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Géraldine Vitellius, Marc Lombes

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1 **Partial glucocorticoid resistance in the pathophysiology of adrenal**  
2 **cortex hyperplasia.**

3 Géraldine VITELLIUS<sup>1,2</sup> and Marc LOMBES<sup>1,3</sup>

4 <sup>1</sup>INSERM UMR\_S U1185, Fac Med Paris-Sud, Univ. Paris-Sud, Université Paris-Saclay, Le  
5 Kremlin Bicêtre, F-94276, France;

6 <sup>2</sup>Service d'Endocrinologie-Diabète-Nutrition, Hôpital Robert Debré, CHU Reims, F-51100,  
7 France;

8 <sup>3</sup>Service d'Endocrinologie et des Maladies de la Reproduction, Hôpitaux Universitaires Paris  
9 Sud, AH-HP, CHU Bicêtre, F-94275, Le Kremlin Bicêtre, France.

10 Address all correspondence and requests for reprints to:

11 Dr Marc Lombès, MD, PhD, Inserm U1185, Faculté de Médecine Paris–Sud,  
12 63, Rue Gabriel Péri, F-94276 Le Kremlin Bicêtre Cedex France.

13 E-mail: marc.lombes@u-psud.fr.

14

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16 **Key words:** *NR3C1*, glucocorticoid signaling, adrenal incidentalomas, glucocorticoid  
17 resistance

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19 **Abstract**

20 The pathophysiology of Bilateral Adrenal Hyperplasia (BAH) remains obscure. Even though  
21 several genetic causes have been associated with this disease, often related to  
22 overactivation of the cAMP signaling pathway, cortisol regulation by autocrine adrenal ACTH  
23 production has also been proposed to enhance corticosteroidogenesis, cortisol secretion and  
24 adrenal cortex growth. Recently, heterozygous glucocorticoid receptor (GR) loss-of-function  
25 mutations have been discovered in patients with BAH, highlighting a potential new genetic  
26 cause of adrenal hyperplasia, as previously reported in a mouse model of GR  
27 haploinsufficiency. However, the molecular mechanisms linking adrenal hyperplasia and GR  
28 haploinsufficiency need to be further elucidated.

## 29 **1 Introduction**

30 Morphological criteria to define adrenal hyperplasia remain scarce. Even though the  
31 prevalence of adrenal mass incidentally discovered during imaging or adrenal incidentaloma  
32 (AI), varies between 1-8% following autopsy studies, it seems to increase with age (1). A  
33 large number of AI are nowadays discovered and frequently referred to a clinical  
34 endocrinologist. Recent guidelines (2) define benign AI with strict radiological criteria (3).  
35 Among AI, 15% are bilateral but mainly non-functional (1). Diagnosis of Bilateral Adrenal  
36 Hyperplasia (BAH) may be considered easy. However, the definition of normal adrenal  
37 glands is still controversial. Initially, normal adrenal glands were defined as a right lateral and  
38 medial limb measurement at 2.8 cm, with the left medial limb at 3.3 cm and lateral at 3.0 cm  
39 (4). More recently, adrenal volume assessed by computerized tomography (CT) was used to  
40 define normality (5). Left adrenal gland volume was estimated at  $4.5 \pm 1.6 \text{ cm}^3$  while the  
41 volume of the right adrenal gland was  $3.8 \pm 1.3 \text{ cm}^3$ . Volume measurements appear to be  
42 more reproducible among investigators compared with linear measurements. The  
43 association between BAH and steroid excess facilitates diagnosis and is important to  
44 recognize given the potential increased morbidity and mortality. Hypercortisolism occurs in  
45 12% of AI, while hyperaldosteronism or catecholamine excess is observed in 2.5% and in 7%  
46 of cases, respectively (2). Metabolic disorders (glucose intolerance, diabetes, hypertension,  
47 dyslipidemia and osteoporosis) can be associated with AI and subclinical hypercortisolism  
48 (2). Malignancy was described in 3% of cases (1) and congenital adrenal hyperplasia in 21%  
49 (6). A new nomenclature of BAH associated with Cushing' syndrome was recently published  
50 (7). When the nodule size is <1cm, thus referring to micronodular adrenals, the main cause  
51 is primary pigmented nodular adrenal disease (PPNAD). However, if their size exceeds 1 cm,  
52 primary bilateral macronodular adrenal hyperplasia (PBMAH) should be considered (8).

53 In this review, we will discuss the pathogenesis of BAH in the context of subclinical  
54 hypercortisolism, highlighting the recently discovered *NR3C1* mutations as recently  
55 discussed by Nicolaidis & Chrousos (9). Adrenal pheochromocytomas or  
56 hyperaldosteronism, carcinoma, metastasis or etiologies of unilateral AI were excluded.

57

## 58 **2. Adrenal hyperplasia.**

### 59 **2.1. Physiology of adrenal cortex**

60 Cortisol release is under the control of hypothalamic corticotropin releasing hormone (CRH).  
61 As illustrated in Fig. 1, CRH stimulates corticotrope cells of the anterior pituitary, which  
62 secrete adrenocorticotrophic hormone (ACTH). ACTH is one of the most potent physiological  
63 modulators of steroidogenesis and binds to its 7-transmembrane G protein-coupled receptor  
64 (GPCR) MC2R, expressed in all zones of the adrenal cortex. ACTH binding to MC2R, in  
65 association with melanocortin-2 receptors accessory proteins (MRAP), activated Gs protein,  
66 followed by activation of adenylyl cyclase, enhancing intracellular cAMP concentration (Fig.  
67 2), thus stimulating Protein Kinase A (PKA) signaling. PKA is a hetero-tetramer composed of  
68 2 catalytic subunits, encoded by three different catalytic subunits of which C $\alpha$  predominates  
69 in the adrenal gland plus 2 regulatory subunits, encoded by four genes (R1 $\alpha$ , R1 $\beta$ , R2 $\alpha$  and  
70 R2 $\beta$ ) (10). Binding of 4 cAMP molecules to PKA regulatory subunit dimers allows release  
71 and activation of the 2 PKA catalytic subunits, which phosphorylate several targets, such as  
72 cAMP response element (CRE) binding protein (CREB), or steroidogenic factor 1 (SF1),  
73 regulating StAR function. Phosphodiesterase, involved in cAMP degradation, act as negative  
74 regulators of this pathway.

75 StAR is the first step of steroidogenesis, involved in cholesterol flux into the inner  
76 mitochondrial membrane (11). Cortisol, the final steroidogenic compound, exerts a negative  
77 feedback loop on hypothalamic CRH and pituitary ACTH (Fig.1).

78 ACTH also plays a major role in the vasculature development *via* local synthesis of VEGF  
79 and in adrenal tissue growth. ACTH may be locally produced by medullary chromaffin cells,  
80 thus playing a major autocrine/paracrine role in the adrenal gland (12). Activation of  
81 cAMP/PKA signaling pathway may lead to hypercortisolism (13). A functional link between  
82 PKA and Wnt pathways was reported, as demonstrated by a cell-specific PKA-activated  
83 downregulation of  $\beta$ -catenin phosphorylation in H295R cells (14).

84

## 85 **2.2. Genetic abnormalities**

86 Every enzyme or protein involved in these pathways could be mutated and responsible for  
87 overactivation of signaling cascades resulting in hypercortisolism and adrenal hyperplasia.  
88 (summarized in Table 1).

### 89 **2.2.1 PPNAD**

90 PPNAD, an autosomal dominant manner disorder, is often observed in patients with  
91 Carney's complex. Seventy percent of index cases are familial, while the remaining cases  
92 are *de novo* germline mutations (15). One hundred and thirty-five pathogenic mutations have  
93 been reported to date. PPNAD is mainly related to germline inactivating mutations of the  
94 *PRKAR1A* gene, associated with constitutive activation of PKA (16), or phosphodiesterases  
95 *PDE11A* and *PDE8B* mutations (17). PPNAD can be associated with hypercortisolism  
96 leading to ACTH-independent Cushing syndrome. Of note, the glucocorticoid receptor (GR)  
97 was shown to be overexpressed in PPNAD nodules, underlying paradoxical dexamethasone  
98 (DXM) induction of cortisol secretion (18).

### 99 **2.2.2 PBMAH**

100 Expression of illicit receptors in the adrenal glands (GIP, LH/HCG, vasopressin,  
101 catecholamines, serotonin 5 HT, angiotensin II or glucagon) was first identified as a leading  
102 cause of regulatory mechanism of cortisol production in PBMAH. The prevalence of these  
103 illegitimate membrane receptors is rather high (up to 77-87% of cases), leading to  
104 cAMP/PKA pathway activation and enhanced corticosteroidogenesis (19). Intra-adrenal  
105 ACTH secretion responsible for an autocrine/paracrine regulation in cortisol production, was  
106 recently reported in PBMAH (20). Activating MC2R mutations (19), or point mutations of the  
107  $\alpha$  subunit types of G proteins responsible for altered GTPase activity (21) leading to  
108 constitutive activation of the cAMP/PKA pathway, have been also described less frequently  
109 in PMBAH. Recently, a new tumor suppressor gene, inactivation of Armadillo Repeat  
110 Containing Family (ARMC5), was discovered after whole genome sequencing of 26 patients  
111 with corticotropin-independent macronodular hyperplasia (22). ARMC5 was described in

112 familial and sporadic forms of PBMAH, but the mechanism by which ARMC5 was  
113 responsible for hypercortisolism and adrenal hyperplasia remains unknown (23). Recently,  
114 Lecoq *et al* (24), identified somatic duplications in the 19q13.32 chromosome region in three  
115 patients. These genetic rearrangements lead to a new genomic environment in which cis-  
116 regulatory regions, containing glucocorticoid response elements of the *ZMYND8* gene, drive  
117 ectopic expression of glucose-dependent insulinotropic polypeptide receptor (GIPR) in two  
118 GIP-dependent cortisol secreting adenomas and one adrenal tumor with PBMAH. PBMAH  
119 was also described in genetic syndromes, as multiple endocrine neoplasia type 1, or APC  
120 syndrome (1).

### 121 **2.2.3 Mutations of steroidogenesis players**

122 Mutations of important players, either transporters (StAR) or enzymes (CYP11A1, HSD3B2,  
123 CYP17A1, POR, CYP11B1 and CYP21A2) involved in steroidogenesis, are responsible for  
124 adrenal hyperplasia associated with a cortisol secretion defect and high levels of ACTH.

125

## 126 **3. Glucocorticoid resistance**

### 127 **3.1. Definition**

128 Glucocorticoid hormones (including Cortisol) regulate various biological functions involved in  
129 development, metabolism, inflammatory processes and stress. Their actions are mediated  
130 mainly by GR, a member of the nuclear receptor superfamily, which is an intracellular  
131 receptor protein acting as a ligand-activated transcription factor. Human GR (hGR) is  
132 encoded by the *NR3C1* gene (MIM#138040). GR may exist in multiple isoforms generated by  
133 alternative splicing: hGR $\alpha$ , hGR $\beta$ , hGR $\gamma$ , hGR-A and hGR-P. Eight alternative initiation sites  
134 into hGR $\alpha$  have been previously described (11, 12), leading to distinct GR protein variants  
135 (GR $\alpha$ -A to D3). GR $\alpha$ -A results from translation of GR $\alpha$  mRNA from the first AUG codon  
136 (Met<sup>1</sup>) while the shorter GR $\alpha$ -B results from translation beginning from the second AUG  
137 codon (Met<sup>27</sup>). Even though numerous GR variants have been discovered by next generation  
138 sequencing (NGS) methods (27), only twenty-eight *NR3C1* loss-of-function mutations have  
139 been clearly associated with a glucocorticoid resistance syndrome (MIM#615962) (28, 29,

140 30) while 30 index cases were studied (28, 29, 30). Eight patients out of 12 in whom an  
141 adrenal CT examination was performed, presented with BAH, bringing into question the role  
142 played by GR mutations and on the involvement of glucocorticoid resistance in the  
143 pathophysiology of adrenal hyperplasia.

144

### 145 **3.2. Role of GR in adrenal cortex**

146 The presence of GR in the normal adrenal gland remains controversial. Several authors  
147 reported a significant contribution of GR in the pathogenesis of hyperplastic glands (18, 31).  
148 Bourdeau *et al* (18) demonstrated, by RT-qPCR and immunohistochemistry, that PPNAD  
149 cells and some PBMAH cells overexpress endogenous GR. Later on, Louiset *et al* (31)  
150 showed, using PPNAD cells, the involvement of GR in a paradoxical response to cortisol by  
151 measuring cortisol release by RIA in culture supernatants (31). They demonstrated that DXM  
152 decreased cortisol production using a PKA inhibitor, indicating that DXM stimulates PKA  
153 catalytic subunits. The GR antagonist RU486 was found to decrease basal glucocorticoid  
154 production, suggesting that cortisol exerts an ultrashort positive feedback on its own  
155 production in PPNAD, *via* GR and supporting GR dependent up-regulation of PKA catalytic  
156 subunit genes (18).

157 More recently, our group demonstrated that GR directly impacts human adrenocortical  
158 H295R cell function by increasing key factors of steroidogenesis, including MC2R  
159 expression, as shown by pharmacological GR inhibition or RNA interference strategy (32).  
160 Finally, the presence of GR was confirmed in human normal adrenal tissue by RT-PCR and  
161 WB (33), while the GR $\alpha$ -D isoform, that had a reduced transcriptional activity compared to  
162 GR $\alpha$ -B, was reported in normal and adrenal cortex tumors (34). Later, it was also  
163 demonstrated that an overexpression of GR variant: A3669G and *BclI* polymorphism, can be  
164 associated with AI. The *BclI* polymorphism was also overrepresented in patients with BAH  
165 (35). To conclude, the involvement of GR in adrenal cortex and in the pathogenesis of  
166 different diseases with adrenal hyperplasia remains unclear, but its link with different  
167 signaling pathways in the adrenal cortex needs to be further elucidated. To address this



168 issue, we recently used CRISPR/Cas 9 technology to introduce a GR loss-of-function  
169 mutation, previously functionally characterized (36), in human H295R cells.

170

#### 171 **4. BAH in patients with *NR3C1* mutations**

172 The French National Hospital Clinical Research Program (PHRC), Muta-GR, was launched  
173 to determine the prevalence of *NR3C1* mutations in a cohort of one hundred patients  
174 presenting with BAH, associated with high blood pressure (HBP) and/or biological  
175 hypercortisolism without Cushing features. Forty patients had abnormal DXM suppression  
176 tests (plasma cortisol concentration remains >50 nmol/L) and high urinary free cortisol levels  
177 sometimes associated with unsuppressed ACTH levels. Five novel heterozygous *NR3C1*  
178 mutations were discovered, among which three have been already functionally characterized  
179 (36, 37), leading to the 5% prevalence of GR mutations. The phenotype of glucocorticoid  
180 resistant patients was much milder than the initial description (Table 2) (38). We recently  
181 unraveled the molecular mechanisms of defective GR-dependent regulation of 11 $\beta$ -HSD2 in  
182 patients carrying GR loss-of-function mutations, associated with  
183 pseudohypermineralocorticoidism (39). These findings emphasize the importance of GR genetic  
184 screening in selected patients enabling their appropriate management and optimized follow-  
185 up. Primary fibroblast cultures were obtained after skin biopsy in four mutated patients  
186 (R469X, R477S, R491X, Q501H GR mutations) and GR haploinsufficiency was further  
187 demonstrated by a reduced DXM induction of FKBP5 mRNA levels, consistent with  
188 glucocorticoid resistance observed in the mutated patients.

189 The study of the first heterozygous GR stop mutation, R469X, was very informative (37).  
190 Indeed, among 8 mutated family members spanning three generations, four had BAH,  
191 among them a 9-y-old child, suggesting the involvement of GR haploinsufficiency in the  
192 pathogenesis of BAH.

193 However, the exact mechanisms by which GR haploinsufficiency could modulate adrenal  
194 hyperplasia remain unknown. Up to now, it was proposed that ACTH overstimulation of the  
195 adrenal gland accounts for BAH in the context of GR loss-of-function mutations, supported

196 by several human studies, including three heterozygous GR mutations associated with BAH  
197 and high ACTH levels (29, 35, 40). This was also reminiscent of the homozygous *Nr3c1*  
198 gene inactivation in mouse models (41) in which 90% of these mice died 1-2 h postnatally,  
199 while an aberrant GR binding site was recovered in different tissues of the 10% of mouse  
200 survivors despite overt glucocorticoid resistance (42). An enlarged adrenal gland size and  
201 disorganized adrenocortical cells were reported in homozygous *GRKO* mice that presented  
202 with high corticosterone and ACTH levels, consistent with altered HPA regulation (41).  
203 However, a heterozygous GR mouse model was recently published in which one functional  
204 GR allele, responsible for GR haploinsufficiency, also had enlarged adrenal glands (29) but  
205 normal ACTH levels, precluding an exclusive role of high circulating ACTH levels in the  
206 pathogenesis of adrenal hyperplasia.

207

## 208 **5. Conclusion**

209 Adrenal hyperplasia is often associated with overactivation of cAMP signaling pathways,  
210 related to well-characterized GPCR or other genetic alterations. Given that GR is expressed  
211 in the adrenal cortex and owing to the recent discovery of heterozygous GR mutations in  
212 BAH patients, it is likely that GR haploinsufficiency might affect adrenal function and  
213 proliferation. The molecular mechanisms involved remain to be elucidated. Genome editing  
214 technology may help unravel these issues.

215 **Disclosure**

216 The authors declare that there is no conflict of interest that could be perceived as prejudicing  
217 the impartiality of this study.

218

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223

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234

235 **Abbreviations:**

236 cAMP, cyclic adenosine monophosphate; ACTH, adrenocorticotrophic hormone; AI, adrenal  
237 incidentaloma; BAH, bilateral adrenal hyperplasia; CAH, congenital adrenal hyperplasia;  
238 CRH, corticotropin releasing hormone; DXM, dexamethasone; DST, DXM suppression test;  
239 GIP, glucose-dependent insulintropic polypeptide; GR, Glucocorticoid receptor; PBMAH,  
240 primary bilateral macronodular adrenal hyperplasia; PHRC, Hospital Clinical Research  
241 Program; PPNAD, primary pigmented nodular adrenal disease; PKA, protein kinase A; UFC,  
242 Urinary-free Cortisol.

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313 R115-138. (doi:10.1530/EJE-13-0308) **This article constitutes an exhaustive review**  
314 **of autocrine/paracrine actors of adrenal hyperplasia.**

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472 **haploinsufficiency.**
- 473

474 **Figure Legends**

475 **Figure 1: Hypothalamus-Pituitary-Adrenal gland axis**

476 Cortisol release is under the control of hypothalamic corticotropin releasing hormone (CRH).  
477 CRH stimulates secretion of pituitary adrenocorticotrophic hormone (ACTH) which is  
478 responsible the production of cortisol by adrenal cortex. Paracrine/autocrine regulation of  
479 cortisol and ACTH secretion in adrenal glands has also been described (20, 33). Cortisol  
480 exerts a negative feedback loop on hypothalamic and pituitary cells.

481

482 **Figure 2: Steroidogenesis in adrenal cells**

483 ACTH binds its MC2R receptor and activates a cAMP pathway leading to PKA activation  
484 through release and activation of the 2 PKA catalytic subunits. Subsequent phosphorylation  
485 of several targets allows StAR activation.

486

487 **Figure 3: Bilateral adrenal hyperplasia in mutated GR patients**

488 Examples of CT images in BAH patients; **(A)** left and right, lateral and medial limb >5mm  
489 without nodules;**(B)** nodules and right and left lateral and medial limb >5 mm; **(C)** Adrenal  
490 macronodules >10 mm.

491

492 **Table 1:Genetic alteration involved in adrenal hyperplasia**

	Gene	Bilateral adrenal hyperplasia
ACTH pathways	PRKAR1A	PPNAD
	PDE11A	PPNAD
	PDE8B	PPNAD and BAH
	MC2R	PBMAH
	GNAS	PBMAH
	ARMC5	PBMAH
	NEM 1	PBMAH
	FH	PBMAH
Steroidogenesis enzyme	StAR	lipoid CAH
	CYP11A1 (P450scc)	lipoid CAH
	HSD3B2	CAH
	CYP17A1	CAH
	POR	CAH
	CYP11B1	CAH
	CYP21A2	CAH
GR pathways	NR3C1	BAH
	chromosome 19q13 microduplication	BAH
Wnt/ $\beta$ catenin pathway	CTNNB1	PBMAH
	APC	PBMAH

493

494 Genes involved in adrenal hyperplasia and corresponding altered signaling pathways.

495 ACTH, adrenocorticotrophic hormone; BAH, bilateral adrenal hyperplasia; CAH, congenital  
 496 adrenal hyperplasia; GR, Glucocorticoid receptor; PBMAH, primary bilateral macronodular  
 497 adrenal hyperplasia; PPNAD, primary pigmented nodular adrenal disease.

498

499 **Table 2: Phenotype comparison between mutated GR patients described in Muta-GR**  
 500 **study (35) and the first mutated GR patient (38)**

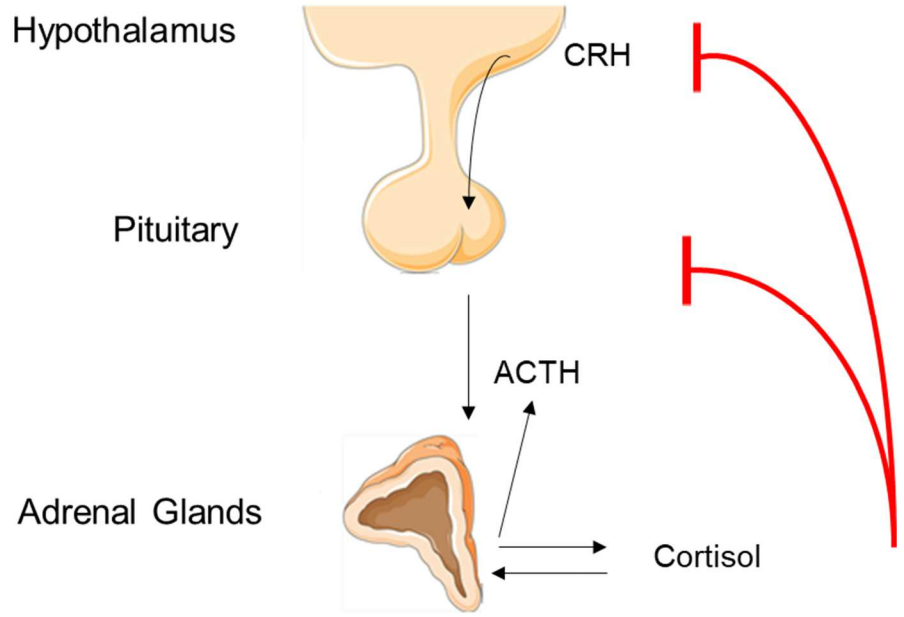
501

	<b>Mutated patients Muta-GR study</b>	<b>First described patient</b>	<b>Normal range</b>
Type of Mutation	Heterozygous	Homozygous	
Age (Years)	53.2 ± 7.7	58	
BMI (kg/m <sup>2</sup> )	30.7 ± 9.5	24.01	<25
SBP (mmHg)	123 ± 4.5	190	<140
DBP (mmHg)	72 ± 4.5	130	<90
Kalemia (mmol/L)	3.6 ± 0.2	↘	3.5-5
Aldosterone (pg/mL)	17.3 ± 9.9	↘	19-117
Renin (pg/mL)	47.2 ± 92.6	Normal	3-16
Adrenal Hyperplasia	Yes	No	
8-AM ACTH (pg/mL)	N	2.1N	<1
UFC (ULN)	1.7 ± 0.65	30-40	<1
DST	–	–	

502

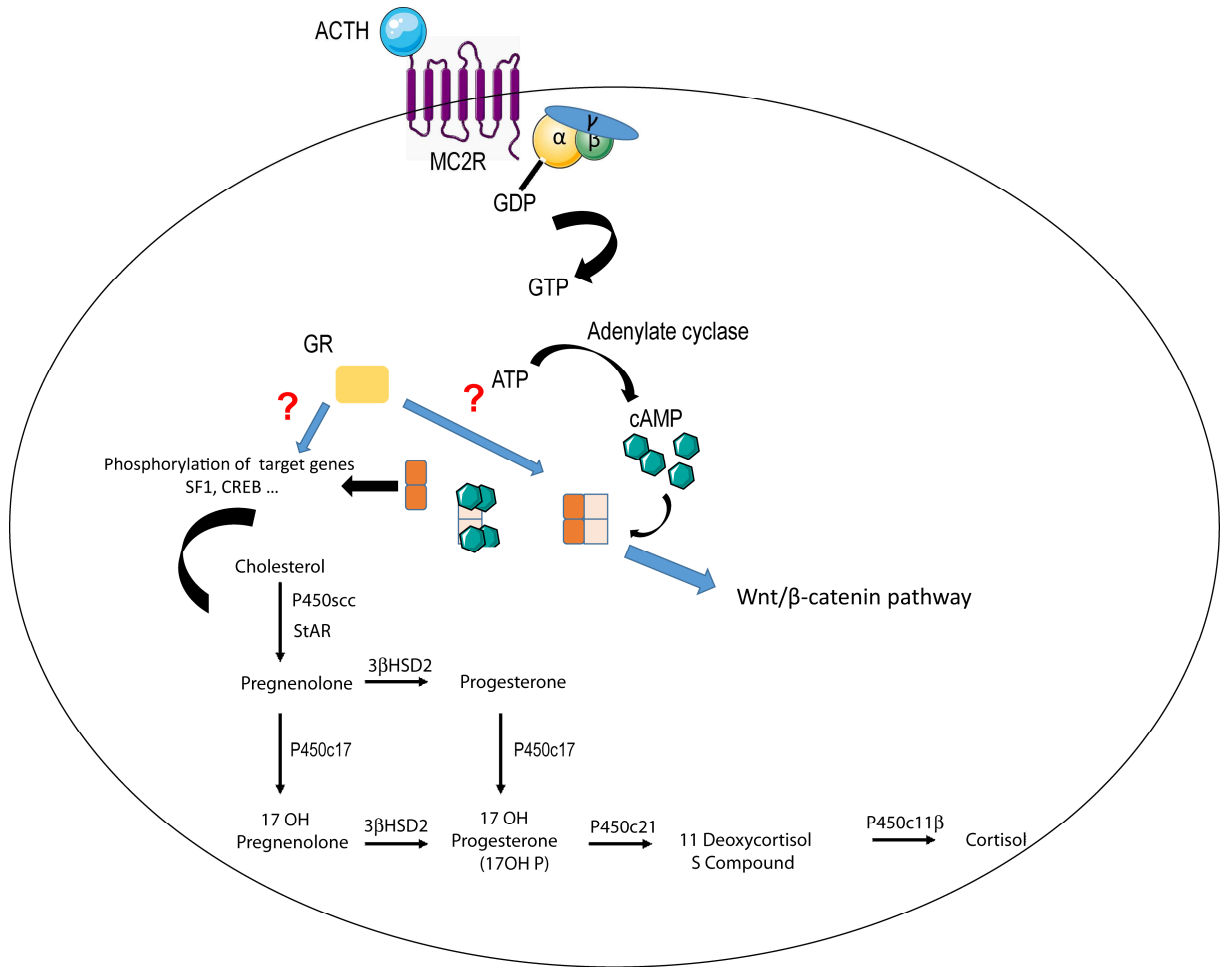
503 BMI: Body mass index; SBP / Systolic Blood Pressure; DBP: Diastolic Blood Pressure; UFC:

504 Urinary Free Cortisol, DST: 1 mg DXM suppression test , –: negative.



505

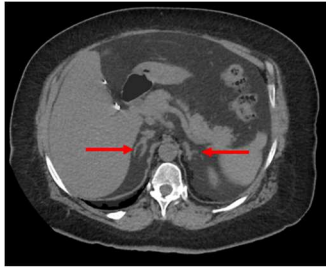
506 **Fig. 1**



507

508 **Fig. 2**

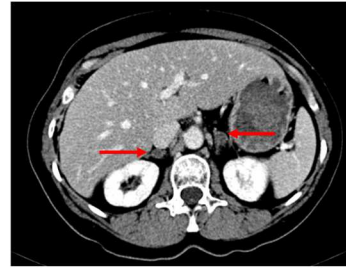
509



Hyperplasia limb



nodular and hyperplasia limb



nodular adrenal

510

511 **Fig. 3**