervised complete linkage clustering was performed on the rows and columns using the Hamming distance as a similarity metric, to investigate interdependency among genetic alterations. The Fisher's exact or χ^2 tests, and Mann-Whitney U test were used to investigate dichotomic and continuous variables, where appropriate. Kruskal-Wallis test, followed by Bonferroni post hoc test, was performed for comparison among groups for non-normally distributed variables. Data are shown as median and ranges, if not otherwise specified (NOS). Statistical analyses were made using GraphPad Prism (version 5.0, La Jolla, CA, USA) and SPSS Software (version 21, SPSS Inc., Chicago, IL, USA). P values <0.05 were considered as statistically significant.

Results

Overview of genetic findings

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

Specific genetic alterations

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

GNAS somatic mutations were identified in 8/74 patients with CPAs (11%), two of them with SCS and six with CS, but in none of the EIAs. In seven patients known activating mutations were found at codon 201, whereas in one patient with CS a novel probably damaging mutation was observed (76A>C, p.Lys58Gln). The 3D *in silico*

Table 2. List of the genes affected by recurrent mutations (in at least 2 samples) among 99 adrenocortical adenomas

Gene symbol	Complete gene name	Mutation	Amino acid substitution	Pph2 (probability)	N of affected samples	CS/SCS *n* = 39/35	EIA *n* = 25
CTNNB1	catenin β 1	133T>C	**p.Ser45Pro**	Possibly (0.905)	39	7/19	13
		134C>T	**p.Ser45Phe**	Probably (0.928)	22	3/10	9
		130C>G	**p.Pro44Ala**[1]	Possibly (0.643)	10	0/6	4
		134C>G	p.Ser45Cys	Probably (0.950)	3	1/1	1
		134C>A	p.Ser45Tyr	Probably (0.950)	1	1/0	0
		133T>G	p.Ser45Ala	Benign (0.403)	1	0/1	0
		110C>T	p.Ser37Phe	Probably (1.000)	1	0/1	0
		122C>A	p.Thr41Asn	Possibly (0.468)	1	1/0	0
		1564G>A	p.Ala522Thr	Benign (0.098)	1	0/1	0
		1602, indel			1	1/0	0
					1	1/1	0
GNAS	GNAS complex locus	602G>A	**p.Arg201His**	Probably (1.000)	8	6/2	0
		601C>A	**p.Arg201Ser**	Probably (1.000)	3	2/1	0
		601C>T	**p.Arg201Cys**	Probably (1.000)	2	2/0	0
		76A>C	p.Lys58Gln	Probably (0.987)	2	1/1	0
					1	1/0	0
PRKACA	catalytic subunit α protein kinase A	589A>G	p.Trp197Arg	Probably (0.969)	3	3/0	0
		95T>A	p.Glu32Val	Benign (0.064)	1	1	0
		731 745del			1	1	0
					1	1	0
COL5A1	collagen type V α 1	935C>A	p.Pro3112Gln	Benign (0.001)	3	1/0	2
		2809G>A	p.Gly937Arg	Probably (0.997)	1	1	0
		3023C>A	p.Thr1008Lys	Probably (0.995)	1	0	1
					1	0	1
CEP76	centrosomal protein 76 kDa	293G>A	p.Thr98Ile	Benign (0.001)	2	2/0	0
		527C>G	p.Gly176Ala	Benign (0.002)	1	1	0
					1	1	0
LPPR3	lipid phosphate phosphatase-related protein type 3	428G>T	p.Ala143Asp	Probably (0.994)	2	1/1	0
		703G>A	p.Arg235Cys	Benign (0.001)	1	1/0	0
					1	0/1	0
REM1	RAS (RAD and GEM)-like GTP-binding 1	796C>A	p.Gln266Lys	Benign (0.001)	2	1/1	0
		700G>T	p.Glu234*		1	1/0	0
					1	0/1	0
IAH1	isoamyl acetate-hydrolyzing esterase 1 homolog	701C>G	p.Ala234Gly	Benign (0.019)	2	2/0	0
		539G>T	p.Val177Leu	Possibly (0.576)	1	1	0
					1	1	0
NID2	nidogen 2	212C>A	p.Arg71Leu	Benign (0.019)	2	1/1	0
		893C>T	p.Arg298His	Benign (0.001)	1	1/0	0
					1	0/1	0
XIRP2	xin actin-binding repeat containing 2	8824C>T	p.Arg2942Cys	Possibly (0.457)	2	1/1	0
		5712G>T	p.Met1904Ile	Benign (0.003)	1	1/0	0
					1	0/1	0
ASH1 liter	ash1 (absent, small, or homeotic)-like	6706G>A	p.Arg2239Trp	Probably (0.999)	2	1/1	0
		2902T>A	p.Lys968*		1	1/0	0
					1	0/1	0
CYP17A1	cytochrome P450, family 17, subfamily A polypeptide 1	6C>T	p.Trp2*		2	1/1	0
		979 981delCTT			1	1/0	0
					1	0/1	0
TNFRSF13C	tumor necrosis factor receptor superfamily, member 13C	91G>T	p.His31Asn	Benign (0.001)	2	1/0	1
		266A>G	p.Leu89Pro	Probably (0.996)	1	1	0
					1	0	1
DST	dystonin	6112G>C	p.Pro2038Ala	Benign (0.002)	2	0/1	1
		13451A>C	p.Met4484Arg	Possibly (0.622)	1	0/1	0
					1	0/0	1
MBP	myelin basic protein	488C>T	p.Trp163*	Benign (0.006)	2	0/2	0
		228G>T	p.Ser76Arg		1	0/1	0
					1	0/1	0
SYNE2	spectrin repeat containing nuclear envelope 2	17381T>A	p.Met5794Lys	Benign (0.001)	2	0/2	0
		16534C>A	p.Leu5512Ile	Benign (0.361)	1	0/1	0
					1	0/1	0
RYR1	ryanodine receptor 1	412G>T	p.Val138Leu	Probably (0.984)	2	0/1	1
		4405C>G	p.Arg1469Gly	Probably (0.987)	1	0/1	0
					1	0/0	1
RYR3	ryanodine receptor 3	AG>GG			2	0/1	1
		13538 13538del			1	0/1	0
					1	0/0	1
PCDHGA6	protocadherin γ subfamily A6	1868C>T	p.Thr623Met	Probably (0.957)	2	0/1	1
		331G>C	p.Glu111Gln	Possibly (0.892)	1	0/1	0
					1	0/0	1
MTHFR	methylene-tetrahydrofolate reductase NAD(P)H	221C>A	p.Arg74Leu	Probably (0.997)	2	1/0	1
		484A>G	p.Tyr162His	Benign (0.027)	1	1	0
					1	0	1
KMT2D	lysine (K)-specific Methyltransferase 2D	16211G>A	p.Ser5404Phe	Probably (0.997)	2	0/0	2
		62G>A	p.Ala21Val	Benign (0.196)	1	0	1
					1	0	1
CADPS2	Ca2 + -dependent secretion activator 2	58G>A	p.Arg20Cys	Benign (0.064)	2	0/0	2
		543 544insTA			1	0	1
					1	0	1

pph2: PolyPhen-2 (Polymorphism Phenotyping v2) algorithm was used to predict the possible impact of an amino acid substitution on the structure and function of a human protein. Results are expressed as probably damaging, possibly damaging and benign, with probability in parentheses).

analysis showed that lysine 58 is near the critical position 201, suggesting a functional significance for p.Lys58Gln substitution, similar to the known GNAS activating mutations (***Supplemental Figure 2***).

Interestingly, we found three novel somatic mutations in *PRKACA* in three patients with CS (p.Trp197Arg, p.245 248.del and p.Glu32Val). Although those mutations occurred outside the known hot-spot region of *PRKACA* in exon 7, the 3D *in silico* analysis pointed towards a potential pathogenic role for two of them. p.Trp197Arg mutation is located at the interface between the catalytic and the regulatory subunit. The exchange of the hydrophobic tryptophan with the hydrophilic, positively charged arginine might lead to alteration in the interaction between the subunits. Moreover, the p.245 248.del affects a region of the catalytic subunit of PKA at the interface with the regulatory subunit, likely inducing a modification that alters the binding of the regulatory to the catalytic subunit. In contrast, the mutation p.Glu32Val, with a hydrophilic, negatively charged glutamate replaced by a hydrophobic valine, is situated outside the interaction region (***Figure 2***).

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

Finally, different potentially relevant "private" mutations were detected, including alterations in genes encoding ionotropic (*GRIA1, GRIA2, GRID1, GRIK2, GRIN1, GRIN3B, GRIP1*) and metabotropic glutamate receptors (*GRM3, GRM4, GRM6*). Moreover, a missense mutation in *ARMC5* (p.Pro866Leu) was observed in a 22-mm unilateral left adenoma associated with CS. However, no second hit at the *ARMC5* gene was observed in this tumor. Finally, a probably damaging frameshift mutation (532 533insG) at *TP53* was detected in a 40-mm, endocrine inactive, oncocytic adenoma. Unfortunately, no follow-up data were available to ascertain the clinical course of this patient during the postoperative period.

Gene enrichment and pathway analysis

The gene enrichment analysis identified 605/706 (86%) mutated genes associated with GO terms. Interestingly, Ca^{2+}-signaling, collagen formation, and extracellular matrix organization were recognized as the most significantly represented pathways (***Supplemental Table 2***). The gene family analysis further showed that eight cytokines and growth factors, 60 transcription factors, including ATRX and MED12, 16 protein kinases, including PRKACA, 14 oncogenes, including CREB1, CREBBP, CTNNB1, and GNAS, and four tumor suppressor genes, including APC and TP53 were included among the mutated genes. None of them were mutated in more than one sample (***Supplemental Table 3***).

Genotype-phenotype correlation and transcriptome analysis

No statistically significant relationship was found between the mutation frequency and clinical data (sex, age, tumor size, and cortisol secretion pattern). We classified patients into three groups according to the known or potential biological consequences of the most frequent mutations: subjects with mutations in genes encoding components of the classic Wnt/β-catenin pathway (CTNNB1, APC, APC2, PCDH15, PCDHA8, PCDHB11, PCDHA10, PKP2), those with alterations in genes encoding components of the cAMP/PKA pathway (GNAS, PRKACA, PRKAR1A, CREB1, CREBBP, ADCY3, GRM3, GRM4, GRM6), and those with mutations in genes encoding components of Ca^{2+}-dependent signaling (CACNA1C, CACNA1E, CACNG8, RYR1, RYR2, RYR3, GRIA1, GRID1, GRIK2, GRIN1, GRIN3B, GRIP1) (***Supplemental Table 4***). The results of the unsupervised binary clustering analysis and the relationship between the genetic landscape of tumors and the clinical phenotype of the three groups of patients are shown in Figure 3A and ***Supplemental Table 5***. Patients with mutations in genes encoding components of the Wnt/β-catenin pathway were older, had larger tumors, and carried a higher total number of mutations than those without these aberrations ($P < .05$). In contrast, patients with mutations in the genes encoding component of the cAMP/PKA pathway were more frequently female and younger, in comparison to subjects not carrying mutations ($P < .01$). Mutations in genes encoding components of Ca^{2+}-dependent signaling were associated with a higher number of mutations when compared to those without ($P = .001$), whereas no difference in clinical and hormonal parameters was evident.

The results of the unsupervised clustering according to the results of the transcriptome analysis are shown in ***Figure 3B*** and ***C***. After considering the expression level, transcriptome profile could clearly identify four groups and well separated patients with CS from those with EIA and SCS, and tumors with mutations of the cAMP/PKA pathway from those with mutations in the Wnt/β-catenin or without mutations in one of those two pathways, showing significant enrichments in calcium-related genes (***Figure 3B***). Surprisingly, restricting the analysis only to genes of

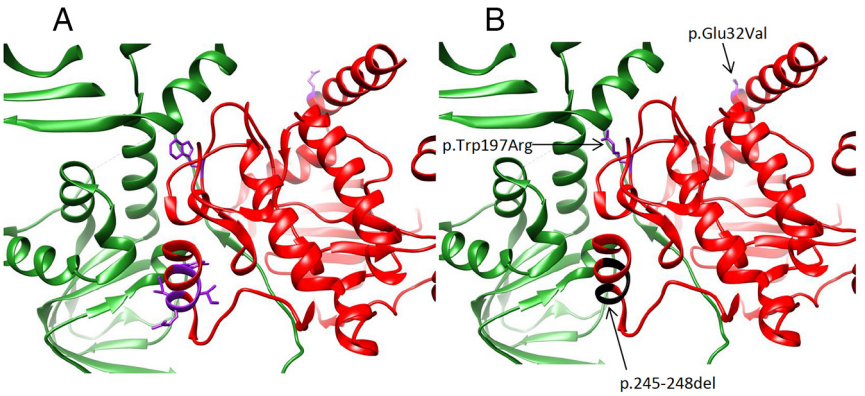

Figure 2. *In silico* analysis of the 3D structure changes of three novel somatic mutations in *PRKACA* gene (589A->G, p.Trp197Arg; 95T->A, p.Glu32Val; and deletion in position 731–745, p.245–248). a) wild type; b) the p.Trp197Arg mutation is at the interface between the catalytic and regulatory subunit. The exchange of the hydrophobic tryptophan with the hydrophilic, positively charged arginine leads to changes in this interaction. The p.245 248.del also affects a region of the catalytic subunit of PKA at the interface with the regulatory subunit. The deletion of this region probably leads to modification of the 3D structure and affects the binding of the regulatory to the catalytic subunit. The mutation p.Glu32Val is situated outside the interaction region between the catalytic and regulatory subunits of PKA or any other reported interaction region of catalytic subunit of PKA.

the Ca^{2+} signaling pathway, the transcriptome profile was also able to clearly divide the patients in four groups. The four clusters showed a good separation in patients with CS vs those with EIA or SCS, as well as tumors with mutations in the cAMP/PKA vs Wnt/β-catenin pathway (***Figure 3C***).

Combined genetic and genomic analysis

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

In 4/14 tumors no somatic mutations were detected by WES. One of those (CS) showed a large amplification at 19p13.2–12 including the genes *AKAP8, CACNA1A, PDE4C* and *PRKACA*. The second tumor (SCS) had amplifications at 7p22.2, which included *PRKAR1B* and 16p13.3. The third sample (EIA), presented a CN gain at chr11p15.5 and several microamplifications, whereas the last one (SCS), did not show any CNA in regions or genes with presumed functional relevance.

Systematic review of genetic data available in unilateral adrenocortical tumors

We compared the genetic findings of the present analysis with WES data available in the literature for ACA (n = 69 CPA) and ACC (n = 176) (***Supplemental Table 6***). The analysis of PRKACA wild-type benign tumors (n = 94 CPA+25 EIAs) showed that mutations in genes involved in cAMP/PKA pathway were present only in CPA (20% of cases), whereas alterations of genes involved in Wnt/β-catenin signaling were mutated in 49% of CPA and in 76% of EIAs. Alterations in genes involved in Ca^{2+}-dependent signaling were found in 14% of CPA and in 16% of EIA.

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

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

Discussion

The present study represents the most comprehensive genetic characterization of unilateral ACA. In this large European series we analyzed also for the first time endocrine inactive adenomas that represent the most frequent but less investigated type of ACAs. By restricting the investigation to patients without mutations in the predominant hot-spot region of *PRKACA* (p.Leu206Arg), WES analysis highlights substantial heterogeneity of the genetic background of cortisol-producing and endocrine inactive

ACAs, and separates well those tumors from aldosterone-producing ACA (27, 28). Overall, we identified 706 somatic mutations with a median of 6 per tumor. Many of the 605 mutated genes encoded components of the cAMP/PKA, the Wnt/β-catenin or, more surprisingly, the Ca^{2+}-dependent signaling pathway.

Among genetic alterations of the cAMP/PKA pathway, *GNAS* somatic mutations were the most frequent, being associated with cortisol production, accordingly with published data (7, 9–10). In addition to the previously reported hot-spot mutations, a novel substitution p.Lys58Gln was found in a patient with CS with potential

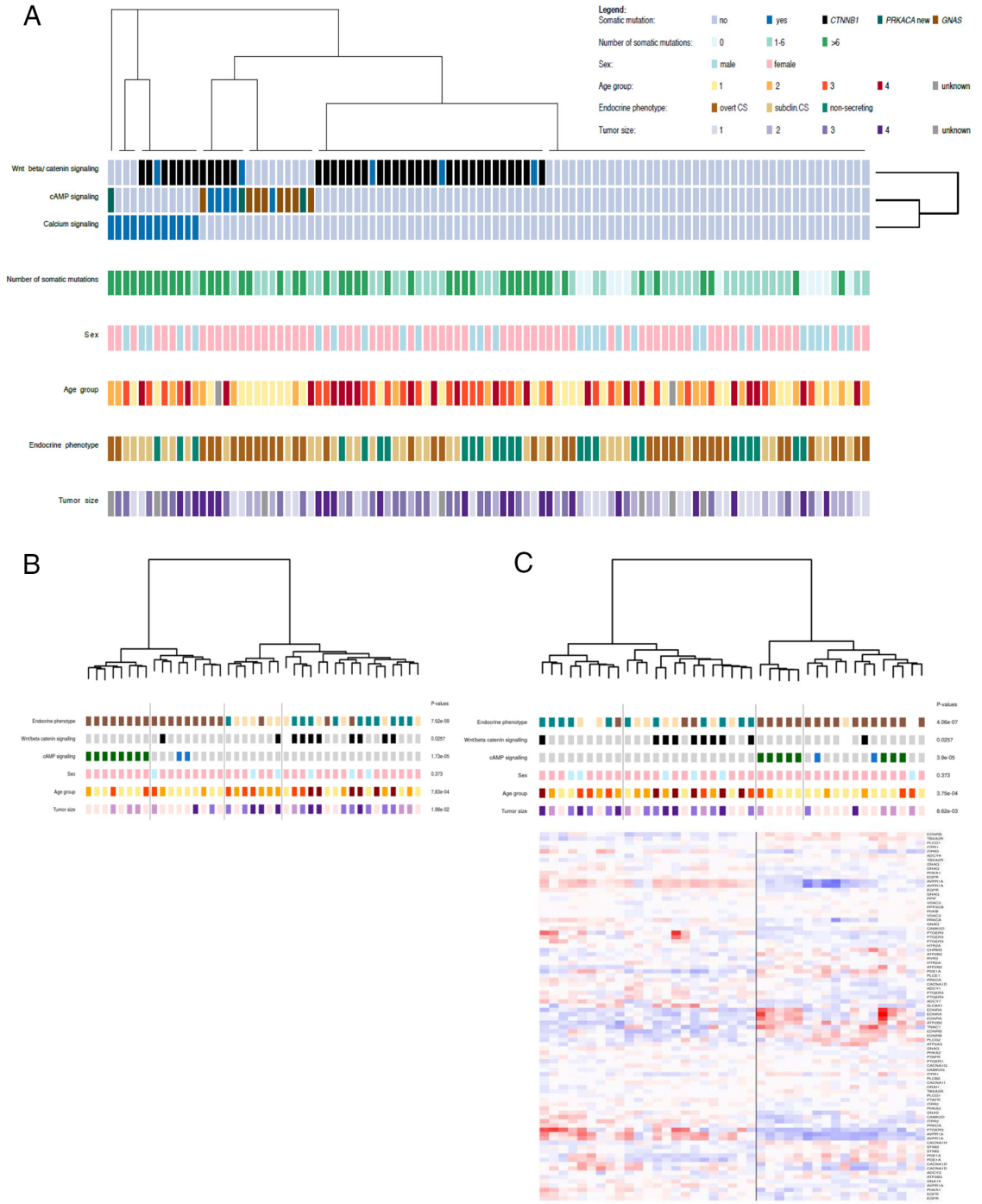

Figure 3. A. Heat map of the most recurrent somatic mutations classified according to their known or potential biological consequences: mutations in genes encoding components of Wnt-β catenin pathway, those in genes encoding members of the cAMP/PKA pathway, and mutations in genes involved in Ca^{2+}-signaling (n = 99 samples). The relationship with the total number of somatic mutations and clinical parameters is also shown. **B and C.** Transcriptome analysis of the cohort of additional 41 adenomas. The unsupervised clustering performed according to the expression level from whole transcriptome profiling is shown in **B**, whereas the clustering restricted to Ca^{2+} signaling-related genes is shown in **C**. The relationship with somatic mutations and clinical parameters, as well as the heat map of under-/overexpressed genes in the two pathways is also shown.

Table 3. Combined analysis between genetic data from the current exome-sequencing cohort and copy number data from previous SNP array profiling in 14 adrenocortical adenomas (17)

ID	Diagnosis	Sex/Age	Tumor size (mm)	N somatic mutations in exons	Recurrent mutations	N of coding copy number alterations in exons	N of gained single genes	Gained chromosomal regions <1.000.000 bps
Samples without somatic mutations								
AD90	CPA (SCS)	M/53	50	0	-	13	14	-
AD91	CPA (SCS)	M/37	25	0	-	2	0	**7p22.3–22.2** (***PRKAR1B***) **16p13.3** (***CACNA1H***)
AD92	CPA (CS)	M/55	40	0	-	5	4	**19p13.2–12** (*AKAP8, CACNA1A,* ***PDE4C***, ***PRKACA***)
AD93	EIA	M/71	65	0	-	26	52	**11p15.5**
Samples with somatic mutations								
AD30	CPA (CS)	F/35	40	9	*PRKACA*	1	1	-
AD39	CPA (CS)	F/43	28	8	*GNAS*	60	71	**16p13.3** (*AXIN1,* ***CACNA1H***) **19p13.3** (***APC2***, *GRIN3B*)
AD26	CPA (SCS)	F/29	20	6	*GNAS*	2	2	-
AD29	CPA (SCS)	M/60	40	11	*DST*	11	6	-
AD31	CPA (SCS)	F/52	60	13	*CTNNB1*	2	0	11q11.2 Y
AD32	CPA (SCS)	F/35	30	7	-	328	134	-
AD33	CPA (SCS)	F/73	30	2	*GNAS*	0	0	
AD25	EIA	F/59	53	8	*CTNNB1, DST*	5	4	**9p11-** (*AKAP2, CACNA1B, GRIN, GRIN3A,* ***PRKACG***)
AD34	EIA	M/49	70	3	*CTNNB1* *RYR1*	152	1149	1p36.33–35.3 1q22–23.1 3p21.31 (*PRKAR2A*) 3q28–29 4p16.3–16.1 (*PDE6B*) 5q35.2–35.3 (*GRM6*) **7p22.3–22.1** (***PRKAR1B***) 7q11.23 7q21.3–22.1 8q24.3 (*GRINA*) 9q32–33.2 (*CACNA1B, GRIN1*) 10q24.31–25.1 (*CYP17A1, PDCD11*) **11p15.5–15.4** (***IGF2, H19***) 11q13.1–13.5 (*PDE2A*) 14q32.2–33 **16p13.3–13.2** (***CACNA1H***, *CREBBP*) 16q24.1 17p13.3–13.1 17q21.31–22 (*CACNA1G*) 17q25.1–25.3 (*PDE6G*) **19p13.3–12** (*AKAP8,* ***APC2***, *CACNA1A, PDE4A,* ***PDE4C***, ***PRKACA***) 19q12–13.41 (*GRIK5, GRIN2D*) **20q13.33** 21q22.3 22q11.21–22 22q13.1–31 (*CACNA1I*) Xp22.33
AD35	EIA	F/53	43	7	*CTNNB1*	12	18	**7p22.3–22.1** (***PRKAR1B***) **11p15.5** (***IGF2, H9***) **16p13.3** (***CACNA1H***) **20q13.3**

In bold: recurrently gained chromosomal regions and genes (observed in more than one sample).

functional relevance in our *in silico* model. Likewise, three novel somatic mutations in *PRKACA* were detected in three CPA associated with CS. Interestingly, *in silico* data provide evidence that the p.Trp197Arg substitution and the p.245–248 deletion may be able to alter the interaction between the catalytic and the regulatory subunit of PKA, similarly to what described for the p.Leu206Arg mutation (11). Moreover, the essential role of the phosphorylation site Trp197 in the binding to PKA regulatory subunit was already described in 1997 (29). In contrast, the localization of the mutation p.Glu32Val outside known interacting regions of the catalytic subunit, do not allow any speculation on the biological relevance of this substitution. Other mutated components of the cAMP pathway included *PRKAR1A, CREB1* (cAMP responsive element binding protein), *CREBBP* (CREB binding protein) and three genes encoding metabotropic glutamate receptors (mGluRs, *GRM3, GRM4, GRM6*). The mutated mGluRs in our cohort belong to the group II and III mGluRs, which are G-protein-coupled receptors involved in regulation of intracellular cAMP levels. Interestingly, mGluR3 has been previously suggested to be involved in the regulation of steroidogenesis in adrenocortical tissues (30). Considering the relationship with the clinical data, mutations in component of the cAMP/PKA pathway occur invariably in young patients with cortisol-secreting tumors. Those results are in line with the data previously published by our group (6, 8) and others (7, 9–10), confirming that additional alterations of the cAMP pathway, apart from the well-known *PRKACA* mutations, are associated with a severe hormonal phenotype and, likely, early diagnosis.

Among mutations affecting genes of the Wnt/β-catenin pathway, as expected, the most common were somatic mutations in *CTNNB1* (39% of cases). They occurred

more frequently in patients with SCS and EIA (54% and 52% of cases, respectively) than in those with CS (18%), as previously reported (15). These findings may further confirm a predominant role of *CTNNB1* mutations in early adrenocortical tumorigenesis. Among the components of the Wnt/β-catenin pathway, genes encoding for the plakophilin (*PKP2),* member of the arm-repeat (armadillo) gene family, the adenomatosis polyposis coli (*APC*) and *APC2*, and four members of the protocadherin family (*PCDH15, PCDHA8, PCDHA10, PCDHB11*) were recognized. Protocadherins play a major role in cell-cell adhesion and interfere with the β catenin signaling proliferation pathway (31). Some members of the protocadherin family have recently been recognized as candidate tumor suppressor genes (31), and somatic mutations have been reported in squamous cell carcinoma, colon adenocarcinoma and melanoma (see COSMIC, http://cancer.sanger.ac.uk/cosmic/gene/analysis). Moreover, protocadherins may play a role in cell-cell adhesion and interfere with the Wnt/β-catenin signaling pathway (32), supporting the hypothesis that alterations of this Wnt/β-catenin regulatory signal may be relevant for adrenocortical tumorigenesis. In this context, it is important to mention that the regulator of Wnt/β-catenin pathway *ZNRF3,* recently reported as one of the most frequently altered genes in ACC (15), was not identified among mutated genes in our ACA series. In general and similarly to what previously reported for *CTNNB1* mutations, the genetic alterations in components of the Wnt/β-catenin pathway were mostly found in older patients with larger and inactive tumors (19).

Among Ca^{2+}-dependent signaling pathways, genes encoding Ca^{2+} receptors (*CACNA1C* and *CACNA1E),* ryanodine receptors (RyRs), ionotropic glutamate receptors (iGluRs) and one glutamate receptor interacting protein (*GRIP1*) were included. The RyRs are intracellular Ca^{2+}-release channels found on the sarcoplasmic reticulum of myocytes and on the endoplasmic reticulum of several nonmuscular organs (33). There is some evidence on the potential role of RyR alterations on adrenal function (34). According to our *in silico* analysis of *RYR1* and *RYR2* mutations and considering that the interaction between transmembrane and cytoplasmic domains of those receptors is an important mechanism in Ca^{2+} release modulation (35), it is well conceivable that the mutations found in our cohort may be biologically relevant. Several genes responsible for regulation of intracellular Ca^{2+} levels are known or suspected to be involved in the pathogenesis of endocrine tumors, such as aldosterone-producing adenomas (*KCJN5, ATP1A1, ATP2B3*, and *CACNA1D*) (27, 28) and GH-secreting pituitary adenomas (36, 37). In contrast, the role of alterations of Ca^{2+} signaling in the pathogenesis of CPA is not well understood, even though it has been demonstrated that adrenal fasciculata cells express high levels of T-type and L-type Ca^{2+} channels that may regulate cortisol secretion (38). Additionally, Ca^{2+} channels could be involved in molecular mechanisms of apoptosis regulation and cancer transformation (39), leading us to speculate on the proliferative role of this pathway in adrenocortical cells. Interestingly, the transcriptome analysis performed on our independent cohort clearly showed that the expression of Ca^{2+} signaling-related genes in ACAs not associated with primary hyperaldosteronism is able to classify patients into meaningful clusters. In fact, the unsupervised clustering restricted to the expression levels of those genes, provided a good separation of patients with CS from those with SCS and EIA, and tumors with mutations in the cAMP-PKA pathway from those with Wnt/β catenin alterations. This finding, together with the identification of somatic mutations in Ca^{2+} signaling genes in our study, provides indirect evidence for a role of Ca^{2+}-related pathways in the tumorigenesis and steroidogenesis of nonaldosterone secreting ACAs. Further studies will be necessary to unravel the specific underlying mechanisms.

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

According to the results of the pathway analysis, components of Ca^{2+} signaling, collagen formation, and extracellular matrix organization were among the most significantly represented. Extracellular matrices (ECM) are secreted molecules composed of glycoproteins, collagens, glycosaminoglycans and proteoglycans that can regulate cell migration, differentiation, proliferation and survival by communicating with intracellular cytoskeleton and growth factor signals (40). Interestingly, a putative role for ECM expression has been hypothesized in the development of human adrenal cortex (41). Moreover, a previous transcriptome study on ACAs identified enrichment in genes related to ECM (42). However, we observed only "private" mutations in ECM and collagen formation pathways and it is unclear whether they derive from pro-

liferative processes or might represent early events in adrenocortical tumorigenesis.

We also performed an unsupervised clustering considering the WES data available for all ACA together (n = 168) and separated for CPAs with or without *PRKACA* mutations (n = 49 and 94, respectively), providing results similar to that obtained in our present series (***Supplemental Figure 4***). In addition, in this very large series, we observed that most genetic alterations in the cAMP/PKA signaling pathway were not associated with alterations at the Wnt/β-catenin or Ca^{2+}-dependent signaling pathway, further confirming their major role in the pathogenesis of CPAs.

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

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

In summary, our study represents the largest sequencing effort on sporadic unilateral adrenocortical adenomas and demonstrates the heterogeneity of the genetic background of ACAs without *PRKACA* p.Leu206Arg mutation. Apart from the known somatic mutations, no other recurrent mutation can alone explain the processes that lead to tumor formation and hormone hypersecretion. However, the provided landscape and the genetic alterations in newly described pathways (ie, Ca^{2+}-dependent signaling) are shedding light on the pathogenesis of adrenocortical tumors and are providing a solid basis for future molecular analysis.

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CURRICULUM VITAE

PERSONAL INFORMATION

Guido Di Dalmazi

Medical doctor
Specialist in Endocrinology and Metabolic diseases

Date of birth: 7th November 1982
Place of birth: Chieti (I)
Nationality: Italian

CURRENT POSITION: Researcher at Division of Endocrinology, University of Bologna, S. Orsola-Malpighi Hospital, Bologna, Italy

EDUCATION

PhD Fellowship **2013 - 2016**
Ludwig-Maximilian University - Medizinische Klinik und Poliklinik IV – Munich (D)
- Thesis: "Genetic and molecular background of adrenocortical tumors"

Specialization in Endocrinology and Metabolism *cum laude* **2008 - 2013**
Alma Mater Studiorum University - S. Orsola-Malpighi Hospital – Bologna (I)
- Thesis: "Long-term follow-up of adrenal incidentalomas"

Bachelor Degree in Medicine and Surgery *cum laude* **2001 – 2007**
Alma Mater Studiorum University – Bologna (I)
- Thesis: "Endocrine and metabolic alterations in patients with narcolepsy and cataplexy"

WORK EXPERIENCE

Researcher/Physician **2016 - present**
S. Orsola-Malpighi Hospital – Bologna (I)

Clinical activity in outpatient clinic **2013**
S. Orsola-Malpighi Hospital – Bologna (I)
- Diagnosis and management of the main endocrine diseases
- Thyroid ultrasonography and fine needle aspiration biopsy
- Management of clinical practice specifically dedicated to patients with adrenal diseases and obesity

A.U.S.L. Imola –Imola (I)
- Diagnosis and management of the main endocrine diseases
- Thyroid ultrasonography and fine needle aspiration biopsy

Temporary research fellowship **2013**
Alma Mater Studiorum University - S. Orsola-Malpighi Hospital – Bologna (I)
- Project: "Evaluation of the treatment with clomifene citrate on testosterone levels in subjects with obesity, hypogonadism, and impaired glucose tolerance or type 2 diabetes"

Internship in the Endocrinology Unit **2007 – 2008**
Alma Mater Studiorum University of Bologna - S. Orsola-Malpighi Hospital – Bologna (I)
- Execution and interpretation of the main functional endocrinological tests

Temporary research fellowship **2007 – 2008**
Alma Mater Studiorum University - S. Orsola-Malpighi Hospital – Bologna (I)
- Project: "Prevalence of hyperandrogenic states in adolescents and young adults"

PRIZES, AWARDS, and GRANTS

Top 100 Abstracts **2016**
59th Symposium of Deutsche Gesellschaft für Endokrinologie
- Genetic landscape of sporadic unilateral adrenocortical adenomas without *PRKACA* p.Leu206Arg mutation

Bruno Allolio-Nebennieren-Preis der DGE **2016**
Deutsche Gesellschaft für Endokrinologie - Shire Deutschland GmbH
- Genetic landscape of sporadic unilateral adrenocortical adenomas without *PRKACA* p.Leu206Arg mutation

Grant application for research project **2015**
Friedrich Baur Stiftung
- Next generation sequencing of a large cohort of sporadic adrenocortical adenomas

Grant application for research project **2015**
Vereins zur Förderung von Wissenschaft und Forschung an der Medizinischen Fakultät der Ludwig-Maximilians-Universität München
- Steroidogenic enzymes expression in adrenocortical adenomas associated with hypercortisolism with different genetic background

ENS@T Award **2014**
13th ENS@T Scientific Meeting – Nice (F)
- Novel somatic mutations in the catalytic subunit of the protein kinase A as a cause of adrenal Cushing´s syndrome: a European multicentric study

ENS@T Travel Grant **2014**
13th ENS@T Scientific Meeting – Nice (F)

YARE Best Presentation Award 2014 **2014**
16th Annual YARE Meeting – Hamburg (D)
- Novel somatic mutations in the catalytic subunit of the protein kinase A as a cause of adrenal Cushing´s syndrome: a European multicentric study

Best oral communication (Orali Top SIE) **2013**
36th National Congress of the Italian Society of Endocrinology (SIE) – Padova (I)
- Cardiovascular diseases and mortality in patients with subclinical Cushing´s syndrome: 15 year follow-up study

TEACHING ACTIVITIES

7th Postgraduate Training Course "Clinical Endocrinology" **2017**
Zagreb University School of Medicine – Zagreb (HR)
- Glucocorticoids and Type 2 Diabetes: From Physiology to Pathology

Radboud Adrenal Master Class **2017**
Radboud University Nijmegen – Nijmegen (NL)
- Advances on genetics of adrenal Cushing´s syndrome

Radboud Adrenal Master Class **2014**
Radboud University Nijmegen – Nijmegen (NL)
- Recent advances on molecular mechanisms underlying Cushing´s syndrome

COURSES

Training workshop on RCTs **2015**
INSERM – Paris (F)

Controversies in endocrinology: making hypothesis and supporting thesis **2013**
Novartis Oncology – Montegridolfo (I)

Writing a scientific paper: from clinical practice to publication **2010**
Avogadro University and IRCCS S. Raffaele – Bologna (I)

Medical writing and congress abstract presentation **2010**
Elsevier and Alma Mater Studiorum University – Bologna (I)

INVITED LECTURES

Altogether to Beat Cushing's syndrome (ABC) meeting – Naples (I) **2017**
- Genetic and molecular background of Cushing's disease

New frontiers in Endocrinology – Modena (I) **2016**
- Update on genetics of Cushing syndrome

Il PDT delle Patologie Surrenaliche nel Policlinico S.Orsola-Malpighi – Bologna (I) **2016**
- Adrenal tumors: update on physiopathology and therapeutic perspectives

2nd IMPROCUSH (Improving Outcome in Cushing´s Syndrome) – Munich (D) **2016**
- Metabolomics profile in Cushing's syndrome

Endo 2016 – Boston, MA (USA) **2016**
- Can We Predict Recovery of the HPA Axis after Cure of Cushing Syndrome?

17th Adrenal Cortex Conference – Boston, MA (USA) **2016**
- Update on genetic causes of adrenal Cushing

European Congress of Endocrinology – Dublin (IRL) **2015**
- Subclinical Cushing´s syndrome and cardiovascular disease

Symposium Gentianum – Fraueninsel (D) **2015**
- Exome sequencing in adrenocortical adenomas: towards a genetic landscape

26th Birkensteiner Hormonkonferenz – Fischbachau (D) **2014**
- Subclinical hypercortisolism: a disease?

3rd Dresdner Gesellschaft für Universitäre Internistische Diabetologie & Endokrinologie Tagung – Dresden (D) **2014**
- PRKACA mutations in Cushing´s syndrome

1st IMPROCUSH (Improving Outcome in Cushing´s Syndrome) – Munich (D) **2014**
- Postoperative adrenal insufficiency in symptomatic and asymptomatic cortisol producing adenoma

Come gestire oggi la patologia surrenalica – Bologna (I) **2011**
- Medical therapy

ORAL PRESENTATIONS

Endo 2016 Late Breaking Abstract – Boston, MA (USA) **2016**
- Plasma metabolomics profile in patients with Cushing´s syndrome

12th German Adrenal Conference – Rostock (D) **2016**
- Plasma metabolomics profile in patients with Cushing´s syndrome

38th National Congress of the Italian Society of Endocrinology (SIE) – Taormina (I) **2015**
- Steroid profiling by liquid chromatography/tandem mass spectrometry in non-secreting adrenocortical adenomas and in those associated with subclinical hypercortisolism: evidence for pathogenetic biomarkers

58th Symposium DGE – Lübeck (D) **2015**
- Steroid profiling by liquid chromatography/tandem mass spectrometry in non-secreting adrenocortical adenomas and in those associated with subclinical hypercortisolism: evidence for pathogenetic biomarkers

16th Annual YARE Meeting – Hamburg (D) 2014
- Novel somatic mutations in the catalytic subunit of the protein kinase A as a cause of adrenal Cushing´s syndrome: a European multicentric study

37th National Congress of the Italian Society of Endocrinology (SIE) – Pisa (I) 2014
- Somatic mutations of the catalytic subunit of the protein kinase A as a cause of adrenal Cushing's syndrome: a European multicentric study

11th German Adrenal Conference – Würzburg (D) 2013
- Adrenal function after adrenalectomy for subclinical and overt Cushing´s syndrome: a systematic review of the literature

36th National Congress of the Italian Society of Endocrinology (SIE) – Padova (I) 2013
- Cardiovascular diseases and mortality in patients with subclinical Cushing´s syndrome: 15 year follow-up study

International and European Congress of Endocrinology (ICE/ECE) – Firenze (I) 2012
- Progressively increased patterns of subclinical hypercortisolism in adrenal incidentalomas differently predict major metabolic and cardiovascular outcomes: a large cross-sectional study

National Italian Congress of Endocrinology and Metabolism – Verona (I) 2012
- Presentation of a clinical case on obesity and diabetes

National Italian Congress of Hypothalamus and Hypophysis (4I) – Ferrara (I) 2009
- Body mass index independent metabolic alterations in narcolepsy

National Congress of Italian Association of Sleep Medicine (AIMS) – Bari (I) 2008
- Endocrine and Metabolic alterations in patients with narcolepsy and cataplexy

REVISOR ACTIVITY

The Lancet Diabetes and Endocrinology
Oncotarget
Journal of Clinical Endocrinology and Metabolism
European Journal of Endocrinology
Endocrine
Hormone and Metabolic Research
Journal of Endocrinological Investigation
Frontiers in Endocrinology
International Journal of Endocrinology

MEMBERSHIPS

European Society of Endocrinology (ESE)
European Network for the Study of Adrenal Tumors (ENS@T)

Italian Society of Endocrinology (SIE)
German Society of Endocrinology (DGE)
Endocrine Society
Young Italian Endocrinologists (EnGioI)
Young Active Researchers in Endocrinology (YARE)

LANGUAGES

Italian: mother tongue
English: excellent knowledge
German: B1 level
Greek: A2 level

IT SKILLS

Microsoft Office (Word, Excel, PowerPoint)
IBM SPSS
File Maker Pro
SoftGenetics Mutation Surveyor
Integrative Genomics Viewer

SCIENTIFIC PUBLICATIONS

Di Dalmazi G, Quinkler M, Deutschbein T, Prehn C, Rayes N, Kroiss M, Berr CM, Stalla G, Fassnacht M, Adamski J, Reincke M, Beuschlein F. Cortisol-related metabolic alterations assessed by mass spectrometry assay in patients with Cushing's syndrome. Eur J Endocrinol 2017, 177: 227-237.

Mosconi C, Vicennati V, Papadopoulos D, Di Dalmazi G, Morselli-Labate AM, Golfieri R, Pasquali R. Can Imaging Predict Subclinical Cortisol Secretion in Patients With Adrenal Adenomas? A CT Predictive Score. AJR Am J Roentgenol 2017, 209: 122-129.

Weigand I, Ronchi CL, Rizk-Rabin M, Di Dalmazi G, Wild V, Bathon K, Rubin B, Calebiro D, Beuschlein F, Bertherat J, Fassnacht M, Sbiera S. Differential expression of the protein kinase A subunits in normal adrenal glands and adrenocortical adenomas. Sci Rep 2017; 7: 49.

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Mezzullo M, Fazzini A, Gambineri A, Di Dalmazi G, Mazza R, Pelusi C, Vicennati V, Pasquali R, Pagotto U, Fanelli F. Parallel diurnal fluctuation of testosterone, androstenedione, dehydroepiandrosterone and 17OHprogesterone as assessed in serum and saliva: validation of a novel liquid chromatography-tandem mass spectrometry method for salivary steroid profiling. Clin Chem Lab Med 2017; [Epub ahead of print]

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Mezzullo M, Fanelli F, Fazzini A, Gambineri A, Vicennati V, Di Dalmazi G, Pelusi C, Mazza R, Pagotto U, Pasquali R. Validation of an LC-MS/MS salivary assay for glucocorticoid status assessment: evaluation of the diurnal fluctuation of cortisol and cortisone and of their association within and between serum and saliva. J Steroid Biochem Mol Biol 2016; 163: 103-112.

Di Dalmazi G, Pasquali R, Beuschlein F, Reincke M. Subclinical hypercortisolism: a state, a syndrome, or a disease? Eur J Endocrinol 2015; 173: M61-M71.

Di Dalmazi G, Fanelli F, Mezzullo M, Casadio E, Rinaldi E, Garelli S, Giampalma E, Mosconi C, Golfieri R, Vicennati V, Pagotto U, Pasquali R. Steroid profiling by LC-MS/MS in non-secreting and subclinical cortisol-secreting adrenocortical adenomas. J Clin Endocrinol Metab 2015; 100: 3529-3538.

Calebiro D, Di Dalmazi G, Bathon K, Ronchi CL, Beuschlein F. cAMP signaling in cortisol producing adrenal adenoma. Eur J Endocrinol 2015; 173: M99-M106.

Di Dalmazi G, Pasquali R. Adrenal adenomas, subclinical hypercortisolism, and cardiovascular outcomes. Curr Opin Endocrinol Diabetes Obes 2015; 22: 163-168.

Lichtenauer UD*, Di Dalmazi G*, Slater EP, Wieland T, Kuebart A, Schmittfull A, Schwarzmayr T, Diener S, Wiese D, Thasler WE, Reincke M, Meitinger T, Schott M, Fassnacht M, Bartsch DK, Strom TM, Beuschlein F. Frequency and clinical correlates of somatic Ying Yang 1 mutations in sporadic insulinomas. J Clin Endocrinol Metab 2015; 100: E776-E782.
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Berr CM, Di Dalmazi G, Osswald A, Ritzel K, Bidlingmaier M, Geyer LL, Treitl M, Hallfeldt K, Rachinger W, Reisch N, Blaser R, Schopohl J, Beuschlein F, Reincke M. Time to recovery of adrenal function after curative surgery for Cushing's syndrome depends on etiology. J Clin Endocrinol Metab 2015; 100: 1300-1308.

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Di Dalmazi G, Kisker C, Calebiro D, Mannelli M, Canu L, Arnaldi G, Quinkler M, Rayes N, Tabarin A, Jullié ML, Mantero F, Rubin B, Waldmann J, Bartsch DK, Pasquali R, Lohse M, Allolio B, Fassnacht M, Beuschlein F, Reincke M. Novel somatic mutations in the catalytic subunit of the protein kinase A as a

cause of adrenal Cushing's syndrome: a European multicentric study. J Clin Endocrinol Metab 2014; 99: E2093-2100.

Di Dalmazi G, Berr CM, Fassnacht M, Beuschlein F, Reincke M. Adrenal Function After Adrenalectomy for Subclinical Hypercortisolism and Cushing's Syndrome: A Systematic Review of the Literature. J Clin Endocrinol Metab 2014; 99: 2637-2645.

Di Dalmazi G, Vicennati V, Garelli S, Casadio E, Rinaldi E, Giampalma E, Mosconi C, Golfieri R, Paccapelo A, Pagotto U, Pasquali R. Cardiovascular events and mortality in patients with adrenal incidentalomas that are either non-secreting or associated with intermediate phenotype or subclinical Cushing's syndrome: a 15-year retrospective study. Lancet Diabetes Endocrinol 2014; 2: 396-405.

Fanelli F, Gambineri A, Belluomo I, Repaci A, Di Lallo VD, Di Dalmazi G, Mezzullo M, Prontera O, Cuomo G, Zanotti L, Paccapelo A, Morselli-Labate AM, Pagotto U, Pasquali R. Androgen profiling by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in healthy normal-weight ovulatory and anovulatory late adolescent and young women. J Clin Endocrinol Metab 2013; 98: 3058-3067.

Di Dalmazi G, Vicennati V, Pasquali R, Pagotto U. The unrelenting fall of the pharmacological treatment of obesity. Endocrine 2013; 44: 598-609.

Gambineri A, Fanelli F, Prontera O, Repaci A, Di Dalmazi G, Zanotti L, Pagotto U, Flacco ME, Guidi J, Fava GA, Manzoli L, Pasquali R. Prevalence of hyperandrogenic states in late adolescent and young women: epidemiological survey on italian high-school students. J Clin Endocrinol Metab 2013; 98: 1641-1650.

Di Dalmazi G, Pagotto U, Pasquali R, Vicennati V. Glucocorticoids and type 2 diabetes: from physiology to pathology. J Nutr Metab 2012; 2012: 525093.

Di Dalmazi G, Vicennati V, Rinaldi E, Morselli-Labate AM, Giampalma E, Mosconi C, Pagotto U, Pasquali R. Progressively increased patterns of subclinical hypercortisolism in adrenal incidentalomas differently predict major metabolic and cardiovascular outcomes: a large cross-sectional study. Eur J Endocrinol 2012; 166: 669-677.

Vicennati V, Repaci A, Di Dalmazi G, Rinaldi E, Golfieri R, Giampalma E, Minni F, Marrano N, Santini D, Pasquali R. Combined aldosterone and cortisol secretion by adrenal incidentaloma. Int J Surg Pathol 2012; 20: 316-319.

Grassi I, Nanni C, Vicennati V, Castellucci P, Allegri V, Montini GC, Pagotto U, Di Dalmazi G, Pasquali R, Fanti S. I-123 MIBG scintigraphy and 68Ga-DOTANOC PET/CT negative but F-18 DOPA PET/CT positive pheochromocytoma: a case report. Clin Nucl Med 2011; 36: 124-126.

Vicennati V, Pasqui F, Cavazza C, Garelli S, Casadio E, Di Dalmazi G, Pagotto U, Pasquali R. Cortisol, energy intake, and food frequency in overweight/obese women. Nutrition, 2011. 27(6):677-680.

Poli F, Plazzi G, Di Dalmazi G, Ribichini D, Vicennati V, Pizza F, Mignot E, Montagna P, Pasquali R, Pagotto U. Body mass index-independent metabolic alterations in narcolepsy with cataplexy. Sleep 2009; 32: 1491-1497.