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THE EXPANDING GENETIC HORIZON OF PRIMARY ALDOSTERONISM

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1 Abstract

2 Aldosterone is the main mineralocorticoid hormone in humans and plays a key role in maintaining 3 water and electrolyte homeostasis. Primary aldosteronism (PA), characterized by autonomous 4 aldosterone overproduction by the adrenal glands, affects 6% of the general hypertensive population 5 and can be either sporadic or familial. Aldosterone producing adenoma (APA) and bilateral adrenal 6 hyperplasia (BAH) are the two most frequent subtypes of sporadic PA, and 4 forms of familial 7 hyperaldosteronism (FH-I to FH-IV) have been identified. Over the last six years the introduction of 8 next-generation sequencing has significantly improved our understanding of the molecular 9 mechanisms responsible for autonomous aldosterone overproduction in both sporadic and familial 10 PA. Somatic mutations in four genes (KCNJ5, ATP1A1, ATP2B3 and CACNA1D), differently 11 implicated in intracellular ion homeostasis, have been identified in nearly 60% of the sporadic APAs. 12 Germline mutations in KCNJ5 and CACNA1H cause FH-III and FH-IV, respectively, while germline 13 mutations in CACNA1D cause the rare PASNA syndrome, featuring primary aldosteronism seizures 14 and neurological abnormalities. Further studies are warranted to identify the molecular mechanisms 15 underlying BAH and FH-II, the most common forms of sporadic and familial PA whose molecular 16 basis has yet to be uncovered.

17

18 Introduction

Aldosterone is the main mineralocorticoid hormone in humans and, under physiological conditions, its secretion is tightly regulated by angiotensin II, extracellular potassium and adrenocorticotrophin (ACTH) (1). Its principal site of action is the distal nephron, where it promotes sodium retention and potassium excretion, playing a key role in maintaining water and electrolyte homeostasis. The autonomous aldosterone overproduction by one or both adrenal glands is a clinical syndrome known as primary aldosteronism (PA), that can affect up to 6% of the general hypertensive population (2).

1 Its main clinical and biochemical features are hypertension, hypokalaemia and elevated aldosterone-2 plasma renin activity ratio (ARR); moreover, PA patients display an increased risk of cardiovascular 3 events and metabolic alterations compared to patients affected by essential hypertension and similar 4 risk profile (3). While the vast majority of affected patients displays a sporadic form, either due to 5 aldosterone producing adenoma (APA) or bilateral adrenal hyperplasia (BAH), 1-6% of cases carry 6 a familial disease (4). Subtype diagnosis is important because patients with an APA are biochemically 7 cured in 94% of cases with adrenalectomy (5) and many patients with familial disease can avoid 8 further diagnostic work-up including adrenal vein sampling (6). Four forms of familial 9 hyperaldosteronism (FH) have been reported so far (FH-I to FH-IV) (7), together with the PASNA 10 (PA, seizures, neurologic abnormalities) syndrome, which is a genetic disease, but not a familial form 11 of PA (8). Until recently, FH-I (or GRA, glucocorticoid remediable aldosteronism) was the only 12 subtype of PA whose genetic basis was clearly elucidated (9). Over the last few years, the 13 development and wide application of next generation sequencing (NGS) (7), together with the 14 development of monoclonal antibodies directed towards aldosterone synthase (CYP11B2) (10), 15 significantly contributed to our understanding of the molecular mechanisms underlying autonomous 16 aldosterone overproduction in both sporadic and familial PA. This review will provide an overview 17 of the most recent genetic acquisitions in the field of PA.

18

19 GENETICS OF FAMILIAL HYPERALDOSTERONISM

20 Familial hyperaldosteronism type I

FH-I or GRA (OMIM # 103900) is transmitted as an autosomal dominant disorder and it is the most
common form of monogenic arterial hypertension (11). This condition is known since 1966, when
Sutherland et al. reported on a father and son displaying the clinical features of PA (hypertension,
hypokalaemia and suppressed PRA) that could be relieved by the administration of the glucocorticoid

dexamethasone (12). Until 1990, less than 100 cases were described (Supplemental Table S1): since
the diagnosis was clinical (based on dexamethasone suppression of aldosterone overproduction), the
majority of the affected patients displayed a florid PA phenotype, with hypertension and
hypokalaemia.

5 The molecular basis of GRA was elucidated by Lifton et al. in 1992 (9) and resides in the chimeric 6 CYP11B1/CYP11B2 gene, resulting from a non-homologous crossing over on chromosome 8q24.3 7 between CYP11B1 gene encoding 11beta-hydroxylase and CYP11B2, encoding aldosterone synthase. 8 The result is a chimeric enzyme, that can synthesize aldosterone under ACTH control, since it 9 contains CYP11B1 regulatory sequences at 5' and coding sequences from CYP11B2 at the 3' 10 (Figure 1). Two residues (Gly288 and Ala320, Figure 1) are necessary to retain aldosterone synthase activity and therefore in all the chimeric genes causing FH-I reported so far, the 11 12 recombination break-point is comprised between CYP11B2 intron 2 and exon 4 (13). While 13 aldosterone synthase expression is limited to the outer zona glomerulosa, the chimeric enzyme is 14 expressed throughout the entire adrenal cortex. In the adrenal zona fasciculata, cortisol is available 15 as substrate and the chimeric enzyme can catalyse its C-18 hydroxylation and C-18 oxidation, 16 resulting in the production of the so called "hybrid steroids", 18OH-cortisol and 18-oxo cortisol (11). 17 The hybrid steroids display weak mineralocorticoid activity (14, 15) but are a hallmark of the disease 18 and, until the description of the chimeric gene, have been regarded as an essential diagnostic feature. 19 After the identification of the chimeric gene, and the subsequent introduction of the long-range 20 polymerase chain reaction strategy for the amplification of the hybrid gene (16) the clinical 21 spectrum of FH-I dramatically changed (Supplemental Table S1). Large kindreds were available for 22 the easy and relatively inexpensive genetic testing, allowing to diagnose the disease in patients with 23 mild hypertension or even in normotensive subjects (17) indicating that the disease can display a 24 variable clinical phenotype. Moreover, a retrospective report from the International Registry for GRA 25 showed that hypokalaemia is infrequent and affected patients display an elevated prevalence of cerebrovascular events at young age (mainly haemorrhagic stroke, as a result of intracranial
 aneurysms rupture) (18) (Supplemental Table S1).

According to the Endocrine Society guideline, genetic testing for FH-I is appropriate in PA patients with a family history and/or early onset (< 20 years) of PA or in case of strokes at a young age (19). Once the diagnosis has been established, a therapy with low doses of an exogenous glucocorticoid (such as dexamethasone 0.125–0.25 mg/day) should be started to suppress ACTH secretion; to avoid glucocorticoid-related adverse effects, adding a mineralocorticoid receptor antagonist should be considered (19).

9 Familial hyperaldosteronism type II

10 Familial hyperaldosteronism type II was reported for the first time as a novel, not glucocorticoid 11 remediable, form of PA in Australia in 1991 (20). It is transmitted with an autosomal dominant pattern 12 in most of the families, but the mode of inheritance is less certain in others. It is the most common 13 form of FH (with a prevalence of 5% among patients with PA) (4) and can be due to either an APA 14 or a BAH. There are no clinical or biochemical characteristics that allow to distinguish patients 15 affected by FH-II from the ones affected by a sporadic form of PA (4). A linkage with 7p22 was 16 reported in some kindreds from 3 continents (21), but notably some families thought to be affected 17 by FH-II where subsequently re-classified as FH-III (22). There is now general agreement that FH-II 18 might be a heterogeneous group of genetic forms of PA whose molecular basis have yet to be 19 elucidated.

The diagnosis is made when at least two first-degree members of the same family are affected by PA, and the other forms of FH have been excluded through avalaible genetic tests (19). Due to its relatively high prevalence, the Endocrine Society guideline recommend that all hypertensive firstdegree relatives of patients with PA should undergo screening test (19).

24 Familial hyperaldosteronism type III

1 The first case of FH-III dates back to 1959 (23), but the disorder was recognized as a distinct clinical 2 entity with a peculiar clinical and biochemical phenotype solely in 2008 (24). The index case was a 3 young boy affected by polyuria, polydipsia, nicturia, headache and severe hypertension (known since 4 the age of 5) (23). PA was diagnosed on the basis of hypertension (maximum recorded reading of 5 300/190 mmHg), hypokalaemia (2.1-3.0 mEq/L), metabolic alkalosis and elevated average urinary 6 aldosterone (67 µg per day, normal range 1-8 µg per day) (23) and at the age of 9 he underwent 7 bilateral adrenalectomy. At histopathological examination, the removed adrenals were bilaterally 8 enlarged, with nodular hyperplasia mainly of the zona fasciculata. The two daughters of the index 9 case presented, at the age of 7 and 4 years, with a similar clinical and biochemical phenotype 10 characterized by resistant hypertension, severe hypokalaemia (1.8-1.9 mEq/L) and elevated plasma 11 aldosterone levels (137-185 ng/dL) despite suppressed plasma renin activity (0.2-0.3 ng/mL/h) (24). 12 Notably, both girls displayed extremely elevated levels of urinary hybrid steroids and FH-I was 13 suspected, but two dexamethasone suppression tests did not confirm the diagnosis. Surprisingly, not 14 only blood pressure and aldosterone failed to be suppressed by dexamethasone administration, but 15 showed an unexpected and paradoxical increase (24). Similarly, in both patients, cortisol levels were 16 not suppressed after dexamethasone administration, indicating a complete deregulation of adrenal 17 cortex functioning. As for the father, bilateral adrenalectomy was required to obtain normalization of 18 blood pressure and plasma potassium. In both cases adrenal glands were markedly enlarged, with a 19 complete loss of normal zonation (24, 25). Immunohistochemical staining and immunofluorescence 20 studies for the main enzymes involved in cortisol and aldosterone biosynthesis revealed that 21 aldosterone synthase is expressed throughout the entire adrenal cortex and frequently co-expressed 22 with CYP17 (17a-hydroxylase), explaining the abnormally high production of hybrid steroids in these 23 patients (25).

FH-III is transmitted as an autosomal dominant disease and its molecular basis was uncovered by
Choi et al. in 2011 (26). Through NGS technology, the authors identified 2 recurrent heterozygous *KCNJ5* somatic mutations (p.Gly151Arg and p.Leu168Arg) in a cohort of 22 sporadic APAs (26).

1 The experimental evidences obtained from sporadic adenomas suggested that inherited mutations in 2 KCNJ5 could cause FH-III and targeted sequencing of the gene revealed a germline p.Thr158Ala 3 mutation that co-segregated with the disease (26). KCNJ5 is located on chromosome 11q24 and 4 encodes the G protein-activated inward rectifier potassium channel 4, (GIRK4), which is expressed 5 in adrenal zona glomerulosa (26, 27), where it contributes to maintain the cell membrane in a 6 hyperpolarized state. The mutations disrupt the selectivity filter of the channel and are responsible 7 for loss of ion selectivity, Na⁺ entry and cell membrane depolarization with subsequent opening of the voltage gated Ca^{2+} channels (26, 28). The increase in intracellular Ca^{2+} activates the signaling 8 9 cascade that leads to CYP11B2 expression and autonomous aldosterone overproduction (27).

10 FH-III (OMIM # 613677) is a rare condition, affecting <1% of patients with PA (7). To date, 6 KCNJ5 germline mutations associated with FH-III have been reported (Figure 2), for a total of 12 families 11 12 and 22 affected family members (29). Notably, none of the further reported cases displayed the 13 peculiar hormonal phenotype described by Geller et al. (24). The majority of the patients presented 14 with an early onset and severe form of PA, requiring bilateral adrenalectomy to control hypertension 15 and hypokalaemia. However, the carriers of the p.Gly151Glu mutation (7 patients form 3 different 16 families) (22, 30) displayed a favourable disease progress: only two of them underwent 17 adrenalectomy (one had bilateral adrenalectomy and the other had 90% left adrenalectomy); none of 18 the patients displayed adrenal hyperplasia at imaging. Similarly, the patient carrying the p.Tyr152Cys 19 mutation displayed a less severe phenotype (31). Interestingly, a case of FH-III (due to the KCNJ5 20 p.Glu145Gln mutation) presenting with severe PA and typical Cushing's syndrome has recently been 21 reported in a Chinese boy (32). In vitro electrophysiological studies showed that the p.Gly151Glu 22 substitution was associated with a particularly severe impairment of the channel functioning, with 23 massive Na⁺ entry, osmotic shock and cell death. It has been postulated that this could at least partially 24 account for the mild clinical presentation and the lack of adrenal hyperplasia observed in these 25 patients (30).

According to the Endocrine Society guideline, testing for germline mutations in *KCNJ5* causing FH III is appropriate in very young patients with PA (19).

3 Familial hyperaldosteronism type IV

4 FH-IV is a familial form of primary aldosteronism (OMIM #617027) caused by germline mutations 5 in the CACNA1H gene located on chromosome 16p13 (33) (Figure 3, panel A) encoding the pore-6 forming α subunit of a T-type calcium channel, Cav3.2. CACNA1H is the second most expressed 7 Ca^{2+} channel gene in the adrenal zona glomerulosa (8, 26), where it is activated at small depolarizing 8 potentials (34). Through NGS analysis, a novel germline CACNA1H mutation (p.Met1549Val) was 9 identified in 5 out of 40 unrelated patients affected by hypertension and PA in childhood (33). The 10 clinical presentation of the index cases was uniform, without any distinctive clinical or biochemical 11 feature and normal appearing adrenal glands at CT scanning (33). Target sequencing of the 12 CACNA1H gene in the family members of the index cases, allowed to identify 5 additional subjects carrying the p.Met1549Val mutation. Of note, two mutation carriers did not receive a diagnosis of 13 14 early onset hypertension and were normotensives as adults, suggesting an incomplete penetrance (33). 15 The in vitro electrophysiological characterization revealed that the p.Met1549Val CACNA1H 16 displays loss of normal inactivation together with a shift of activation to more hyperpolarized potentials (33), alterations that are very likely to cause an increase in intracellular Ca²⁺ concentration 17 18 in adrenal zona glomerulosa cells. To elucitade the role of the mutation in autonomous aldosterone 19 overproduction, the p.Met1549Val mutant channel was expressed in HAC15 adrenocortical cells, 20 resulting in a 7.1-fold increase in CYP11B2 transcription and a 3.7-fold increase in aldosterone 21 production compared to the cells expressing the wild-type channel (35).

Subsequently, four additional germline *CACNA1H* mutations were identified in patients with PA (p.Met1549Ile, p.Ser196Leu, p.Pro2083Leu and p.Val1951Glu) (36). The p.Met1549Ile substitution was a *de novo* event identified in a sporadic PA patient and both p.Ser196Leu and p.Pro2083Leu mutations were detected in pairs of brothers/sisters affected by PA. Interestingly, the

p.Val1951Glu was identified in a patient affected by apparently sporadic APA, who was cured by
unilateral adrenalectomy (36). These data indicate that *CACNA1H* might represent a susceptibility
gene for PA development that could present with a wide range of clinical phenotypes (36).

4 PASNA syndrome

5 PASNA (primary aldosteronism with seizures and neurologic abnormalities, OMIM #615474) is a 6 clinical syndrome characterized by primary aldosteronism and neurological symptoms. It is caused 7 by gain-of-function mutations in the *CACNA1D* gene located on the chromosome 3p14.3 (8) (Figure 8 3, panel B), coding for the α 1D subunit of a L-type voltage gated calcium channel (Cav 1.3), which 9 is expressed in adrenal zona glomerulosa cells. Electrophysiological in vitro studies, showed that the 10 mutations cause channel activation at less depolarized potentials and altered channel inactivation, 11 with subsequent abnormal calcium signalling (8).

Two paediatric patients affected by PASNA syndrome due to *de novo CACNA1D* germline mutations (p.Gly403Asp and p.Ile770Met) have been reported (8). The index cases presented with early onset severe hypertension, hypokalaemia and neurological manifestations, including seizures and cerebral palsy. In one of the two patients, blood pressure was successfully controlled by the calcium channel blocker amlodipine, raising the possibility that calcium channel blockers might represent a specific treatment for individuals affected by APAs carrying a *CACNA1D* somatic mutation.

Interestingly, a new missense *CACNA1D* germline mutation (p.Val104Leu) was identified in a patient
affected by autism and epilepsy with a phenotype partially overlapping with that observed in patients
with PASNA syndrome (37).

21

22 GENETIC OF SPORADIC PRIMARY ALDOSTERONISM

Until recently, genetic studies on sporadic PA were mainly focused on genetic variants potentially
able to increase the susceptibility to the disease or affect the clinical phenotype, including CYP11B2,

α-adducin and bradykinin B2 receptor polymorphisms (7). The introduction of NGS technology
 allowed the identification of aldosterone stimulating somatic mutations in a significant proportion of
 sporadic APAs (7).

4 Germline mutations in sporadic PA

5 While the molecular determinants of autonomous aldosterone overproduction have been at least 6 partially unravelled, the molecular basis of bilateral hyperaldosteronism and adrenal cell proliferation 7 (in both APA and BAH) are still poorly elucidated. It was postulated by Choi et al. (26) that the 8 intracellular calcium influx induced by *KCNJ5* mutations, other than driving aldosterone secretion, 9 might promote cell proliferation. However, subsequent studies demonstrated that the expression of 10 mutant GIRK4 has a negative effect on HAC15 adrenocortical cells growth (28) suggesting that a 11 second hit might be necessary for APA formation (38).

KCNJ5 sequencing in peripheral blood DNA from 251 patients affected by sporadic bilateral hyperaldosteronism revealed three heterozygous missense germline mutations. The mutations (p. p.Arg52His, p.Glu246Lys, and p.Gly247Arg) are not associated with FH-III and are not located in proximity of the selectivity filter of the channel (39). Electrophysiological studies conducted in Xenopus oocytes showed that the expression of both the p.Arg52His and p.Glu246Lys substitutions resulted in cell membrane depolarization, while the p.Gly247Arg mutation did not alter the resting potential (39).

ARMC5 gene maps on 16p11 and encodes the armadillo repeat containing 5, whose function is currently unknown, but is likely to act as a tumor-suppressor gene. Somatic and germline mutations in *ARMC5* are frequently found in macronodular adrenal hyperplasia and Cushing syndrome (40) and in a significant proportion of PA patients of African American descent (41). However, another study failed to confirm this association in patients of European ancestry (42). Similarly, a potential role of ARMC5 in FH-II has been recently ruled out (43).

1 KCNJ5 somatic mutations

2 Following the seminal report by Choi et al. (26), several centres from four continents investigated the 3 prevalence of KCNJ5 somatic mutations in APAs. In the largest study conducted in a Western 4 population, comprising 474 adrenal adenomas collected through the European Network for the Study 5 of Adrenal Tumours (ENS@T), the prevalence of KCNJ5 mutations resulted to be 38% (44), while 6 the largest study conducted in East Asia reported, in a cohort of 168 samples, a 78% prevalence (45). 7 According to a recent meta-analysis including 13 studies for a total of 1,636 patients, the overall 8 prevalence of KCNJ5 mutations is 43%, with wide variation across centres (46). The prevalence 9 appears to be consistently higher in East Asian populations compared to Western populations, but 10 also in those centres where strict criteria for adrenal vein sampling interpretation were used (47). Sequencing analysis allowed to identify 15 further KCNJ5 somatic mutation associated with sporadic 11 12 unilateral PA (48).

13 Comprehensive clinical, biochemical and histopathological studies showed that the adenomas 14 carrying KCNJ5 mutations are more prevalent in females than in males (46) and are associated with 15 younger age at diagnosis and higher preoperative aldosterone levels (46). Moreover, adenomas 16 carrying KCNJ5 mutations express lower levels of aldosterone synthase compared to APAs carrying 17 mutations in ATP1A1, ATP2B3 or CACNA1D and are composed mainly of zona fasciculata-like cells 18 (49) expressing CYP17A1 (50). These characteristics might at least partially account for the high 19 amount of hybrid steroids detected in APA patients carrying KCNJ5 mutations (51) and, considering 20 the high prevalence of KCNJ5 mutations in the East Asian patients, explain the potential diagnostic 21 value of 18-oxocortisol in subtype differentiation in this specific subpopulation (52).

In vitro pharmacological studies showed that mutated GIRK4 exhibited a different pharmacological profile compared to the wild type, in particular the calcium channel blocker verapamil strongly inhibits the p.Leu168Arg mutant channel, suggesting a potential therapeutic use of this drug (53). Even more surprisingly, mutant GIRK4, but not the wild-type channel, is effectively inhibited by a series of molecules belonging to the macrolide class of antibiotics and by synthetic derivatives lacking
 the antibiotic activity (54). In light of this recent finding, a murine model of PA due to a germline
 KNCJ5 mutation would be an extremely valuable tool for further pharmacological studies.

4 ATP1A1 and ATP2B3 somatic mutations

5 The application of the NGS technology to a series of sporadic KCNJ5 wild-type APAs led to the 6 identification of four different somatic mutations affecting ATP1A1 (55, 56) and two different in-7 frame deletions of ATP2B3 (56). ATP1A1 is located on chromosome 1p21 and encodes the 8 sodium/potassium-transporting ATPase subunit alpha-1, while the ATB2B3 gene is located on 9 chromosome Xq28 and encodes the plasma membrane calcium-transporting ATPase 3. The 10 sodium/potassium ATPase exchanges three cytoplasmic sodium ions for two extracellular potassium 11 ions against the concentration gradient thus maintaining the resting membrane potential and the cellular excitability; Ca²⁺-ATPase3 removes one cytosolic Ca²⁺ in exchange for two H⁺ and plays a 12 key role in calcium homeostasis. In vitro studies showed that the mutant Na⁺/K⁺ ATPase exhibits 13 14 reduced K⁺ affinity and disturbed gating properties, resulting in lowered intracellular pH, but, surprisingly not in cell membrane depolarization (55, 57). On the contrary, the mutant Ca^{2+} -ATPase3 15 16 strongly depolarized the plasma membrane, as a consequence of a complete loss of its physiological pump function, that become permeable to cations, permitting Na^+ and Ca^{2+} influx (58). 17

A total of 13 different ATP1A1 and 9 ATPB3 somatic mutations have been reported so far (48). *ATP1A1* somatic mutations account for 5.3% of the sporadic APAs while *ATP2B3* mutations have
been identified in 1.7% of the samples (44).

21 CACNAID somatic mutations

Since its original description (8, 56), a total of 31 different *CACNA1D* mutations have been reported (48), accounting for 9.3% of the sporadic APAs (44). APAs carrying *CACNA1D* mutations are composed mainly of zona-glomerulosa-like cells (49, 50) and are smaller compared with those with *KCNJ5* or no mutations (44, 49). Accordingly, somatic *CACNA1D* mutations are the most frequent
 genetic alteration in CYP11B2-positive cortical micro-nodules in cross-sectional imagine-negative
 PA (59).

4 CTNNB1 somatic mutations

5 *CTNNB1* gene is located on chromosome 3 and encodes β -catenin, which is part is part of a complex 6 of proteins that constitute adherens junctions. β -catenin plays a key role in adrenocortical function: 7 inactivation of β -catenin, using the Cre-loxP transgenic strategy, causes adrenal aplasia in newborn 8 mice (60) while its constitutive activation in murine adrenal cortex results in increased aldosterone 9 production (61). Similarly, mice lacking the WNT inhibitor SFRP2 display increased aldosterone 10 production, supporting the evidence that SFRP2 down-regulation in APAs is likely to cause WNT/β-11 catenin constitutive activation (62). Activating CTNNB1 mutations have been detected in both benign 12 and malignant adrenocortical tumours (63). Somatic mutations in CTNNB1 have been identified in 13 around 3% of sporadic APAs (64, 65) and have been associated to female gender and relatively large 14 adenomas. APAs associated CTNNB1 mutations are located on exon 3 (64, 65) and result in aberrant 15 activation of Wnt signaling, by altering specific residues that are involved in β -catenin degradation.

16 Somatic mutations in aldosterone producing cell clusters

The adrenal zona glomerulosa, composed of compact cells forming nests, is the exclusive site of CYP11B2 expression and aldosterone production (1), however the APAs are composed mainly of zona-fasciculata like cells (large cells with lipid-laden cytoplasm) and only less frequently display a zona-glomerulosa like phenotype (49). Moreover, histological examination of the removed adrenal glands, following a diagnosis of unilateral PA, revealed significant heterogeneity in both the nodules and the adjacent adrenal cortex (49, 66).

The recent development of specific monoclonal antibodies able to distinguish between the highly
 homologous CYP11B1 and CYP11B2 allowed a more specific characterization of both normal

1 adrenals and unilateral PA, opening a new scenario that goes beyond the classical view of 2 adrenocortical zonation (10). Histological examination and immunohistochemical staining of adrenal 3 specimens revealed the presence of subcapsular nests of adrenocortical cells extending in the zona 4 fasciculata and strongly expressing CYP11B2, named aldosterone producing cell clusters (APCCs) 5 (67). The APCCs were found in both normal adrenals and in the cortex adjacent to an APA (68) and 6 a significant proportion of them carries somatic mutations in ATPIA1 and CACNA1D genes (but not 7 in KCNJ5), supporting the hypothesis that they might display autonomous aldosterone 8 overproduction and progress to overt PA over time (68). In particular, APCCs are likely to progress 9 to CT-negative PA, as suggested by the elevated prevalence of CACNA1D mutations in this particular 10 subtype of PA (59), but less likely to CT-detectable adenomas (which more frequently harbour 11 somatic mutations in KCNJ5).

12 Clinical correlates indicated that APCCs number and size increase with age, (69) paralleled by a 13 progressive transition towards a discontinuous CYP11B2 expression pattern in older-age adrenal 14 glands which might account for the age-related changes in renin and aldosterone physiology (70).

15

16 CONCLUSIONS

The last six years have witnessed major advances in the field of both sporadic and familial PA. Three novel familial forms have been characterized and somatic mutations, altering intracellular ion homeostasis, drive aldosterone overproduction in around 60% of sporadic APAs. Notably, some of the somatic mutations have also been detected in APCCs, which might represent the precursors of CT-undetectable PA. In the next future, steroid profiling and targeted inhibition of mutated GIRK4 are very likely to change the classical clinical approach to patients affected by PA due to an aldosterone producing adenoma.

24 FIGURE LEGENDS

1 Figure 1. Schematic representation of the CYP11B1/CYP11B2 chimeric gene. The chimeric gene, 2 expressed throughout the entire adrenal cortex (dashed circle), originates from an unequal crossing 3 over between the highly homologous CYP11B1 and CYP11B2 genes coding for 11β-hydroxylase and 4 aldosterone synthase, respectively. The crossing over break-points are comprised between CYP11B1 5 intron 2 and exon 4 so that the chimeric gene contains the promoter region of CYP11B1 (regulated 6 by ACTH) and a coding region of CYP11B2. The two CYP11B2 residues Gly288 and Ala320 7 are responsible for 18-hydroxylation and 18-oxidation respectively and are therefore indispensable 8 to retain aldosterone synthase activity.

9 Figure 2. Germline mutations in GIRK4 associated with FH-III. FH-III causing *KCNJ5* germline
10 mutations (black dots) are located near or within the selectivity filter of the GIRK4 channel. N
11 indicates the N-terminus and C indicates the C-terminus.

Figure 3. Panel A - Germline mutations in Cav3.2 causing FH-IV. *CACNA1H* encodes the poreforming alpha subunit (Cav3.2) of a T-type calcium channel. Cav3.2 is composed of four repeated domains (I–IV), with six transmembrane segments each (S1–S6). The germline mutations associated with FH-IV are indicated as black dots and are located in S4 segment of domain I, S6 segment of domain III, and in the C-terminal cytoplasmic domain. N indicates the N-terminus and C indicates the C-terminus.

Panel B - Germline mutations in Cav1.3 causing PASNA syndrome. *CACNA1D* encodes the α1 (pore-forming) subunit (Cav1.3) of an L-type voltage-gated calcium channel. The α1 subunit is composed of four repeated domains (I–IV), with six transmembrane segments each (S1–S6). The two germline mutations associated with PASNA syndrome are indicated as black dots and are located in S6 segment of domains I and II. N indicates the N-terminus and C indicates the C-terminus.

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