1 **Inherited forms of primary hyperaldosteronism: new genes, new phenotypes and proposition of a**

2 **new classification**

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Abstract

Primary aldosteronism is a common cause of endocrine hypertension. It results from the excess production of aldosterone by the adrenal cortex and is related to increased morbidity and mortality. Most cases of PA are sporadic but inherited patterns of the disease have been reported in the literature. Four forms of familial hyperaldosteronism (FH-I- FH-IV) are currently recognized, and the genetic basis has been clarified in recent years. In FH-I patients, aldosterone excess is produced by a *CYP11B1/CYP11B2* fusion gene and it is suppressed by glucocorticoid treatment. FH-II is caused by mutations in the inwardly rectifying chloride channel *CLCN2*. FH-III is caused by mutations in *KCNJ5*, a gene coding for an inward rectifier K^+ channel and mutations in the T-type calcium channel subunit *CACNA1H* cause FH-IV. In this review we summarize the knowledge on inherited forms of primary aldosteronism, the genetic alterations that cause them and the implications it may have for the classification. Based on current evidence, we propose the term "familial hyperaldosteronism" to refer only to inherited forms of primary aldosteronism with a known genetic basis.

Primary aldosteronism (PA) is the clinical manifestation of a heterogeneous group of adrenal disorders that are characterized by an excessive production of aldosterone, which becomes relatively independent of the angiotensin-renin system regulation. Over time, sustained levels of aldosterone lead to increased blood pressure and elevated potassium excretion, therefore patients with PA are hypertensive, in many cases hypokalemic, and at higher risk of stroke, renal complications, metabolic and cardiovascular mortality than patients with essential hypertension. Once classified as a rare disease, PA is now considered the most common cause of endocrine hypertension, with an estimated prevalence of about 4-6% in the general population with hypertension and up to 10-20% in the subset of patients with resistant hypertension [1–3].

Most diagnosed cases of PA are sporadic and are mainly caused by aldosterone overproduction by both adrenal glands (bilateral adrenal hyperplasia) or by unilateral aldosterone-producing adenomas (APA). Other causes include unilateral hyperplasia and very rarely, adrenocortical carcinomas. In some cases, PA affects several members of the same family in the inherited or familial forms of hyperaldosteronism (FH). Current guidelines recognize three well established types of FH, namely FH-I to FH-III [4], however data from genetic analyses reveal a more complex situation, with at least 4 different inheritable forms of PA and possibly still more yet to be discovered.

Genes associated with inherited forms of PA

The genetics of PA has remained obscure for a long time. Although infrequent, the early onset and the heritability favored the study of familial PA as an approach to understand the pathophysiology of the more common sporadic forms. The identification of the first genetic alteration causative for a particular subtype of PA by linkage analysis on affected relatives, the chimera *CYP11B1/CYP11B2* [5], was an outstanding discovery but subsequent investigation quickly revealed that it was not present in sporadic forms [6,7]. The failure to find new causative genes and the introduction of next generation sequencing techniques turned the focus to sporadic patients.

Now that hundreds of APAs have been sequenced, it is well known that *KCNJ5*, *CACNA1D*, *ATP1A1* and *ATP2B3* genes are mutated in about 50% of adenomas (reviewed in [8] and [9]) and that ion channels and pumps exert an important role on aldosterone signaling through the control of Ca^{2+} influx [10]. Following the trend of next generation sequencing of sporadic cases, the study of patients with early-onset PA has uncovered that some of those genes also exert an important role in inherited forms. Thus, *KCNJ5* germline mutations cause FH-III and *CACNA1H* mutations have been found in families with FH-IV and *de novo* germline mutations in *CACNA1D* have been reported in patients with early onset of PA, seizures and neurologic abnormalities (PASNA). In addition, two recent studies in patients with early-onset PA have shown mutations in *CLCN2* associated with FH-II. Table 1 summarizes the genes associated with PA and the main clinical features and Figure 1 depicts the molecular mechanisms.

CYP11B1/CYP11B2 chimera: Familial Hyperaldosteronism Type I (FH-I)

FH-1 was first reported in 1966 by Sutherland and colleagues [11]. They reported two hypertensive relatives, a father and a son, with a condition that mimicked the symptoms of sporadic PA (increased aldosterone, low renin activity and hypokalemia) but with the particularity of suppression of aldosterone production by a 2 mg/day dexamethasone treatment. Thus FH-I is often referred to as glucocorticoid-remediable aldosteronism (GRA). The basis of the glucocorticoid suppression was discovered in 1992, after linkage analysis identified the genetic cause as a chimeric fusion on chromosome 8 containing an unequal recombination between the highly homologous genes *CYP11B1* (11-β-hydroxylase) and *CYP11B2* (aldosterone synthase) [5]. The exact point of cross-over can be different in each reported family but always contains the promoter and the first exons of *CYP11B1* and most of the coding region of *CYP11B2*, resulting in an enzyme with aldosterone synthase activity with expression under the control of the adrenocorticotropic hormone (ACTH) instead of angiotensin II and potassium. As a consequence, aldosterone synthase is expressed in the zona fasciculata rather than in the zona glomerulosa, resulting in

88 the ectopic production of aldosterone and the production of the hybrid steroids 18-oxocortisol and 18-

89 hydroxycortisol [5,12].

90 FH-I is considered as a rare subgroup of PA that represents less than 1% of all cases, increasing to 3% in

91 children with hypertension [13–15]. It is characterized by the development of bilateral adrenal

92 hyperplasia, occasionally adrenal nodules, with variable clinical and biochemical features [14,16]. FH-I

93 follows an autosomal dominant inheritance pattern and is generally associated with early onset severe

94 hypertension and an increased risk of stroke; however, different degrees of severity have been reported,

95 including cases of mild hypertension and normotensive individuals [16–18].

96 The Endocrine Society guideline recommends testing for FH-I in patients with an early onset of PA (<20

97 years old) and in those with a familial occurrence of PA or stroke at a young age (<40 years old) [4]. The

98 correct diagnosis is clinically relevant because aldosterone excess can be controlled successfully through

99 glucocorticoid therapy [19]. Prior to the existence of targeted molecular tests, the diagnosis was made

100 through clinical and biochemical evaluation. Dexamethasone suppression of aldosterone and levels of

101 hybrid steroids were used to establish a diagnosis of FH-I [20,21] until the introduction of techniques to

102 specifically detect the presence of the *CYP11B1/CYP11B2* chimeric gene either by Southern blotting or by

103 the recommended technique employing a long-chain PCR amplification [4, 21,22].

104 In patients with FH-I aldosterone production is abrogated under glucocorticoid treatment, and partial

105 suppression of ACTH is enough to correct the hypertension associated with FH-I. Accordingly, low doses

106 of dexamethasone are recommended to achieve normotension whilst preventing undesired cushingoid

107 features [19]. Mineralocorticoid receptor antagonists (spironolactone or eplerenone) can be used as a

108 second line of therapy to block possible non-genomic effects of aldosterone on target organs, or in

109 children to avoid possible side effects of dexamethasone treatment [24].

110

111 *CLCN2: Familial Hyperaldosteronism Type II (FH-II)*

112 FH-II was first described by Gordon et al. in 1991, a year before the genetic cause of FH-I was published.

113 They described 6 relatives from 3 independent affected families who presented with PA caused by either

114 APA or BAH and a lack of suppression of aldosterone production by fludrocortisone or

115 dexamethasone[25]. Several families were reported by the same group shortly thereafter [26,27].

116 Until very recently, the genetic cause of FH-II remained elusive. Early targeted genetic studies showed a

117 lack of mutations on genes related to steroidogenic production or tumorigenesis, such as *CYP11B2*, the

118 angiotensin receptor *AT1R* or *TP53*. Later on, genetic linkage analysis of non-related families highlighted

119 a locus at chromosome 7p22 that segregates with the disease in some families, but not in all cohorts [28–

120 30]. However both targeted sequencing of different genes in that region as well as next-generation

121 sequencing of the complete locus have failed to find mutations [29,31–33]. Scholl and colleagues

122 analyzed the genomic DNA by exome sequencing of three members from one of the FH-II families

123 described by Stowasser *et al*. in 1992 [27]. The authors identified a germline mutation in the gene *CLCN2*

124 that segregated with the disease. This variant, p.Arg172Gln, was confirmed subsequently in five additional

125 family members, four of them with aldosterone-to-renin ratio suggestive of PA [34]. Because the

126 discovery family was one of the first families diagnosed with FH-II, Scholl *et al*. proposed the use of that

127 term only for inherited PA due to *CLCN2* mutations [34]. The authors also reported the same mutation in

128 three additional unrelated individuals, as well as rare germline *CLCN2* variants (p.Met22Lys, p.Tyr26Asn,

129 p.Lys362del and Ser865Arg, with allele frequencies below 10^{-5}) in four additional unrelated patients [34].

130 Simultaneously, Fernandes-Rosa and colleagues identified another germline *CLCN2* mutation in a 9-

131 years-old patient by exome-sequencing sequencing of genomic DNA from 12 patients with young-onset

132 hypertension and PA. In that case, p.Gly24Asp was a *de novo* mutation. Two additional variants were

133 found in two cases from a cohort of 100 patients with idiopathic bilateral adrenal hyperplasia (p.Arg66Gln

134 and p.Pro48Arg, with minor allele frequencies of $3x10^{-5}$ and 1.7×10^{-4}, respectively) [35]. Both studies

135 showed that *CLCN2* mutations were related to PA diagnosed at early age and absent in patients with

136 essential hypertension [34,35].

137 *CLCN2* gene is located in chromosome 3q27 and encodes the inwardly rectifying chloride channel ClC2, a

138 member of the CLC voltage-gated Cl^- channels family. ClC2 is broadly expressed in mammalian cells,

139 especially in brain, gut, kidney, heart and liver [36]. Mutations inactivating *CLCN2* cause leukodystrophy,

140 in some cases with azoospermia, and *Clcn2* knockout mice also develop early postnatal retinal

141 degeneration [37–39]. Scholl *et al.* and Fernandes-Rosa *et al.* have shown that ClC2 is also expressed in

142 the adrenal gland. Furthermore, germline mutations that associate with PA result in gain of function of the

143 Cl^- channel, causing an efflux of Cl^- ions that leads to the depolarization of the plasma membrane, the

144 consequent opening of voltage-gated Ca^{2+} channels, the accumulation of cytosolic Ca^{2+} and the activation

145 of *CYP11B2* transcription [34,35].

146 Before the recent discovery of *CLCN2* mutations, screening for FH-II was based on the diagnosis of PA in

147 at least two first-degree members of the same family and the absence of known germline mutations. Thus,

148 this familial form was thought to be the most prevalent, representing about 3-6% of all PA cases [11,54].

149 Nevertheless, Korah and Scholl pointed out that this estimation may be misleading: considering the

150 prevalence of hypertension in the general population (about 30%) and the PA prevalence in the general

151 population with hypertension (about 5%), the probability for an index case to have at least a first-degree

152 relative with PA just by chance is ~5.9% [40]. Accordingly, it is likely that some of the described FH-II

153 families were in fact coincidental cases of sporadic idiopathic PA. This observation may explain, at least

154 partially, the apparent heterogeneity reported in previous studies. To avoid confusion, and to base the

155 classification on a simple and transparent genetic basis, similar to other genetic diseases, we propose to

156 use the term "familial hyperaldosteronism" only when an inherited genetic cause is established.

157 Following this reasoning, the number of true FH-II families is probably much lower than previously

158 reported. In their studies, Scholl *et al*. and Fernandes-Rosa *et al*. identified *CLCN2* mutations in about

159 10% of cases with young-onset PA without known germline mutations and 2% with bilateral adrenal

160 hyperplasia [34,35] suggesting a lower frequency than previous estimates. Further efforts are needed to

161 determine the actual prevalence of FH-II.

162

163 *KCNJ5: Familial Hyperaldosteronism Type III (FH-III)*

164 FH-III was described by Geller *et al.* in three family members, a father and his two young daughters, who

165 developed hyperaldosteronism with hypokalemia and severe hypertension at very early age, together with

166 marked bilateral adrenal enlargement. High levels of the hybrid steroids 18-oxocortisol and 18-

167 hydroxycortisol were detected in urine samples but the disorder was distinguishable from FH-I by the

168 glucocorticoid resistance of the hyperaldosteronism and the lack of suppression of aldosterone production

169 on dexamethasone suppression testing. Hypertension and hypokalemia were refractory to medical therapy

170 and disease control was achieved only after bilateral adrenalectomy [41]. Careful examination of the

171 adrenals revealed disorganized zonation, a reduction in the thickness of the zona glomerulosa, an enlarged

172 zona fasciculata and the presence of cells that co-express enzymes which are usually expressed in distinct

173 zones, such as CYP11B1 and CYP11B2 and also CYP17 and CYP11B2. The co-expression of CYP17 and

174 CYP11B2 is the likely basis for the production of hybrid steroids [12,41,42].

175 It was not until 2011 that the genetic etiology of FH-III was clarified. By means of exome sequencing,

176 Choi *et al*. identified a heterozygous germline mutation located on chromosome 11q24 in the patients

177 reported by Geller and colleagues, as well as in sporadic cases of PA [43]. The affected gene was *KCNJ5*,

178 which codes for the G-protein-activated inward rectifier K^+ channel 4 (Kir3.4). This protein forms homo-

179 and heterotetramers with other Kir family members to constitute the functional G-protein-activated

180 inwardly rectifying potassium channel, which contributes to the control of membrane polarity in the zona

181 glomerulosa [44]. The mutation identified in Geller's cases (p.Thr158Ala) was associated with a loss in

182 K^+ selectivity and an increased influx of Na^+ into the cytoplasm, leading to membrane depolarization and

183 the elevation of intracellular Ca^{2+} levels, which ultimately triggers aldosterone production through the

184 activation of Ca^{2+}-related signaling pathways [45].

185   Since the link between inherited PA and *KCNJ5*, several familial cases with different mutations in that

186   gene have been published, mostly in or next to the selectivity filter [46–51], and the term FH-III is used

187   for familial cases with PA due to germline *KCNJ5* mutations, regardless of the phenotype. Indeed, the

188   clinical features of the affected cases vary all along the PA spectrum, from mild and treatment-responding

189   forms to severe PA with progressive disease, including symptoms mimicking diabetes insipidus and a

190   recent report showing development of Cushing´s syndrome in one patient with FH-III [50]. This

191   variability seems to be dependent on the type of the grounding *KCNJ5* mutations, among other

192   factors[46]. Thus, p.Gly151Glu mutations seem to associate with a milder phenotype and stable disease

193   [46,49], while p.Gly151Arg, p.Thr158Ala, p.Ile157Ser and p.Tyr152Cys mutations relate to a more

194   severe hyperaldosteronism [52]. Other infrequent germline alterations of *KCNJ5* (some of them *de novo*)

195   and a rare non-synonymous SNP (rs7102584) have been described. The mutation p.Glu145Gln affects a

196   salt bridge close to the selectivity filter, while mutations p.Arg52His, p.Glu246Lys, p.Gly247Arg and the

197   SNP Glu282Gln were located elsewhere in the protein [53,54]. Except of the p.Gly247Arg, those variants

198   altered channel functionality and increased aldosterone production compared with the wild-type protein.

199   The prevalence of FH-III has not been established systematically but it is estimated to be present in <1%

200   of all PA cases [47]. The Endocrine Society guideline recommends testing for FH-III by sequencing

201   peripheral blood for mutations in *KCNJ5* in those patients with a very early onset of PA [4]. Because of

202   the variety of presentations, treatment for FH-III depends on the severity of the disease. Milder cases can

203   be well controlled with spironolactone, while adrenalectomy is currently the best option to treat resistant

204   forms successfully [52].

205

206   *CACNA1H: Familial Hyperaldosteronism Type IV (FH-IV)*

207   FH-IV was reported by Scholl and coworkers in a cohort of 40 patients diagnosed with PA in early

208   childhood (at age 10 years or below) and without mutations in any common known PA genes. By whole

209   exome sequencing analysis, a recurrent mutation in the gene *CACNA1H* was identified in five unrelated

210   patients, four males and one female [55]. Shortly thereafter Daniil et al. reported the presence of different

211   mutations in the same gene in two unrelated individuals who were diagnosed originally with FH-II, as

212   well as an adult male case with a *de novo* mutation and an adult female patient with an APA and a

213   germline mutation in the same gene [56]. Patients showed no apparent signs of seizures, cardiac

214   arrhythmia or muscular or neurological alterations that have been commonly linked to other disorders

215   caused by *CACNA1H* germline mutations or by another Ca^{2+} channel subunit, *CACNA1D* [57], although

216   one of the patients was diagnosed with minor mental retardation and multiplex developmental disorder

217   [56]. So far, eight families with FH-IV have been described.

218 The gene *CACNA1H* is located on chromosome 16 and encodes the T-type (low voltage activated)

219 calcium channel subunit Cav3.2. This protein is expressed in the zona glomerulosa [55,57] and, as other

220 Cav3 family members, is activated by small depolarizing changes in the membrane potential [58].

221 Germline *CACNA1H* mutations have been associated with several diseases including epilepsy, autism and

222 amyotrophic lateral sclerosis [59–61]. In their studies, Scholl et al. and Daniil et al. reported six index

223 cases with germline mutations affecting the residue Met1549, four cases with an inherited p.Met1549Val

224 substitution, one with a *de novo* p.Met1549Val and one with a *de novo* p.Met1549Ile [55,56]. This residue

225 is located in the transmembrane segment S6 of the repeat domain III of Cav3.2, forming a conserved

226 methionine-phenylalanine-valine (MFV) tripeptide motif that controls channel inactivation [62].

227 Functional experiments have demonstrated that mutations in Met1549 result in a decrease in the

228 inactivation of Cav3.2 compared with the wild-type protein. As a consequence, the channel remains open

229 longer with an increase in Ca^{2+} influx, which activates the expression of *CYP11B2* and other steroidogenic

230 genes [55,56,63]. Noteworthy, treatment with a T-type calcium channel blocker abrogated the aberrant

231 CYP11B2 activation and aldosterone production in HAC15 cells overexpressing Cav3.2 p.Met1549Val

232 mutant channels, which indicates that drugs of this class could be useful in the treatment of patients with

233 FH-IV [63].

234 In their study, Daniil et al. reported 3 additional variants: p.Ser196Leu, located in the voltage sensor

235 region on the transmembrane segment S4 of the repeat domain I of Cav3.2, in a male patient and his sister;

236 p.Pro2083Leu, located in the C-terminal cytoplasmic domain, in another index case and his brother; and a

237 *de novo* p.Val1951Glu, also located in the C-terminal domain, in a patient with an APA (no familial

238 history available). All mutations altered Cav3.2 function and enhanced aldosterone production to a greater

239 or a lesser degree [56].

240 Although further studies are needed, available data suggests FH-IV may be a rare form of FH. It follows

241 an autosomal dominant pattern of heritability but with reduced penetrance, particularly in adults. Indeed,

242 some family members with mutations in p.Met1549 were affected with resistant hypertension and PA and

243 others displayed milder or even normotensive phenotype, suggesting that other factors, such as genetic

244 modifiers, somatic mosaicism or the age of the patient, could restrain the gene defect [55]. The type and

245 location of the mutation may also play a role in the pathophysiology of FH-IV, resembling what has been

246 described for *KCNJ5* [46]. This fact could also explain the differences on disease presentation among the

247 index cases: some of them were florid cases of PA at their early childhood but without evidence of adrenal

248 hyperplasia; while other patients were diagnosed in their adulthood, nodularity was detected bilaterally in

249 one patient and an APA was diagnosed in another case [55,56].

250

Other germline mutations described in patients with PA

Although not considered established causes of FH, it is worth mentioning that germline mutations in *CACNA1D* and *ARMC5* have been reported in patients with PA.

CACNA1D codes for Cav1.3, an L-type (high-voltage activated) Ca^{2+} channel subunit and is recurrently mutated in about 10% of sporadic APAs. Most sporadic alterations cause gain of function and lead to an increase of Ca^{2+} influx and the consequent overproduction of aldosterone [57]. Recently, Scholl and coworkers identified two *de novo* mutations in two unrelated cases diagnosed with PASNA (PA associated with seizures and neurological abnormalities) [57]. Although the severe comorbidities of affected individuals make the heritability of PASNA very unlikely, it is tempting to speculate that other *CACNA1D* mutations that cause a milder phenotype could be involved in a still not described familial form of PA, in the same way that has been proposed for *KCNJ5* in FH-III.

ARMC5 encodes an apoptosis regulator that belongs to the armadillo/β-catenin-like repeat superfamily. Inactivating mutations in *ARMC5* have been reported in both sporadic and inherited primary bilateral macronodular hyperplasia, an adrenocortical disease associated with cortisol excess [64–66]. Mutated ARMC5 promotes cell survival and cortisol production *in vitro* [64,65]. Interestingly, germline *ARMC5* variants have been identified in patients with apparent sporadic cases of PA [67,68], suggesting a possible inherited predisposition for nodule formation prior to the hormonal-producing phenotype. Nevertheless, the deleterious effect of those mutations is still quite unclear, as most variants are predicted to be unlikely pathogenic [69]. Thus, further studies must confirm or refuse the possible role of *ARMC5* germline mutations in the etiology of PA.

New genes, new phenotypes - We need a new classification!

In the recent years, our knowledge on inherited forms of PA has progressed substantially [8,70]. FH classification has evolved from two clinically distinct forms (FH-I and FH-II) described in the previous Endocrine Society guideline [71] to at least four genetically defined types in which patients are grouped based on the presence of causative mutations (FH-I/*CYP11B1/B2* chimera, FH-II/*CLCN2*, FH-III/*KCNJ5* and FH-IV/*CACNA1H*). Despite substantial scientific advances, some questions remain unanswered. Firstly, the clinical heterogeneity within groups of FH related to variable disease presentation and incomplete penetrance suggest a possible modulation of genetic causes by non-genetic factors. This hypothesis could explain why relatives with germline mutations are apparently asymptomatic. Secondly, the prevalence of FH-II and FH-IV families is still uncertain. Evidence suggests that the frequency of *CLCN2* and *CACNA1H* mutations is low. Thus, extensive studies are needed to determine the actual

283 prevalence and the clinical relevance of these subtypes. Lastly, it must be elucidated whether apparent

284 familial cases without known mutations truly follow inherited patterns of PA. Further next-generation

285 sequencing studies will gain insight into the molecular causes of PA and probably to contribute to the

286 establishment of new FH types. Misclassification of sporadic PA cases as FH should be avoided. For that

287 reason, we discourage the use of non-genetic criteria for the screening and classification of FH and

288 propose the term "familial hyperaldosteronism" only to be used when known germline mutations are

289 detected.

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302 **Figure 1. Genetic causes of familial hyperaldosteronism.** Summary of the known molecular

303 mechanisms that lead to familial hyperaldosteronism types I to IV. FH-I is produced by an asymmetrical

304 recombination between *CYP11B1* and *CYP11B2*, resulting in the expression of a chimeric enzyme under

305 the control of ACTH stimulation. FH-II is caused by germline mutations in the chloride channel *CLCN2*

306 that decrease intracellular Cl^-. FH-III is produced by germline mutations in *KCNJ5* that affect the

307 selectivity of the channel, allowing Na^+ conductance. Both reduction of intracellular Cl^- and increase in

308 Na^+ cause plasma membrane depolarization and open voltage-gated Ca^{2+} channels, elevating cytosolic

309 Ca^{2+}. FH-IV is caused by germline mutations in *CACNA1H* that facilitate Ca^{2+} entry. In all cases, increase

310 of intracellular Ca^{2+} triggers *CYP11B2* transcription and aldosterone synthesis.

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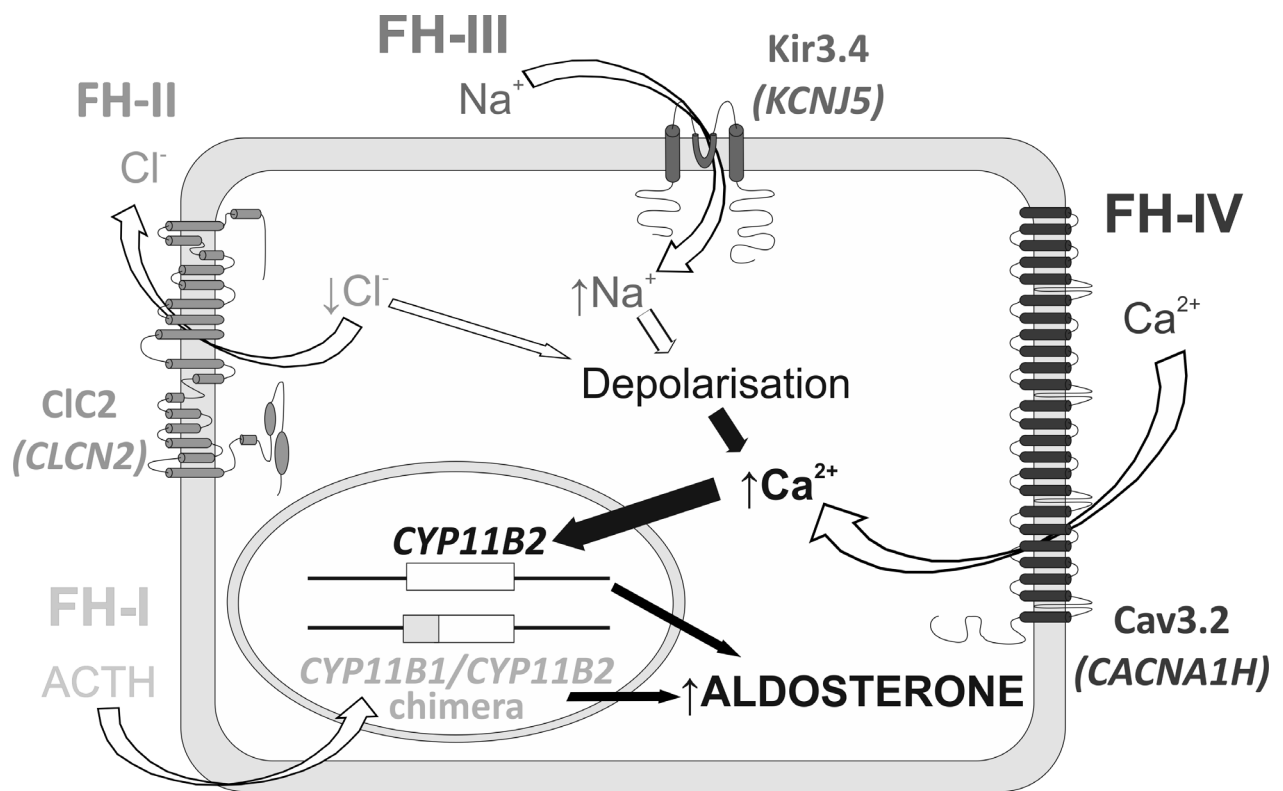
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