Genetic Spectrum and Clinical Correlates of Somatic Mutations in Aldosterone-Producing Adenoma

Fabio Luiz Fernandes-Rosa, Tracy Ann Williams, Anna Riester, Olivier Steichen, Felix Beuschlein, Sheerazed Boulkroun, Tim M. Strom, Silvia Monticone, Laurence Amar, Tchao Meatchi, Franco Mantero, Maria-Verena Cicala, Marcus Quinkler, Francesco Fallo, Bruno Allolio, Giampaolo Bernini, Mauro Maccario, Gilberta Giacchetti, Xavier Jeunemaitre, Paolo Mulatero, Martin Reincke and Maria-Christina Zennaro

Hypertension. published online May 27, 2014;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0194-911X. Online ISSN: 1524-4563

Original Article

Genetic Spectrum and Clinical Correlates of Somatic Mutations in Aldosterone-Producing Adenoma

Fabio Luiz Fernandes-Rosa,* Tracy Ann Williams,* Anna Riester,* Olivier Steichen, Felix Beuschlein, Sheerazed Boulkroun, Tim M. Strom, Silvia Monticone, Laurence Amar, Tchao Meatchi, Franco Mantero, Maria-Verena Cicala, Marcus Quinkler, Francesco Fallo, Bruno Allolio, Giampaolo Bernini, Mauro Maccario, Gilberta Giacchetti, Xavier Jeunemaitre, Paolo Mulatero,† Martin Reincke,† Maria-Christina Zennaro

Abstract—Primary aldosteronism is the most common form of secondary hypertension. Somatic mutations in *KCNJ5*, *ATP1A1*, *ATP2B3*, and *CACNA1D* have been described in aldosterone-producing adenomas (APAs). Our aim was to investigate the prevalence of somatic mutations in these genes in unselected patients with APA (n=474), collected through the European Network for the Study of Adrenal Tumors. Correlations with clinical and biochemical parameters were first analyzed in a subset of 199 patients from a single center and then replicated in 2 additional centers. Somatic heterozygous *KCNJ5* mutations were present in 38% (180/474) of APAs, whereas *ATP1A1* mutations were found in 5.3% (25/474) and *ATP2B3* mutations in 1.7% (8/474) of APAs. Previously reported somatic *CACNA1D* mutations as well as 10 novel *CACNA1D* mutations were identified in 44 of 474 (9.3%) APAs. There was no difference in the cellular composition of APAs or in *CYP11B2*, *CYP11B1, KCNJ5*, *CACNA1D*, or *ATP1A1* gene expression in APAs across genotypes. Patients with *KCNJ5* mutations were more frequently female, diagnosed younger, and with higher minimal plasma potassium concentrations compared with *CACNA1D* mutation carriers or noncarriers. *CACNA1D* mutations were associated with smaller adenomas. These associations were largely dependent on the population structure of the different centers. In conclusion, recurrent somatic mutations were identified in 54% of APAs. Young women with APAs are more likely to be *KCNJ5* mutation carriers; identification of specific characteristics or surrogate biomarkers of mutation status may lead to targeted treatment options. **(*Hypertension*. 2014;64:00-00.)** ● **Online Data Supplement**

Key Words: adrenal cortex ■ aldosterone ■ aldosteronism ■ mineralocorticoids ■ mutation ■ potassium channels

Arterial hypertension is a major cardiovascular risk factor that affects 10% to 40% of the adult population in industrialized countries. Detection of secondary forms of hypertension is particularly important because it allows the targeted management of the underlying disease. Primary aldosteronism (PA) is the most common form of secondary hypertension with an estimated prevalence of ≈10% in referred patients and 4% in primary care but as high as 20% in patients with resistant hypertension.[1–5] PA is caused by the excessive production of aldosterone from the adrenal cortex, resulting in hypertension associated with high plasma aldosterone levels, low plasma renin activity, and varying degrees of hypokalemia and metabolic alkalosis. Long-term consequences include an increased risk of myocardial infarction, stroke, and atrial fibrillation.[6,7]

Received February 23, 2014; first decision March 13, 2014; revision accepted April 29, 2014.

From the INSERM, UMRS_970, Paris Cardiovascular Research Center, Paris, France (F.L.F.-R., S.B., L.A., T.M., X.J., M.-C.Z.); University Paris Descartes, Sorbonne Paris Cité, Paris, France (F.L.F.-R., S.B., L.A., T.M., X.J., M.-C.Z.); Service de Génétique (F.L.F.-R., X.J., M.-C.Z.), Unité Hypertension artérielle (L.A.), and Service d'Anatomie Pathologique (T.M.), Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Paris, France; Divisions of Internal Medicine and Hypertension, Department of Medical Sciences (T.A.W., S.M., P.M.) and Endocrinology, Diabetes, and Metabolism, Department of Medical Sciences (M.M.), University of Torino, Torino, Italy; Medizinische Klinik und Poliklinik IV, Ludwig-Maximilians-University, Munich, Germany (A.R., F.B., M.R.); Faculty of Medicine, Sorbonne Universités, Université Pierre et Marie Curie Paris 06, Paris, France (O.S.); Institute of Human Genetics, Helmholtz Zentrum München, Neuherberg, Germany (T.M.S.); Institute of Human Genetics, Technische Universität München, Munich, Germany (T.M.S.); Endocrine Unit, Department of Medicine (F.M., M.-V.C.) and Department of Medicine (F.F.), University of Padova, Padova, Italy; Clinical Endocrinology, Campus Mitte, University Hospital Charité, Berlin, Germany (M.Q.); Department of Medicine I, Endocrine and Diabetes Unit, University Hospital Würzburg, Würzburg, Germany (B.A.); Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy (G.B.); and Division of Endocrinology, Azienda Ospedaliero-Universitaria Ospedali Riuniti Umberto I-GM Lancisi-G Salesi, Università Politecnica delle Marche, Ancona, Italy (G.G.).

*These authors contributed equally to this work

†These authors contributed equally to this work

The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.114.03419/-/DC1.

Correspondence to Maria-Christina Zennaro, INSERM, U970, Paris Cardiovascular Research Center (PARCC), 56 rue Leblanc, 75015 Paris, France. E-mail maria-christina.zennaro@inserm.fr

Hypertension **is available at http://hyper.ahajournals.org** **DOI: 10.1161/HYPERTENSIONAHA.114.03419**

Aldosterone-producing adenoma (APA) and bilateral adrenal hyperplasia (also known as idiopathic hyperaldosteronism) are the most frequent subtypes of PA together accounting for ≈95% of all cases.[8]

In recent years, major advances in the knowledge of the genetic basis of APA development have been made. The activation of the calcium signaling pathway is central for the regulation of aldosterone production, in particular by increasing the expression of *CYP11B2*, encoding aldosterone synthase. Zona glomerulosa (ZG) cells are sensitive to changes in extracellular potassium concentration (K^+). The ZG cell membrane is selectively permeable to a wide range of K^+ concentrations, and the depolarization of these cells leads to calcium (Ca^{2+}) entry through voltage-dependent calcium channels.[9] Somatic and inherited mutations of *KCNJ5*, coding for the potassium channel GIRK4, have been implicated in the formation of APA and in familial hyperaldosteronism type 3.[10,11] More recently, somatic mutations in *ATP1A1* (coding for the α1 subunit of the Na^+/K^+-ATPase) and *ATP2B3* (coding for the plasma membrane Ca^{2+}-ATPase, type 3) were identified in APA.[12] Mutations in *KCNJ5* and *ATP1A1* affect adrenal ZG cell membrane potential and intracellular ionic homeostasis, with chronic depolarization leading to opening of voltage-dependent calcium channels and activation of calcium signaling and aldosterone production.[10,12,13] Finally, somatic mutations in the *CACNA1D* encoding the $Ca_v1.3$ channel (calcium channel, voltage dependent, L type, α-1d subunit) have been identified in APA. The altered residues are located in particular segments bordering the channel pore.[14,15] These changes result in channel activation and opening at lower voltages, leading, like *ATP2B3* mutations, to increased intracellular calcium concentrations.

A large collection of APA samples, endowed with comprehensive clinical and biological information, collected through reference centers is organized within the European Network for the Study of Adrenal Tumors (ENS@T). The aim of the present study was to establish the prevalence of *KCNJ5*, *CACNA1D*, *ATP1A1*, and *ATP2B3* mutations in this European cohort and to explore the clinical and biological correlates of the mutation status across the spectrum of APA.

Subjects and Methods

An expanded Methods section is available in the online-only Data Supplement.

Patients

Patients with PA were recruited between 1994 and 2012 from 9 different centers belonging to the ENS@T APA working group (http://www.ENSAT.org). An extended phenotype was available for patients from Paris, Munich, and Torino.[11,12,16,17] Case detection and subtype identification of PA were performed according to institutional and Endocrine Society guidelines.[18–22] In patients diagnosed with PA, a thin slice computed tomographic scan or MRI of the adrenal or an adrenal venous sampling (AVS) was performed to differentiate between unilateral and bilateral aldosterone hypersecretion. Baseline clinical and biochemical characteristics of the patients are indicated in Table S1 in the online-only Data Supplement. Further details are available in the online-only Data Supplement.

DNA Isolation and Sequencing

Details are available in the online-only Data Supplement.

Pathological Analysis

Details are available in the online-only Data Supplement.

Statistical Analyses

Quantitative variables are reported as medians and interquartile range and compared with the Kruskal–Wallis (3 groups) or Mann–Whitney tests (2 groups). Categorical variables are reported as percentages and compared with Fisher exact test. Because of the low number of patients with mutations in *ATP1A1* and *ATP2B3*, extended phenotypes in patients from Paris were only compared between patients with *KCNJ5* or *CACNAD1* mutations or no mutations. A P value <0.05 was considered significant for global comparisons between the 3 groups; significant differences were further explored with 3 post hoc pairwise comparisons, for which a P value <0.05/3 was considered significant. We tried to replicate the significant pairwise differences in patients from the other 2 centers with large cohorts (Munich and Torino), first in each center and then by overall comparisons stratified by center (stratified linear regression for quantitative variables and Mantel–Haenszel test for categorical variables).

Results

Spectrum of Somatic Mutations in APA

Hot spot regions for mutation in *KCNJ5*, *CACNA1D*, *ATP1A1*, and *ATP2B3* were successfully sequenced in 474 somatic APA DNA samples from 9 different centers. Genetic screening of mutations in *KCNJ5*, *ATP1A1*, and *ATP2B3* was partially described in previous studies (online-only Data Supplement).[11,12,17] In addition, whole exome sequencing was performed in 25 paired somatic and germline DNA samples. Somatic mutations were found in 54.2% of APAs with 38.0% of *KCNJ5* mutations, 9.3% of *CACNA1D* mutations, 5.3% of *ATP1A1* mutations, and 1.7% of *ATP2B3* mutations (Table 1). There was no difference in the distribution of mutations across different centers (P=0.21). From the 25 patients who underwent exome sequencing, somatic mutations affecting one of the *KCNJ5*, *CACNA1D*, *ATP1A1*, and *ATP2B3* genes were identified in 11 patients. The somatic mutations were screened in paired germline DNA, and no mutation was observed.

Somatic *KCNJ5* mutations were found in 180 APA samples (Table S3). The recurrent mutations p.Gly151Arg (c.451G>A or c.415G>C) and p.Leu168Arg (c.503T>G) accounted for 62.7% (113/180) and 36.1% (65/180) of *KCNJ5* mutations, respectively. There was no difference in the frequency

Table 1. Type and Frequency of Somatic Mutations in Aldosterone-Producing Adenomas Across Different Centers

Center	No. With Successful Sequencing	*KCNJ5* Mutations (%)	*CACNA1D* Mutations (%)	*ATP1A1* Mutations (%)	*ATP2B3* Mutations (%)	Total (%)
Paris	199	74 (37.2)	27 (13.6)	9 (4.5)	3 (1.5)	113 (56.7)
München	93	32 (34.4)	7 (7.5)	7 (7.5)	4 (4.3)	50 (53.7)
Torino	73	29 (39.7)	4 (5.4)	5 (6.8)	1 (1.3)	39 (53.4)
Padova A	37	14 (37.8)	2 (5.4)	1 (2.7)	0	17 (45.9)
Berlin	23	11 (47.8)	1 (4.3)	1 (4.3)	0	13 (56.5)
Ancona	21	10 (47.6)	2 (9.5)	2 (9.5)	0	14 (66.6)
Padova B	11	3 (27.2)	0	0	0	3 (27.2)
Würzburg	9	4 (44.4)	1 (11.1)	0	0	5 (55.5)
Pisa	8	3 (37.5)	0	0	0	3 (37.5)
Total	474	180 (38.0)	44 (9.3)	25 (5.3)	8 (1.7)	257 (54.2)

of p.Gly151Arg and p.Leu168Arg across different centers (*P*=0.33). The cohort also included 2 patients harboring 2 additional previously described somatic mutations (p.Thr158Ala/c.472A>G and p.Trp126Arg/c.376T>C).[17]

CACNA1D mutations were found in 44 APAs from 7 centers. Nineteen *CACNA1D* mutations were identified; these mutations are located in exons 6, 8A, 8B, 14, 16, 23, 27, and 32 (Table S4). Among these mutations, we identified 9 previously described *CACNA1D* mutations[14,15] (please note that mutations p.Phe747Leu and p.Ile750Met described here and in Azizan et al[14] correspond to p.Phe767Leu and p.Ile770Met in Scholl et al[15] because of the use of different reference sequences as indicated in Table S4). The mutations p.Gly403Arg (c.1207G>C), p.Phe747Leu (c.2239T>C), and p.Val1338Met (c.4012 G>A) were the most prevalent, being found in 8, 6, and 6 APAs, respectively. Six novel *CACNA1D* mutations were identified: p.Tyr741Cys (c.2222A>G) and p.Ile750Phe (c.2248A>T) located in exon 16; p.Val979Asp (c.2936T>A), p.Val981Asn (c.2943G>C), p.Ala998Val (c.2993C>T), and p.Ala998Ile (c.2992-2993GC>AT) located in exon 23. Four additional new mutations were identified in exons not previously reported to carry somatic *CACNA1D* mutations, with whole exome sequencing: p.Leu655Pro (c.1964T>C) and p.Ser652Leu (c.1955C>T) located in exon 14 and p.Val1151Phe (c.3451G>T) and p.Ile1152Asn (c.3455T>A) located in exon 27 (Figure 1). The new mutations are located in regions conserved across different species (Figure S1). All but 2 novel mutations were present in single samples; the mutations p.Ala998Ile and p.Val1151Phe were present in 3 and 2 samples, respectively.

ATP1A1 mutations were found in 25 APAs from 6 centers (Table S5). The mutation p.Leu104Arg (c.311T>G) identified in 17 APAs is the most prevalent. Another 3 mutations were observed, p.Phe100_Leu104del (c.299_313delTCTCAATGTTACTGT) in 5 samples, p.Val332Gly (c.995T>G) in 2 samples, and p.Gly99Arg (c.295G>A) in 1 sample. Novel somatic *ATP1A1* mutations were not identified. Somatic *ATP2B3* mutations were found in 8 APAs (Table S6). Three different in frame deletion mutations were observed affecting the region comprised between residues 424 and 427. The deletion p.Leu425_Val426del (c.1272-1277delGCTGGT or c.1273-1278delCTGGTC), identified in 5 APAs, was the most prevalent. The mutation p.Val426_Val427del (c.1277_1282delTCGTGG) was present in 1 sample. A new mutation p.Val424_Leu425del (c.1270-1275del GTGCTG) was identified in 2 patients from the Paris cohort.

Correlation of Mutation Status With Morphological and Molecular Characteristics of APA

To verify the hypothesis that mutations in different genes are associated to a specific cellular composition of APA, we analyzed the cellular composition of 78 APAs from the Paris cohort. APAs are generally composed of zona fasciculata (ZF)–like cells or ZG-like cells in variable proportions; sometimes, smaller eosinophilic cells resembling cells from the zona reticularis are also observed. ZF cells are large cells with pale-staining nuclei and acidophilic and clear cytoplasm with abundant lipid droplets (Figure S2A). ZG cells are small cells with basophilic nuclei and clear cytoplasm with few intracellular fat droplets (Figure S2B). Seventy-two percent of APAs were composed of a majority (>50%) of ZF-like cells. Overall, ZF-like cells represented the main cellular component of APA: ZF-like cells median, 70 (50–90); ZG-like cells median, 10 (10–35); *P*<0.001 (Figure S2C). Analysis of the cellular composition of APA according to the mutation status did not show any difference in the percentage of ZF-like and ZG-like cells across groups (Figure S2D and S2E).

To verify the hypothesis that the mutation status is associated to different levels of *CYP11B2* or *CYP11B1* expression, we retrieved *CYP11B2* and *CYP11B1* mRNA expression from a transcriptome study performed on 92 APAs with complete genotypes.[11] This analysis included 45 APAs with *KCNJ5* mutations, 10 APAs with *CACNA1D* mutations, 5 APAs with *ATP1A1* mutations, and 31 APAs without mutations. There was no difference in *CYP11B2* or *CYP11B1* mRNA expression as a function of the mutation status (Figure 2A and 2B). In addition, we also investigated *KCNJ5*, *CACNA1D*, and *ATP1A1* mRNA expression according to the mutation status. No difference in gene expression was observed across groups with different mutations (Figure 2C–2E).

Combined information on mutation status, *CYP11B2* and *CYP11B1* mRNA expression, and cellular composition was available for 31 APAs. There was no correlation between *CYP11B2* expression and cellular composition in the different groups of APA carrying *KCNJ5*, *CACNA1D*, or no mutations (Figure S3A and S3B). Similarly, no correlation was observed between *CYP11B1* expression and cellular composition in APAs carrying *KCNJ5* or no mutations; although APAs carrying *CACNA1D* mutations showed a weak correlation of *CYP11B1* expression with the percentage of ZG-like and ZF-like cells, no definitive conclusions can be drawn because of the small numbers in this group (Figure S3C and S3D).

Genotype–Phenotype Correlations

The comparison of patients from Paris with *KCNJ5* or *CACNAD1* mutations or no mutation showed that patients with *KCNJ5* mutations were more often females, were diagnosed younger, and had higher minimal plasma potassium concentrations than patients from the 2 other groups (Table 2). Patients with *CACNAD1* mutations had smaller adenomas compared with those from the 2 other groups. Overall, significant differences between the 3 groups were also observed for systolic blood pressure and AVS lateralization ratio, but pairwise differences were not statistically significant. There was no association between the mutation status and preoperative plasma aldosterone or renin levels, the aldosterone to renin ratio, or the number of medications taken before surgery. There was also no association with postoperative blood pressure outcome as measured by blood pressure and treatment score at follow-up, cure, or improvement of hypertension.

In replication analyses, patients with *KCNJ5* mutations were significantly more often females than those with no mutation in the Munich cohort (69% versus 21%; Fisher *P*<0.001) but not in the Torino cohort (56% versus 31%; *P*=0.07), resulting in a significant overall difference

Table 2. Extended Phenotype of Patients From Paris With Different Mutation Status

Clinical and Biological Parameters	No Mutation (A) (n=86)	*KCNJ5* (B) (n=74)	*CACNA1D* (C) (n=27)	*ATP1A1* (n=9)	*ATP2B3* (n=3)	*P* Value Overall	A vs B	A vs C	B vs C
Females	45%	72%	33%	11%	67%	<0.001	0.001	0.37	0.001
Age at HTN diagnosis, y	39.5 (31–45)	36.5 (28–43)	39 (35–44)	40 (35–46)	38 (38–38)	0.33			
Age at PA diagnosis, y	45.5 (40–51)	40 (35–45)	46 (39–51)	50 (43–53)	44 (42–53)	0.001	<0.001	0.95	0.007
Lowest plasma K, mmol/L	3.0 (2.7–3.3)	3.3 (3.0–3.6)	3.0 (2.8–3.3)	3.1 (2.5–3.2)	1.9 (1.7–3.3)	<0.001	<0.001	0.69	0.009
Systolic BP, mm Hg	144.5 (134–158)	138 (131–153)	150 (138–164)	146 (138–151)	162 (157–167)	0.05	0.08	0.24	0.03
Diastolic BP, mm Hg	90 (85–97)	90 (80–99)	92 (85–95)	88 (84–95)	107 (81–111)	0.51			
Treatment score, n	2 (1–3)	2 (1–3)	2 (1–3)	2 (1–2)	3 (2–3)	0.52			
eGFR, mL/min per 1.73 m^2	92 (82–113.5)	94.5 (84–114)	91 (69–103)	94 (74–107.5)	92 (92–92)	0.45			
Urinary aldosterone, nmol/24 h	93.5 (64–147)	78 (63–112)	115 (75–142)	124 (80–223)	330 (59–575)	0.15			
Plasma aldosterone, pmol/L	812 (511–1176)	827 (626–1148)	685 (520–1012)	1102 (894–2157)	2193 (1249–9576)	0.27			
Plasma rennin, mU/L	1.7 (1.0–3.1)	1.7 (1.0–2.7)	2.9 (1.2–4.7)	1.9 (1.3–5.2)	1.0 (1.0–8.7)	0.16			
ARR, pmol/mU	149 (92–203)	165 (122–230)	126 (95–195)	218 (179–431)	252 (250–1915)	0.10			
AVS lateralization ratio	16 (10–35)	23 (13–45)	10 (7–32)	19 (13–58)	53 (6–99)	0.03	0.05	0.19	0.02
Solitary adenoma	79%	92%	81%	89%	100%	0.06			
Largest adenoma, mm	12 (10–20)	16 (13–20)	9 (8–12)	10 (10–18)	20 (12–25)	<0.001	0.02	0.002	<0.001
SBP at FU, mm Hg	127 (118–135)	125 (117–134)	126 (122–135)	133 (124–134)	136.5 (133–140)	0.62			
Diastolic BP at FU, mm Hg	82 (75–87)	82 (76.5–88)	82 (76–87)	86 (79–89)	87 (84–90)	0.77			
Treatment score at FU, n	0 (0–1)	0 (0–1)	1 (0–2)	1 (0–2)	0 (0–0)	0.10			
K at FU, mmol/L	4.2 (3.9–4.4)	4.1 (3.9–4.5)	4.2 (4.0–4.3)	4.5 (4.1–4.7)	4.6 (4.0–5.2)	0.87			
ARR at FU, pmol/mU	9.3 (4.8–16.1)	9.8 (6.6–19.7)	9.6 (4.1–18.1)	5.9 (2.3–6.7)	…	0.63			
Adjusted change in SBP, mm Hg*	26 (16–38)	26 (13–35)	25 (17–37)	21 (10–30)	45 (44–46)	0.58			
Hypertension cure	56%	58%	32%	44%	50%	0.08			
Significant BP improvement†	85%	78%	76%	78%	100%	0.45			

Statistical analysis has been done on groups A, B, and C only. Characteristics of *ATP1A1* and *ATP2B3* mutation carriers are presented for completeness but were not included in statistical comparisons because of small sample sizes. Categorical data reported as number (percentage) and compared with Fisher exact test; quantitative data reported as median (interquartile range) and compared with the Kruskal–Wallis test (overall *P*, significant if <0.05) or Mann–Whitney test (pairwise *P*, significant if <0.05/3). ARR indicates aldosterone-to-renin ratio; AVS, adrenal venous sampling; BP, blood pressure; eGFR, estimated glomerular filtration rate; FU, follow-up; HTN, hypertension; K, potassium; PA, primary aldosteronism; and SBP, systolic blood pressure.

*Change in SBP adjusted for change in treatment score by ANCOVA.

†Hypertension cure or adjusted change in SBP >20 mm Hg.

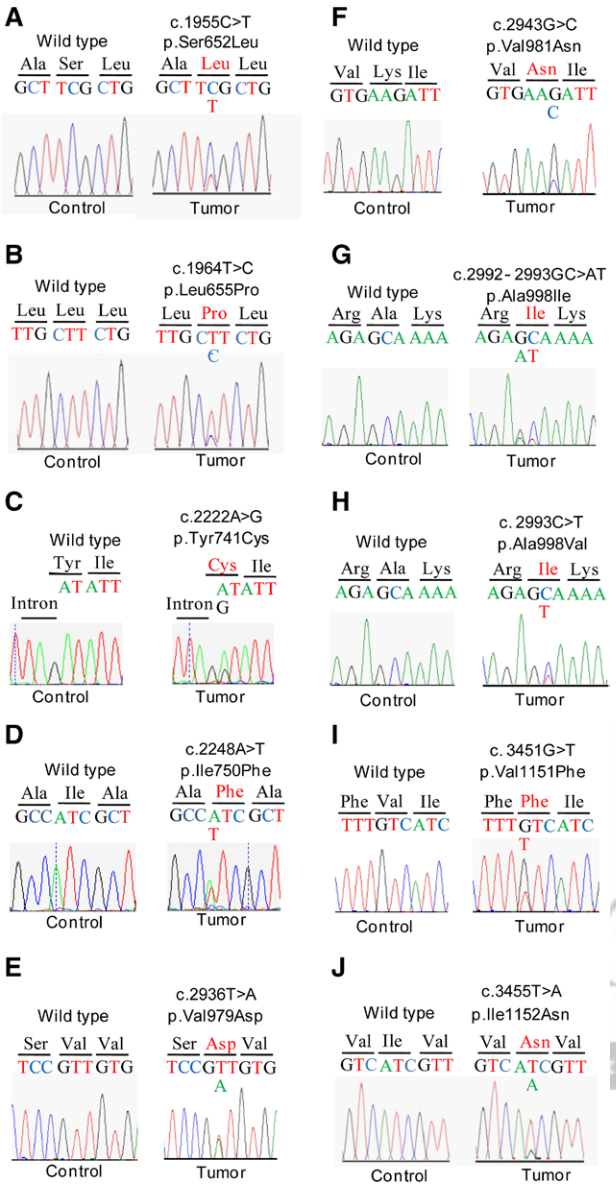


Figure 1. New mutations identified in *CACNA1D*. Sanger sequencing chromatograms of new mutations found in *CACNA1D* (NM_001128839.2). **A**, pSer652Leu. **B**, P.Leu655Pro. **C**, p.Tyr741Cys. **D**, p.Ile750Phe. **E**, p.Val979Asp. **F**, p.Val981Asn. **G**, p.Ala998Ile. **H**, p.Ala998Val. **I**, p.Val1151Phe. **J**, p.Ile1152Asn.

(*P*=0.001 with the Mantel–Haenszel test controlling for center). Median age at diagnosis was lower in patients with *KCNJ5* mutations compared with those with no mutation in the Munich cohort (47 versus 58 years; *P*<0.001) but not in the Torino cohort (50 versus 51 years; *P*=0.50), resulting in a nonsignificant overall difference (*P*=0.61 with stratified linear regression controlling for center). Median minimal plasma potassium was similar in patients with *KCNJ5* mutations or no mutation in both cohorts from Munich (2.8 versus 2.9 mmol/L; *P*=0.08) and Torino (3.0 versus 3.0 mmol/l; *P*=0.40), resulting in a nonsignificant overall difference (*P*=0.08). There was no difference in median adenoma size between patients with *CACNAD1* mutations or *KCNJ5* mutations or no mutation in the 2 replication cohorts and overall.

Discussion

Here, we report the prevalence of recurrent somatic mutations in *KCNJ5, ATP1A1, ATP2B3*, and *CACNA1D* in a large cohort of 474 patients recruited through ENS@T and the analysis of association of the mutation status with clinical and biological parameters of the disease. *CACNA1D* mutations were the second most prevalent in 474 APAs enrolled in this study with a frequency of 13.6% in the Paris cohort. This is much higher than the previously described prevalence of *CACNA1D* in 5% to 7.8% of APAs.[14,15] *CACNA1D* codes for the pore-forming $Ca_v1.3$ subunit of the L-type voltage-gated calcium channel. $Ca_v1.3$ contains 4 repeated domains (I–IV), each with 6 transmembrane segments (S1–S6) and a membrane-associated loop between S5 and S6. S5, S6, and the interposed loop line the channel pore.[23] In previous studies, *CACNA1D* mutations were identified in genomic regions encoding transmembrane S6 segments (p.Gly403Arg, p.Ile750Met, p.Phe747Leu) known to form the channel activation gate, or in regions coding for the transmembrane S4 segment involved in voltage sensing (p.Arg990His) and the cytoplasmic S4–S5 linker coupling the voltage-sensing domain to the pore (p.Pro1336Arg, p.259Asp).[14,15] We identified 10 new *CACNA1D* mutations, including mutations in exons different from those described in previous studies. These mutations are located in regions encoding transmembrane segments S4, S5, and S6 and cytoplasmic segments S4 and S5. These regions are predicted to be involved in voltage sensing and may increase the intracellular concentration of Ca^{2+}.[23] Although in vitro studies would be necessary to define the exact consequences of mutations located in this region on channel function, they are likely to alter channel function in a manner similar to that described previously.[23] Importantly, the identification of new mutations in different regions of $Ca_v1.3$ implies the necessity of a large genotyping of *CACNA1D* in APAs, including exons 14 and 27.

Although there was no statistical difference in the distribution of the mutations across the different centers participating in this study, *CACNA1D* mutations were highest in the Paris cohort, whereas *ATP2B3* mutations were highest in the Munich cohort. These differences may depend on the different characteristics of the populations investigated in these studies, in particular with regard to sex, age at diagnosis of PA, severity of the disease, in terms of aldosterone production and blood pressure levels, and size of adenoma. Given the large retrospective nature of the Paris cohort including samples diagnosed before introduction of systematic AVS for diagnostic workup, we were able to further explore the occurrence of somatic mutations depending on the diagnostic procedure for subtype identification. The distribution of mutations was significantly different depending on whether AVS or computed tomography was used (*P*=0.002; Table S7), suggesting that smaller adenomas harboring a *CACNA1D* mutation may be missed at diagnosis in the absence of AVS. Remarkably, mutations in *ATP2B3* were not identified in 2 recent studies investigating somatic mutations in APA by whole exome sequencing,[14,15] indicating that these mutations may be specifically associated with a subtype of APA. Because of the small number of patients with ATPase mutations in each single

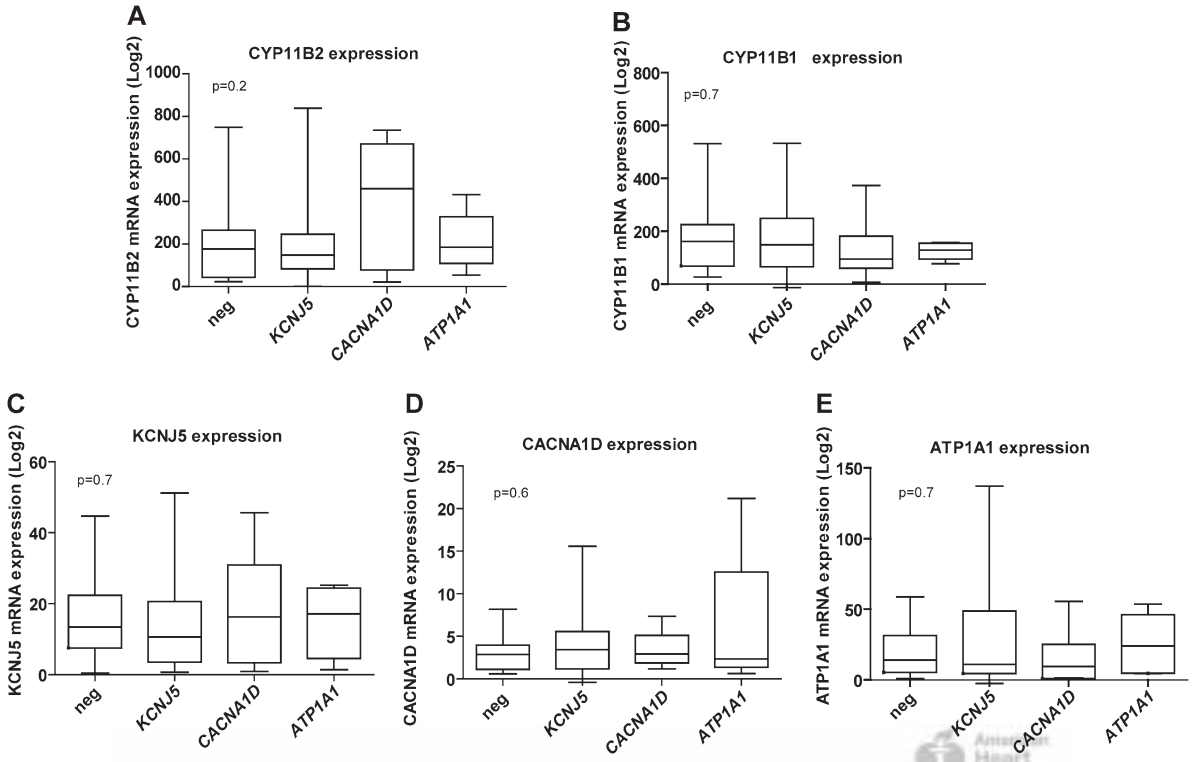


Figure 2. Gene expression as a function of the mutation status. **A**, *CYP11B2* mRNA expression. **B**, CYP11B1 mRNA expression. **C**, *KCNJ5* mRNA expression. **D**, *CACNA1D* mRNA expression. **E**, *ATP1A1* mRNA expression. Aldosterone-producing adenomas carrying *ATP2B3* mutations were not included in statistical comparisons because of small sample sizes.

cohort, analysis of clinical and biological correlates could not be performed in our study design.

APAs present a marked histological heterogeneity, being composed of cells with characteristics of different zones of the adrenal cortex.[24,25] Based on these observations, some authors have proposed that there may be more than 1 type of APA and that their cellular origins might be different. Some studies have identified a higher proportion of ZF-like cells in APA harboring *KCNJ5* mutations and higher proportion of ZG-like cells in APA harboring *CACNA1D* and *ATP1A1* mutations.[14,25] In our study, histological analysis of 78 APAs did not reveal any difference in the proportion of ZF or ZG-like cells across groups with different mutation status, the majority of APAs being mainly composed of ZF-like cells. Heterogeneity between samples from different centers may explain absent replication of previously described correlations between mutation status and morphological characteristics of the APA.[14,25] Although *CYP11B2* expression seemed somewhat lower in APA carrying *KCNJ5* mutations compared with *CACNA1D* mutations, similar to previous reports,[17,25] it was not statistically different across mutation groups when taking into account nonmutated tumor samples and independent of the cellular phenotype (Figure S3A and S3B). This supports previous data by our group showing that despite their ZF-like cellular phenotype, APAs are composed of cells expressing ZG markers such as *CYP11B2*, Dab2, and CD56.[26]

In this study, we have explored the clinical and biological correlates of somatic mutations in APA. Given the large individual sample sizes available from 3 different centers, we have performed an exploratory study in the Paris cohort followed by replication analyses in the Torino and Munich cohorts. This approach was chosen to obtain strong correlates that were independent of recruitment biases relative to single centers. In the Paris cohort, we confirmed the previously described association of *KCNJ5* mutations with female sex and a younger age of diagnosis of PA.[11] *KCNJ5* mutations were also associated with higher minimal recorded potassium. These results are slightly different from our previous observations in a group of 309 patients, in which *KCNJ5* mutations were associated with higher preoperative plasma aldosterone levels in a grouped analysis including all centers.[11] Because the centers participating in the 2 studies are not completely overlapping, different results may reflect a strong individual contribution by 1 single center to the associations reported previously. In addition, the group without mutations analyzed previously included patients carrying *CACNA1D* and *ATPase* mutations, which were unknown at that time.

In the Paris cohort, adenoma size was associated to the mutation status. APAs with *CACNA1D* mutations were smaller than APAs without mutation or with *KCNJ5* mutations. These observations partially confirm results described previously that compared *KCNJ5*-mutant APAs versus *CACNA1D*- and *ATP1A1*-mutant APAs,[14] in which APAs with *CACNA1D* mutations or *ATP1A1* mutations were smaller than APAs with *KCNJ5* mutations.

The associations observed in the Paris cohort were partially replicated for age and sex, but not for minimal recorded potassium concentration, systolic blood pressure, or adenoma size. Again, these results likely reflect the different population characteristics observed among the 3 largest cohorts. While

patients from the Munich cohort are more frequently males, older, and with lower minimal recorded potassium, patients from the Torino cohort present higher preoperative blood pressure, plasma aldosterone and aldosterone-to-renin ratio, and larger adenoma (Table S1). Patients from the Paris cohort are significantly younger than the patients from the other 2 cohorts. Although guidelines for the management of PA have standardized screening procedures and treatment among specialized centers,[22] some patients analyzed in the present study were diagnosed before the publication and implementation of the guidelines in each center. In particular, AVS, which represents the gold standard method for subtype identification in PA, has been implemented only recently for the systematic screening of patients with PA in all centers. On the contrary, differences in the type of reference center recruiting the patients (hypertension versus endocrinology) and the criteria for patients' referral (hypokalemia or resistant hypertension) may explain the differences in the population structure, frequency of mutations, and clinical correlations observed among centers.

Perspectives

KCNJ5, *CACNA1D*, *ATP1A1*, and *ATP2B3* mutations were identified in >50% of APAs in the ENS@T cohort. The mutation status of APA was associated to specific clinical and biochemical characteristics of PA, but this association was largely dependent on the population structure. There was marked variability in clinical and biochemical characteristics of patients with PA across centers, possibly reflecting different referral procedures, ethnic background, and the retrospective study design. Nevertheless, young women are more likely to be *KCNJ5* mutation carriers. It will be particularly relevant in the future to identify additional features or surrogate biomarkers associated with the mutation status for assessing the usefulness of stratifying patients for targeted treatment before surgery involving molecules such as verapamil specifically blocking mutated *KCNJ5* potassium channels[27] or L-type calcium channel blockers specifically inhibiting $Ca_v1.3$ in patients carrying *CACNA1D* mutations.[15]

Acknowledgments

We thank Pierre-François Plouin (Hypertension Unit, Hôpital Européen Georges-Pompidou) and the COMETE (COrtico and MEdullary adrenal Tumors) network, Paris, France, for providing tissue samples from APA. We thank Brigitte Mauracher for excellent technical assistance and Susanne Schmid, Friederike Konrad and Nina Nirschl, Munich, Germany, for clinical characterization of the patients.

Sources of Funding

This work was funded through institutional support from INSERM and by the Agence Nationale pour la Recherche (ANR Physio 2007, No. 013-01; Genopat 2008, No. 08-GENO-021), the Fondation pour la Recherche sur l'Hypertension Artérielle (Appel d'offre 2007), the Fondation pour la Recherche Médicale (ING20101221177), the Programme Hospitalier de Recherche Clinique (PHRC grant AOM 06179), and by grants from INSERM and Ministère Délégué à la Recherche et des Nouvelles Technologies. F.L. Fernandes-Rosa was supported by fellowship from Fondation Lefoulon-Delalande. The work was also funded by grants from the Italian Ministry of University, Scientific and Technological Research (COFIN No. 2006060985), Compagnia di San Paolo, and Fondi Regione Piemonte Ricerca Finalizzata 2008 I and II. The German Conn's Registry-Else-Kröner Hyperaldosteronism Registry is supported by a grant of the Else Kröner-Fresenius Stiftung to M. Reincke, who is also supported by the Deutsche Forschungsgemeinschaft (Re 752/20–1). The ENS@T registry is supported by a grant of the European Science Foundation (ESF-ENS@T) and has received funding from the Seventh Framework Programme (FP7/2007–2013) under grant agreement n° 259735.

Disclosures

None.

References

1. Rossi GP, Bernini G, Caliumi C, et al; PAPY Study Investigators. A prospective study of the prevalence of primary aldosteronism in 1,125 hypertensive patients. *J Am Coll Cardiol*. 2006;48:2293–2300.
2. Hannemann A, Bidlingmaier M, Friedrich N, et al. Screening for primary aldosteronism in hypertensive subjects: results from two German epidemiological studies. *Eur J Endocrinol*. 2012;167:7–15.
3. Mulatero P, Stowasser M, Loh KC, Fardella CE, Gordon RD, Mosso L, Gomez-Sanchez CE, Veglio F, Young WF Jr. Increased diagnosis of primary aldosteronism, including surgically correctable forms, in centers from five continents. *J Clin Endocrinol Metab*. 2004;89:1045–1050.
4. Pitt B, Remme W, Zannad F, Neaton J, Martinez F, Roniker B, Bittman R, Hurley S, Kleiman J, Gatlin M; Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study Investigators. Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. *N Engl J Med*. 2003;348:1309–1321.
5. Zannad F, McMurray JJ, Krum H, van Veldhuisen DJ, Swedberg K, Shi H, Vincent J, Pocock SJ, Pitt B; EMPHASIS-HF Study Group. Eplerenone in patients with systolic heart failure and mild symptoms. *N Engl J Med*. 2011;364:11–21.
6. Savard S, Amar L, Plouin PF, Steichen O. Cardiovascular complications associated with primary aldosteronism: a controlled cross-sectional study. *Hypertension*. 2013;62:331–336.
7. Mulatero P, Monticone S, Bertello C, Viola A, Tizzani D, Iannaccone A, Crudo V, Burrello J, Milan A, Rabbia F, Veglio F. Long-term cardio- and cerebrovascular events in patients with primary aldosteronism. *J Clin Endocrinol Metab*. 2013;98:4826–4833.
8. Amar L, Plouin PF, Steichen O. Aldosterone-producing adenoma and other surgically correctable forms of primary aldosteronism. *Orphanet J Rare Dis*. 2010;5:9.
9. Bayliss DA, Barrett PQ. Emerging roles for two-pore-domain potassium channels and their potential therapeutic impact. *Trends Pharmacol Sci*. 2008;29:566–575.
10. Choi M, Scholl UI, Yue P, et al. K+ channel mutations in adrenal aldosterone-producing adenomas and hereditary hypertension. *Science*. 2011;331:768–772.
11. Boulkroun S, Beuschlein F, Rossi GP, et al. Prevalence, clinical, and molecular correlates of KCNJ5 mutations in primary aldosteronism. *Hypertension*. 2012;59:592–598.
12. Beuschlein F, Boulkroun S, Osswald A, et al. Somatic mutations in ATP1A1 and ATP2B3 lead to aldosterone-producing adenomas and secondary hypertension. *Nat Genet*. 2013;45:440–444, 444e1.
13. Oki K, Plonczynski MW, Luis Lam M, Gomez-Sanchez EP, Gomez-Sanchez CE. Potassium channel mutant KCNJ5 T158A expression in HAC-15 cells increases aldosterone synthesis. *Endocrinology*. 2012;153:1774–1782.
14. Azizan EA, Poulsen H, Tuluc P, et al. Somatic mutations in ATP1A1 and CACNA1D underlie a common subtype of adrenal hypertension. *Nat Genet*. 2013;45:1055–1060.
15. Scholl UI, Goh G, Stölting G, et al. Somatic and germline CACNA1D calcium channel mutations in aldosterone-producing adenomas and primary aldosteronism. *Nat Genet*. 2013;45:1050–1054.
16. Küpers EM, Amar L, Raynaud A, Plouin PF, Steichen O. A clinical prediction score to diagnose unilateral primary aldosteronism. *J Clin Endocrinol Metab*. 2012;97:3530–3537.
17. Williams TA, Monticone S, Schack VR, et al. Somatic ATP1A1, ATP2B3, and KCNJ5 mutations in aldosterone-producing adenomas. *Hypertension*. 2014;63:188–195.
18. Letavernier E, Peyrard S, Amar L, Zinzindohoué F, Fiquet B, Plouin PF. Blood pressure outcome of adrenalectomy in patients with primary hyperaldosteronism with or without unilateral adenoma. *J Hypertens*. 2008;26:1816–1823.

19. Reincke M, Rump LC, Quinkler M, Hahner S, Diederich S, Lorenz R, Seufert J, Schirpenbach C, Beuschlein F, Bidlingmaier M, Meisinger C, Holle R, Endres S; Participants of German Conn's Registry. Risk factors associated with a low glomerular filtration rate in primary aldosteronism. *J Clin Endocrinol Metab*. 2009;94:869–875.
20. Mulatero P, Monticone S, Veglio F. Diagnosis and treatment of primary aldosteronism. *Rev Endocr Metab Disord*. 2011;12:3–9.
21. Rossi GP. Diagnosis and treatment of primary aldosteronism. *Endocrinol Metab Clin North Am*. 2011;40:313–332, vii.
22. Funder JW, Carey RM, Fardella C, Gomez-Sanchez CE, Mantero F, Stowasser M, Young WF Jr, Montori VM; Endocrine Society. Case detection, diagnosis, and treatment of patients with primary aldosteronism: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab*. 2008;93:3266–3281.
23. Catterall WA. Signaling complexes of voltage-gated sodium and calcium channels. *Neurosci Lett*. 2010;486:107–116.
24. Neville AM, O'Hare MJ. Histopathology of the human adrenal cortex. *Clin Endocrinol Metab*. 1985;14:791–820.
25. Azizan EA, Lam BY, Newhouse SJ, Zhou J, Kuc RE, Clarke J, Happerfield L, Marker A, Hoffman GJ, Brown MJ. Microarray, qPCR, and KCNJ5 sequencing of aldosterone-producing adenomas reveal differences in genotype and phenotype between zona glomerulosa- and zona fasciculata-like tumors. *J Clin Endocrinol Metab*. 2012;97:E819–E829.
26. Boulkroun S, Samson-Couterie B, Golib-Dzib JF, Amar L, Plouin PF, Sibony M, Lefebvre H, Louiset E, Jeunemaitre X, Meatchi T, Benecke A, Lalli E, Zennaro MC. Aldosterone-producing adenoma formation in the adrenal cortex involves expression of stem/progenitor cell markers. *Endocrinology*. 2011;152:4753–4763.
27. Tauber P, Penton D, Stindl J, Humberg E, Tegtmeier I, Sterner C, Beuschlein F, Reincke M, Barhanin J, Bandulik S, Warth R. Pharmacology and pathophysiology of mutated KCNJ5 found in adrenal aldosterone-producing adenomas. *Endocrinology*. 2014;155:1353–1362.

Novelty and Significance

What Is New?

- Recurrent somatic mutations in *KCNJ5*, *ATP1A1*, *ATP2B3*, and *CACNA1D* were identified in 54% of 474 patients recruited through reference centers organized within the European Network for the Study of Adrenal Tumors (ENS@T).
- Heterozygous *KCNJ5* mutations were present in 38% of aldosterone-producing adenomas (APAs), whereas *ATP1A1* mutations were found in 5.3% and *ATP2B3* mutations in 1.7% of APAs. *CACNA1D* mutations were identified in 9.3% of APAs.
- Ten novel *CACNA1D* mutations were identified.

What Is Relevant?

- Patients with *KCNJ5* mutations were more frequently female, diagnosed younger, and with higher minimal plasma potassium concentrations compared with *CACNA1D* mutation carriers or noncarriers.
- *CACNA1D* mutations were associated with smaller adenomas.
- Associations of the mutation status with clinical and biological parameters were largely dependent on the population structure of the different centers.
- The identification of new mutations in different regions of $Ca_v1.3$ implies the necessity of a large genotyping of *CACNA1D* in APAs.

Summary

Recurrent somatic mutations are present in more than half of APAs in a large European cohort. Young women with APA are more likely to be *KCNJ5* mutation carriers. Identification of specific characteristics or surrogate biomarkers of mutation status may lead to targeted treatment options before surgery.

Online Supplements

GENETIC SPECTRUM AND CLINICAL CORRELATES OF SOMATIC MUTATIONS IN ALDOSTERONE-PRODUCING ADENOMA

Fabio Luiz Fernandes-Rosa[1,2,3]*; Tracy Ann Williams[4]*, Anna Riester[5]*, Olivier Steichen[6], Felix Beuschlein[5], Sheerazed Boulkroun[1,2], Tim M Strom[7,8], Silvia Monticone[4], Laurence Amar[1,2,9], Tchao Meatchi[1,2,10], Franco Mantero[11], Maria-Verena Cicala[11], Marcus Quinkler[12], Francesco Fallo[13], Bruno Allolio[14], Giampaolo Bernini[15], Mauro Maccario[16], Gilberta Giacchetti[17], Xavier Jeunemaitre[1,2,3], Paolo Mulatero[4^], Martin Reincke[5^], Maria-Christina Zennaro[1,2,3]

Affiliations:
[1]INSERM, UMRS_970, Paris Cardiovascular Research Center, Paris, France
[2]University Paris Descartes, Sorbonne Paris Cité, Paris, France
[3]Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Service de Génétique, Paris, France
[4]Division of Internal Medicine and Hypertension, Department of Medical Sciences, University of Torino, Torino, Italy
[5]Medizinische Klinik und Poliklinik IV, Ludwig-Maximilians-University, 80336 Munich, Germany
[6]Sorbonne Universités, UPMC Univ Paris 06, Faculty of Medicine, F-75006, Paris, France
[7]Institute of Human Genetics, Helmholtz Zentrum München, Neuherberg, Germany
[8]Institute of Human Genetics, Technische Universität München, Munich, Germany
[9]Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Unité Hypertension artérielle, Paris, France
[10]Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Service d'Anatomie Pathologique, Paris, France
[11]Endocrine Unit, Department of Medicine,University of Padua,Italy
[12]Clinical Endocrinology, Campus Mitte, University Hospital Charité, Berlin, Germany
[13]Department of Medicine, University of Padova, 35128 Padova, Italy
[14]Department of Medicine I, Endocrine and Diabetes Unit, University Hospital Würzburg, 97080 Würzburg, Germany
[15]Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy
[16]Division of Endocrinology, Diabetes, and Metabolism, Department of Medical Sciences, University of Torino, Torino, Italy
[17]Division of Endocrinology, Azienda Ospedaliero-Universitaria Ospedali Riuniti UmbertoI-GM Lancisi-G Salesi, Università Politecnica delle Marche, Ancona, Italy
*These authors contributed equally to this work
^These authors contributed equally to this work

Address correspondence to:
Maria-Christina Zennaro, MD, PhD
INSERM, U970
Paris Cardiovascular Research Center – PARCC
56, rue Leblanc,
75015 Paris – France
Tel : +33 (0)1 53 98 80 42
Fax : + 33 (0)1 53 98 79 52
e-mail : maria-christina.zennaro@inserm.fr

Subjects and Methods

Patients

The diagnosis of adrenocortical adenoma was histologically confirmed after surgical resection. A final diagnosis of APA, diagnosed by CT scanning and AVS, was considered "proven" when the following conditions were satisfied: 1) histological demonstration of adenoma; 2) normalization of hypokalemia, if present; 3) cure or improvement of hypertension; 4) normalization of ARR and/or suppressibility of aldosterone under saline load.[1,2] All patients gave written informed consent for genetic and clinical investigation within each individual institution. Procedures were in accordance with institutional guidelines.

For part of the patients included in this study, the genetic screening of mutations in *KCNJ5*, *ATP1A1* and *ATP2B3* was previously described. In particular, sequences of those genes were already reported for all patients from the Torino, Ancona, Padova B, Pisa, Würzburg and Berlin cohorts.[3-5] For the Paris cohort, 134 patients have been previously published [3,4] and 65 new patients were included in this study. 21 new patients were included in the Munich cohort, while 72 patients have previously been investigated for mutation in the *KCNJ5*, *ATP1A1* and *ATP2B3* genes.[3,4] None of the 474 patients has previously been screened for *CACNA1D* mutations.

Genotype-phenotype correlations were performed in patients from three centers, Paris, Munich and Torino. In Paris and Munich, plasma active renin was determined by chemiluminescent immunoassay (LIAISON; Diasorin, Saluggia, Italy). Plasma and urinary aldosterone concentrations were determined by RIA (Coat-A-Count; Siemens Medical Solutions Diagnostics, Erlangen, Germany). For the Torino cohort, PRA and aldosterone were measured by RIA using commercially available kits from DIASORIN (Saluggia, Italy). The cutoff values for the ARR were 64 pmol/mU for the Paris cohort, 12 ng/mU (33pmol/mU) for the Munich cohort and 40 ($ng/dl \cdot ng*ml^{-1}/h^{-1}$) (135 pmol/mU) for the Torino cohort. All three centers use a cutoff of 4 for the lateralization index.

DNA isolation and sequencing

Tumor DNA was extracted using QIAamp DNA midi kit (Qiagen, Courtaboeuf Cedex, France), or Maxwell 16 LEV Blood DNA Kit (Promega, Madison, WI, US); DNA from peripheral blood leukocytes was prepared using salt-extraction. Whole exome sequencing was performed with DNA of 25 APAs and of matched blood leukocytes as described in Beuschlein et al.[4] *KCNJ5*, *ATP1A1*, and *ATP2B3* DNA were amplified using intron-spanning primers as reported previously.[4,6] *CACNA1D* DNA was amplified using the intron-spanning primers described in supplemental table S2. PCR was performed on 100 ng of DNA (4 µl of RT product) in a final volume of 25 µl containing 0.75 mM $MgCl_2$, 400 nM of each primer, 200 µM deoxynucleotide triphosphate and 1.25 U Platinum Taq DNA Polymerase (Invitrogen). Cycling conditions were as previously described with an annealing temperature of 58°C for *KCNJ5*, *ATP1A1* and *ATP2B3*, and 60°C for *CACNA1D*. Direct sequencing of PCR products was performed using the ABI Prism Big Dye Terminator® v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI Prism 3700 DNA Analyzer (Applied Biosystems).

Pathological analysis

Histological annotations were performed on APA and peritumoral adjacent tissue. Cellular composition of APA was determined by Haematoxylin-Eosin-Safran (HES) staining.

Cellular composition was determined by examining for known features of zona fasciculata (ZF) cells, in particular a large vacuolar cytoplasm with a central regular nucleus. All microscopic examinations were performed with a Leica microscope.

References

1. Mulatero P, Bertello C, Verhovez A, Rossato D, Giraudo G, Mengozzi G, Limerutti G, Avenatti E, Tizzani D, Veglio F. Differential diagnosis of primary aldosteronism subtypes. *Curr Hypertens Rep*. 2009;11:217-223.
2. Rossi GP, Bernini G, Caliumi C, Desideri G, Fabris B, Ferri C, Ganzaroli C, Giacchetti G, Letizia C, Maccario M, Mallamaci F, Mannelli M, Mattarello MJ, Moretti A, Palumbo G, Parenti G, Porteri E, Semplicini A, Rizzoni D, Rossi E, Boscaro M, Pessina AC, Mantero F. A prospective study of the prevalence of primary aldosteronism in 1,125 hypertensive patients. *J Am Coll Cardiol*. 2006;48:2293-2300.
3. Boulkroun S, Beuschlein F, Rossi GP, Golib-Dzib JF, Fischer E, Amar L, Mulatero P, Samson-Couterie B, Hahner S, Quinkler M, Fallo F, Letizia C, Allolio B, Ceolotto G, Cicala MV, Lang K, Lefebvre H, Lenzini L, Maniero C, Monticone S, Perrocheau M, Pilon C, Plouin PF, Rayes N, Seccia TM, Veglio F, Williams TA, Zinnamosca L, Mantero F, Benecke A, Jeunemaitre X, Reincke M, Zennaro MC. Prevalence, Clinical, and Molecular Correlates of KCNJ5 Mutations in Primary Aldosteronism. *Hypertension*. 2012;59:592-598.
4. Beuschlein F, Boulkroun S, Osswald A, Wieland T, Nielsen HN, Lichtenauer UD, Penton D, Schack VR, Amar L, Fischer E, Walther A, Tauber P, Schwarzmayr T, Diener S, Graf E, Allolio B, Samson-Couterie B, Benecke A, Quinkler M, Fallo F, Plouin PF, Mantero F, Meitinger T, Mulatero P, Jeunemaitre X, Warth R, Vilsen B, Zennaro MC, Strom TM, Reincke M. Somatic mutations in ATP1A1 and ATP2B3 lead to aldosterone-producing adenomas and secondary hypertension. *Nat Genet*. 2013;45(4):440-444.
5. Williams TA, Monticone S, Schack VR, Stindl J, Burrello J, Buffolo F, Annaratone L, Castellano I, Beuschlein F, Reincke M, Lucatello B, Ronconi V, Fallo F, Bernini G, Maccario M, Giacchetti G, Veglio F, Warth R, Vilsen B, Mulatero P. Somatic ATP1A1, ATP2B3, and KCNJ5 Mutations in Aldosterone-Producing Adenomas. *Hypertension*. 2014;63:188-195.
6. Choi M, Scholl UI, Yue P, Bjorklund P, Zhao B, Nelson-Williams C, Ji W, Cho Y, Patel A, Men CJ, Lolis E, Wisgerhof MV, Geller DS, Mane S, Hellman P, Westin G, Akerstrom G, Wang W, Carling T, Lifton RP. K+ channel mutations in adrenal aldosterone-producing adenomas and hereditary hypertension. *Science*. 2011;331:768-772.

Table S1. Baseline phenotypes of included patients in the three largest centres.

Clinical and biological parameters	Paris (n=205)		Munich (n=93)		Torino (n=73)		p value
	N	Value	N	Value	N	Value	
Female sex	205	107 (52%)	93	35 (38%)	73	41 (56%)	0.03
Age at PA diagnosis (ys)	205	44 (37, 50)	92	54 (44, 60)	73	50 (44, 55)	< 0.001
Lowest plasma K (mmol/l)	203	3.1 (2.8, 3.4)	89	2.8 (2.5, 3.1)	73	3.0 (2.5, 3.3)	< 0.001
Systolic BP (mmHg)	200	145 (133, 157)	90	146 (137, 158)	73	170 (155, 180)	< 0.001
Diastolic BP (mmHg)	200	91 (83, 97)	90	91 (86, 99)	73	100 (100, 115)	< 0.001
Treatment score (n)	199	2 (1, 3)	62	2.5 (2, 3)	72	3 (2, 3)	0.02
Plasma aldosterone (pmol/l)	191	820 (553, 1176)	91	676 (529, 1110)	73	1321 (942, 1800)	< 0.001
Plasma renin (mU/l)	191	1.7 (1.0, 3.6)	91	3.3 (2.0, 9.7)	73	1.6 (1.6, 2.5)	< 0.001
ARR (pmol/mU)	191	155 (105, 217)	91	114 (56, 189)	73	264 (188, 360)	< 0.001
Largest adenoma (mm)	205	15 (10, 19)	93	15 (10, 20)	73	15 (10, 20)	0.42

No: number of observations; PA: primary aldosteronism; K: potassium; BP: blood pressure; ARR: aldosterone to renin ratio. Categorical data reported as number (percentage) and compared with Fischer's exact test; quantitative data reported as median (interquartile range) and compared with the Kruskal-Wallis test. Conversion factor used for plasma aldosterone: 1 ng/l = 2.77 pmol/l ; conversion factor used for plasma renine: 1 ng/ml/h = 8.2 mU/l.

Table S2. Primers used for *CACNA1D* sequencing

CACNA1D		
Exon 6*	**Foward**	GTAAAGGAGGCATGGTTAGG
	Reverse	TGGCTCAGTAAATGTGCTGGT
Exon 8A*	**Foward**	GCCTTGATGACTCTGTGTG
	Reverse	CCAGCAAAGCTTGTGTGGT
	Forward‡	TTGAATTGCCCTGGGTGTAT
	Reverse‡	AATGTCTGGCAACCCCTCTT
Exon 8B†	**Foward**	AGCTGCAACTGGGGCTC
	Reverse	GCAGCTAGGAGACACGCAG
Exon 14*	**Foward**	GTCCTGCATGGGTGTTCTGA
	Reverse	ACGAAGTGCTTTTCGGGGAA
Exon 16*	**Foward**	TAACACTTGGGACGGTCAC
	Reverse	CCATGATCCACAAAGCAGC
Exon 23*	**Foward**	CACGCTAACTGTGCAGGGA
	Reverse	TCAGCTCTGCCCAGAAGAG
Exon 27*	**Foward**	CCAATCTACAACCACCGCGT
	Reverse	GACCAAGGGACAGAAGCCAA
Exon 32*	**Foward**	ACGGTTCTTCCTCACTGTCG
	Reverse	CTTCAGCAGAGGCATTTGGCT

* NM_001128839.2; † NM_000720.3, ‡ used in Munich and Padova A

Table S3. Distribution of Somatic *KCNJ5* mutations across different centers

***KCNJ5* mutation**	**Paris**	**Munich**	**Torino**	**Padova A**	**Berlin**	**Ancona**	**Padova B**	**Würzburg**	**Pisa**	**Total**
p.Trp126Arg	0	0	1	0	0	0	0	0	0	1
p.Gly151Arg	46	18	19	8	5	9	3	3	2	113
p.Thr158Ala	0	0	1	0	0	0	0	0	0	1
p.Leu168Arg	28	14	8	6	6	1	0	1	1	65

Table S4. Distribution of somatic CACNA1D mutations across different centers

CACNA1D mutation	Paris	Munich	Torino	Padova A	Berlin	Ancona	Padova B	Würzburg	Pisa	Total
p.Val259Asp*	1	0	0	0	0	0	0	0	0	1
p.Gly403Arg*	5	2	0	0	0	0	0	1	0	8
p.Gly403Arg (8B)†	2	1	0	0	0	0	0	0	0	3
p.Ser652Leu*	0	0	0	1	0	0	0	0	0	1
p.Leu655Pro*	0	1	0	0	0	0	0	0	0	1
p.Tyr741Cys*	0	0	1	0	0	0	0	0	0	1
p.Phe747Val*	0	1	0	0	0	0	0	0	0	1
p.Phe747Leu*	3	0	0	0	1	2	0	0	0	6
p.Ile750Met*	3	0	1	0	0	0	0	0	0	4
p.Ile750Phe*	0	0	1	0	0	0	0	0	0	1
p.Val979Asp*	1	0	0	0	0	0	0	0	0	1
p.Lys981Asn*	1	0	0	0	0	0	0	0	0	1
p.Ala998Ile*	3	0	0	0	0	0	0	0	0	3
p.Ala998Val*	1	0	0	0	0	0	0	0	0	1
p.Val1151Phe*	1	1	0	0	0	0	0	0	0	3
p.Ile1152Asn*	0	1	0	0	0	0	0	0	0	0
p.Pro1336Arg*	1	0	0	0	0	0	0	0	0	1
p.Val1338Met*	5	0	0	1	0	0	0	0	0	6
p.Met1354Ile*	0	0	1	0	0	0	0	0	0	1

*NM_001128839.2; † NM_000720.3

Table S5. Distribution of somatic *ATP1A1* mutations across different centers

ATP1A1 mutation	Paris	Munich	Torino	Padova A	Berlin	Ancona	Padova B	Würzburg	Pisa	Total
p.Gly99Arg	0	0	1	0	0	0	0	0	0	1
p.Phe100_Leu104del	3	2	0	0	0	0	0	0	0	5
p.Leu104Arg	5	4	4	1	1	2	0	0	0	17
p.Val332Gly	1	1	0	0	0	0	0	0	0	2

Table S6. Distribution of somatic *ATP2B3* mutations across different centres

ATP2B3 mutation	Paris	Munich	Torino	Padova A	Berlin	Ancona	Padova B	Würzburg	Pisa	Total
p.Leu424_Val425del	2	0	0	0	0	0	0	0	0	2
p.Leu425_Val426del	1	3	1	0	0	0	0	0	0	5
p.Val426_Val427del	0	1	0	0	0	0	0	0	0	1

Table S7. Distribution of somatic mutations in the Paris cohort depending on the diagnostic procedure for subtype identification.

Gene	AVS (%)	CT (%)
KCNJ5	34 (28)	40 (53)
CACNA1D	23 (19)	4 (5)
ATP1A1	7 (6)	2 (3)
ATP2B3	2 (2)	1 (1)
Non mutated	57 (46)	29 (38)
Total	123 (100)	76 (100)

Fisher's exact test p=0.002

Figure S1. **New mutations identified in Cav1.3 (NP_001122311.1)**. Alignment and conservation of residues p.Ser652, p.Leu655, p.Tyr741, p.Ile750, p.Val979, p.Val981, p.Ala998, p.Val1551, and p.Ile1152 encoded by *CACNA1D* orthologs.

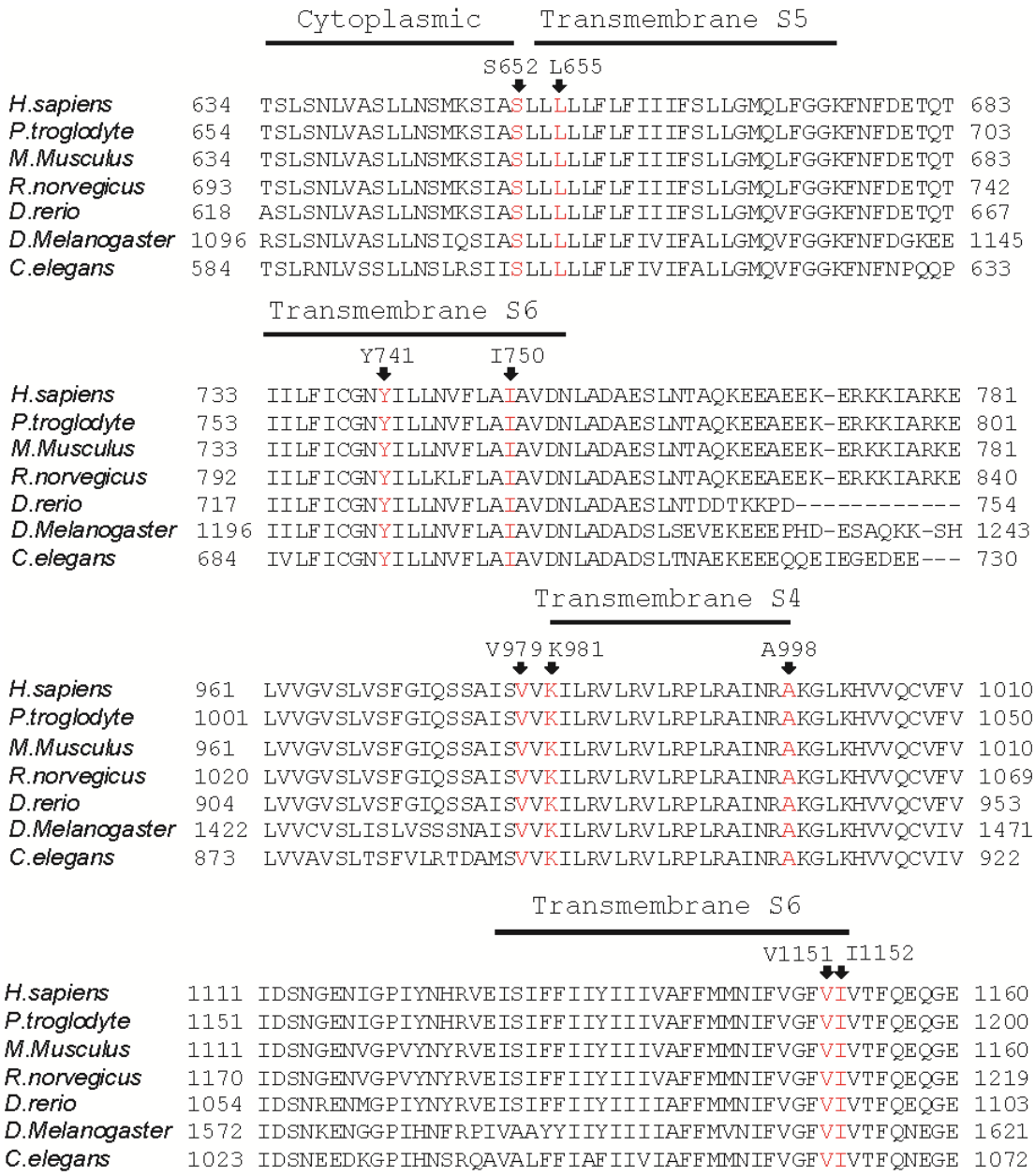


Figure S2. Cellular composition of APA. **A**. HES staining in APA with predominance of zona fasciculata-like cells composed by columns of large and clear cells with pale-staining nuclei. **B**. HES staining in APA with predominance of zona glomerulosa-like cells composed of small cells with darkly basophilic nuclei and pale clear cytoplasm. **C**. Analysis of the percentage of zona glomerulosa-like cells and zona fasciculata-like cells in 78 APAs. **D**. Proportion of zona glomerulosa-like cells among different mutation status. **E**. Proportion of zona fasciculata-like cells among different mutation status.

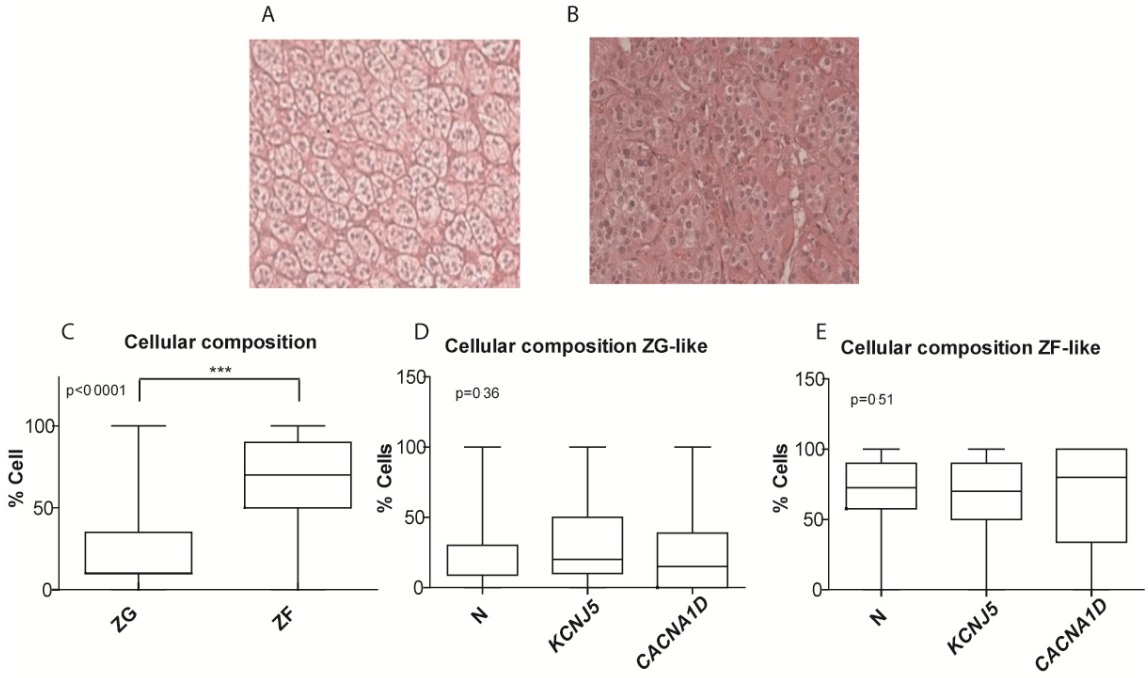
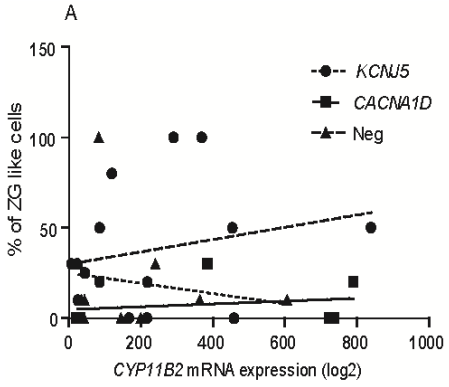
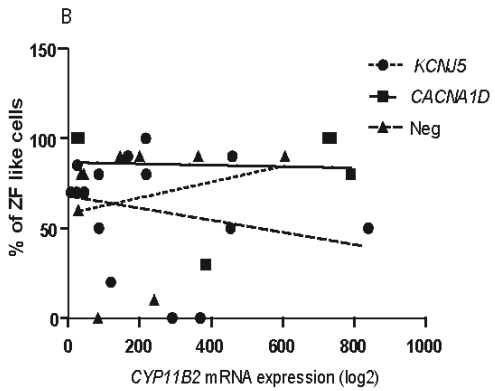


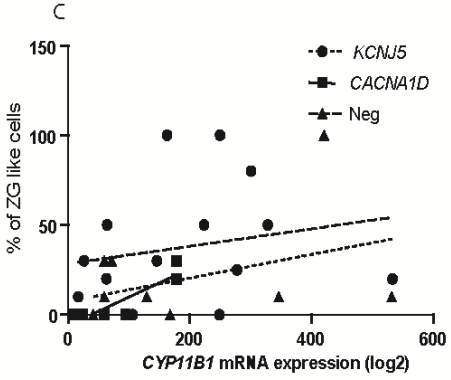
Figure S3. *CYP11B2* and *CYP11B1* expression as a function of the cellular composition of APA. A. Spearman correlation of *CYP11B2* mRNA expression and % of ZG-like cells. **B**. Spearman correlation of *CYP11B2* mRNA expression and % of ZF-like cells. **C**. Spearman correlation of *CYP11B1* mRNA expression and % of ZG-like cells. **D**. Spearman correlation of CYP11B1 mRNA expression and % of ZF-like cells. APA carrying *ATP1A1* and *ATP2B3* mutation were not included in statistical comparisons because of small sample sizes.



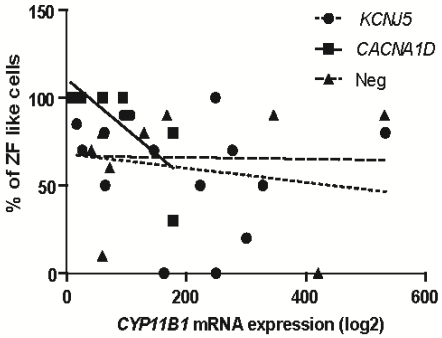
	n	r	p
KCNJ5	15	0.17	0.53
CACNA1D	6	0.3	0.5
Neg	10	-0.08	0.8



	n	r	p
KCNJ5	15	-0.19	0.5
CACNA1D	6	-0.3	0.5
Neg	10	0.5	0.15



	n	r	p
KCNJ5	15	0.31	0.25
CACNA1D	6	0.84	0.03
Neg	10	0.2	0.27



	n	r	p
KCNJ5	15	-0.3	0.2
CACNA1D	6	-0.84	0.03
Neg	10	0.34	0.32