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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**

19 Aldosterone is the main mineralocorticoid hormone in humans and, under physiological conditions,
20 its secretion is tightly regulated by angiotensin II, extracellular potassium and adrenocorticotrophin
21 (ACTH) (1). Its principal site of action is the distal nephron, where it promotes sodium retention and
22 potassium excretion, playing a key role in maintaining water and electrolyte homeostasis. The
23 autonomous aldosterone overproduction by one or both adrenal glands is a clinical syndrome known
24 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**

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

1 dexamethasone (12). Until 1990, less than 100 cases were described (Supplemental Table S1): since
2 the diagnosis was clinical (based on dexamethasone suppression of aldosterone overproduction), the
3 majority of the affected patients displayed a florid PA phenotype, with hypertension and
4 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
11 synthase activity and therefore in all the chimeric genes causing FH-I reported so far, the
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

1 cerebrovascular events at young age (mainly haemorrhagic stroke, as a result of intracranial
2 aneurysms rupture) (18) (Supplemental Table S1).

3 According to the Endocrine Society guideline, genetic testing for FH-I is appropriate in PA patients
4 with a family history and/or early onset (< 20 years) of PA or in case of strokes at a young age (19).
5 Once the diagnosis has been established, a therapy with low doses of an exogenous glucocorticoid
6 (such as dexamethasone 0.125– 0.25 mg/day) should be started to suppress ACTH secretion; to avoid
7 glucocorticoid-related adverse effects, adding a mineralocorticoid receptor antagonist should be
8 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.

20 The diagnosis is made when at least two first-degree members of the same family are affected by PA,
21 and the other forms of FH have been excluded through available genetic tests (19). Due to its
22 relatively high prevalence, the Endocrine Society guideline recommend that all hypertensive first-
23 degree 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 (17 α -hydroxylase), explaining the abnormally high production of hybrid steroids in these
23 patients (25).

24 FH-III is transmitted as an autosomal dominant disease and its molecular basis was uncovered by
25 Choi et al. in 2011 (26). Through NGS technology, the authors identified 2 recurrent heterozygous
26 *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
8 the voltage gated Ca²⁺ channels (26, 28). The increase in intracellular Ca²⁺ activates the signaling
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*
11 germline mutations associated with FH-III have been reported (Figure 2), for a total of 12 families
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).

1 According to the Endocrine Society guideline, testing for germline mutations in *KCNJ5* causing FH-
2 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
13 carrying the p.Met1549Val mutation. Of note, two mutation carriers did not receive a diagnosis of
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
17 potentials (33), alterations that are very likely to cause an increase in intracellular Ca^{2+} concentration
18 in adrenal zona glomerulosa cells. To elucidate 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).

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

1 p.Val1951Glu was identified in a patient affected by apparently sporadic APA, who was cured by
2 unilateral adrenalectomy (36). These data indicate that *CACNAIH* might represent a susceptibility
3 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 *CACNAID* gene located on the chromosome 3p14.3 (8) (Figure
8 3, panel B), coding for the $\alpha 1D$ 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).

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

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

21

22 **GENETIC OF SPORADIC PRIMARY ALDOSTERONISM**

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

1 α -adducin and bradykinin B2 receptor polymorphisms (7). The introduction of NGS technology
2 allowed the identification of aldosterone stimulating somatic mutations in a significant proportion of
3 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).

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

19 *ARMC5* gene maps on 16p11 and encodes the armadillo repeat containing 5, whose function is
20 currently unknown, but is likely to act as a tumor-suppressor gene. Somatic and germline mutations
21 in *ARMC5* are frequently found in macronodular adrenal hyperplasia and Cushing syndrome (40) and
22 in a significant proportion of PA patients of African American descent (41). However, another study
23 failed to confirm this association in patients of European ancestry (42). Similarly, a potential role of
24 *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).
11 Sequencing analysis allowed to identify 15 further *KCNJ5* somatic mutation associated with sporadic
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).

22 In vitro pharmacological studies showed that mutated GIRK4 exhibited a different pharmacological
23 profile compared to the wild type, in particular the calcium channel blocker verapamil strongly
24 inhibits the p.Leu168Arg mutant channel, suggesting a potential therapeutic use of this drug (53).
25 Even more surprisingly, mutant GIRK4, but not the wild-type channel, is effectively inhibited by a

1 series of molecules belonging to the macrolide class of antibiotics and by synthetic derivatives lacking
2 the antibiotic activity (54). In light of this recent finding, a murine model of PA due to a germline
3 *KCNJ5* 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
12 cellular excitability; Ca^{2+} -ATPase3 removes one cytosolic Ca^{2+} in exchange for two H^{+} and plays a
13 key role in calcium homeostasis. In vitro studies showed that the mutant $\text{Na}^{+}/\text{K}^{+}$ ATPase exhibits
14 reduced K^{+} affinity and disturbed gating properties, resulting in lowered intracellular pH, but,
15 surprisingly not in cell membrane depolarization (55, 57). On the contrary, the mutant Ca^{2+} -ATPase3
16 strongly depolarized the plasma membrane, as a consequence of a complete loss of its physiological
17 pump function, that become permeable to cations, permitting Na^{+} and Ca^{2+} influx (58).

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

21 ***CACNA1D* somatic mutations**

22 Since its original description (8, 56), a total of 31 different *CACNA1D* mutations have been reported
23 (48), accounting for 9.3% of the sporadic APAs (44). APAs carrying *CACNA1D* mutations are
24 composed mainly of zona-glomerulosa-like cells (49, 50) and are smaller compared with those with

1 *KCNJ5* or no mutations (44, 49). Accordingly, somatic *CACNA1D* mutations are the most frequent
2 genetic alteration in CYP11B2-positive cortical micro-nodules in cross-sectional imagine-negative
3 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**

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

23 The recent development of specific monoclonal antibodies able to distinguish between the highly
24 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 *ATP1A1* 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**

17 The last six years have witnessed major advances in the field of both sporadic and familial PA. Three
18 novel familial forms have been characterized and somatic mutations, altering intracellular ion
19 homeostasis, drive aldosterone overproduction in around 60% of sporadic APAs. Notably, some of
20 the somatic mutations have also been detected in APCCs, which might represent the precursors of
21 CT-undetectable PA. In the next future, steroid profiling and targeted inhibition of mutated G1R4
22 are very likely to change the classical clinical approach to patients affected by PA due to an
23 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 11 β -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.

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

18 **Panel B - Germline mutations in Cav1.3 causing PASNA syndrome.** *CACNA1D* encodes the $\alpha 1$
19 (pore-forming) subunit (Cav1.3) of an L-type voltage-gated calcium channel. The $\alpha 1$ subunit is
20 composed of four repeated domains (I–IV), with six transmembrane segments each (S1–S6). The two
21 germline mutations associated with PASNA syndrome are indicated as black dots and are located in
22 S6 segment of domains I and II. N indicates the N-terminus and C indicates the C-terminus.

23

24

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