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1 **Identification of predictive criteria for pathogenic variants of the Primary Bilateral**
2 **Macronodular Adrenal Hyperplasia gene *ARMC5* in 352 unselected patients.**

3

4 **Authors:**

5 Lucas Bouys¹, Anna Vaczlavik^{1,2}, Anne Jouinot^{1,2,3}, Patricia Vaduva^{1,4}, Stéphanie Espiard⁵,
6 Guillaume Assié^{1,2}, Rossella Libé^{1,2}, Karine Perlemoine¹, Bruno Ragazzon¹, Laurence
7 Guignat², Lionel Groussin^{1,2}, Léopoldine Bricaire², Isadora Pontes Cavalcante¹, Fidéline
8 Bonnet-Serrano^{1,6}, Hervé Lefebvre⁷, Marie-Laure Raffin-Sanson⁸, Nicolas Chevalier⁹,
9 Philippe Touraine¹⁰, Christel Jublanc¹¹, Camille Vatier¹², Gérald Raverot¹³, Magalie
10 Haissaguerre¹⁴, Luigi Maione¹⁵, Matthias Kroiss^{16,17}, Martin Fassnacht¹⁶, Sophie Christin-
11 Maitre¹², Eric Pasmant^{1,18}, Françoise Borson-Chazot¹³, Antoine Tabarin¹⁴, Marie-Christine
12 Vantyghem⁵, Martin Reincke¹⁷, Peter Kamenicky¹⁵, Marie-Odile North¹⁸, Jérôme Bertherat^{1,2}
13 and the COMETE and ENSAT networks groups and collaborators.

14

15 COMETE and ENSAT networks groups and collaborators: Ana Agapito, Laurence Amar,
16 Anna Angelousi, Wiebke Arlt, Felix Beuschlein, Eric Bruckert, Thierry Brue, Laure Cazabat,
17 Olivier Chabre, Trevor Cole, Delphine Drui, Anne-Paule Gimenez-Roqueplo, Anne-Marie
18 Guedj, Véronique Kerlan, Marcus Quinkler, Isabelle Raingard, Patrice Rodien, Igor
19 Tauveron, Massimo Terzolo, Delphine Vezzosi, Géraldine Vitellius.

20

21 **Affiliations:**

22 ¹Université de Paris, Institut Cochin, Inserm U1016, CNRS UMR8104, F-75014, Paris,
23 France

24 ²Department of Endocrinology and National Reference Center for Rare Adrenal Disorders,
25 Hôpital Cochin, Assistance Publique Hôpitaux de Paris, F-75014, Paris, France

26 ³Institut Curie, INSERM U900, MINES ParisTech, PSL-Research University, CBIO-Centre
27 for Computational Biology, F-75005, Paris, France

28 ⁴Department of Endocrinology, Diabetology and Nutrition, CHU Rennes, F-35000, Rennes,
29 France

30 ⁵Department of Endocrinology, Diabetology, Metabolism and Nutrition, CHRU Lille, Inserm
31 U1190, F-59000, Lille, France

32 ⁶Unit of Hormonology, Hôpital Cochin, Assistance Publique Hôpitaux de Paris, F-75014,
33 Paris, France

34 ⁷Department of Endocrinology, Diabetes and Metabolic Diseases, CHU Rouen, F-76000,
35 Rouen, France

36 ⁸Department of Endocrinology, Diabetology and Nutrition, Hôpital Ambroise Paré,
37 Assistance Publique Hôpitaux de Paris, F-92100, Boulogne-Billancourt, France

38 ⁹Department of Endocrinology, Diabetology and Reproduction, CHU Nice, F-06000, Nice,
39 France

40 ¹⁰Department of Endocrinology and Reproduction, Hôpital Pitié-Salpêtrière, Assistance
41 Publique Hôpitaux de Paris, F-75013, Paris, France

42 ¹¹Department of Endocrinology and Metabolism, Hôpital Pitié-Salpêtrière, Assistance
43 Publique Hôpitaux de Paris, F-75013, Paris, France

44 ¹²Department of Endocrinology, Diabetology and Reproduction, Hôpital Saint-Antoine,
45 Assistance Publique Hôpitaux de Paris, F-75012, Paris, France

46 ¹³Department of Endocrinology, Groupement Hospitalier Est, Hospices Civils de Lyon, F-
47 69500, Bron, France

48 ¹⁴Department of Endocrinology, Diabetology and Nutrition, Hôpital Haut-Lévêque, CHU
49 Bordeaux, F-33000, Bordeaux, France

50 ¹⁵Université Paris-Saclay, Inserm, Physiologie et Physiopathologie Endocriniennes,
51 Department of Endocrinology and Reproduction, Reference Center for Rare Pituitary
52 Diseases, Hôpital Bicêtre, Assistance Publique Hôpitaux de Paris, F-94275, Le Kremlin-
53 Bicêtre, France

54 ¹⁶Department of Internal Medicine I, Division of Endocrinology and Diabetes, University
55 Hospital of Würzburg, University of Würzburg, 97080, Würzburg, Germany

56 ¹⁷Medizinische Klinik und Poliklinik IV, Klinikum der Universität München, 80336, Munich,
57 Germany

58 ¹⁸Unit of Oncogenetics, Hôpital Cochin, Assistance Publique Hôpitaux de Paris, F-75014,
59 Paris, France

60

61

62 **Corresponding author:** Prof Jérôme Bertherat, Department of Endocrinology and National
63 Reference Center for Rare Adrenal Disorders, Hôpital Cochin, Assistance Publique Hôpitaux
64 de Paris, 27 rue du Faubourg Saint-Jacques, F-75014, Paris, France;
65 jerome.bertherat@aphp.fr

66

67

68 **Short title:** *ARMC5* in Macronodular Adrenal Hyperplasia.

69

70

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72 *ARMC5*, Cushing syndrome, genetic screening, tumor suppressor gene.

73

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75 **Word count:** 4493.

76

77 **Abstract**

78

79 **Objective:** Primary Bilateral Macronodular Adrenal Hyperplasia (PBMAH) is a
80 heterogeneous disease characterized by adrenal macronodules and variable levels of cortisol
81 excess, with not clearly established clinical diagnostic criteria. It can be caused by *ARMC5*
82 germline pathogenic variants. We aimed to identify predictive criteria for *ARMC5* variants.

83 **Methods:** We included 352 consecutive index patients from 12 European centers, sequenced
84 for germline *ARMC5* alteration. Clinical, biological and imaging data were collected
85 retrospectively.

86 **Results:** 52 patients (14.8%) carried *ARMC5* germline pathogenic variants and showed a
87 more distinct phenotype than non-mutated patients for cortisol excess (24-hour urinary free
88 cortisol 2.32 vs 1.11-fold ULN, respectively, $p < 0.001$) and adrenal morphology (maximal
89 adrenal diameter 104 vs 83 mm, respectively, $p < 0.001$); and were more often surgically or
90 medically treated (67.9 vs 36.8%, respectively, $p < 0.001$). *ARMC5*-mutated patients showed a
91 constant bilateral adrenal involvement and at least a possible autonomous cortisol secretion
92 (defined by a plasma cortisol after 1 mg dexamethasone suppression above 50 nmol/L) while
93 these criteria were not systematic in wild-type patients (78.3%). The association of these two
94 criteria holds a 100% sensitivity and a 100% negative predictive value for *ARMC5* pathogenic
95 variant.

96 **Conclusion:** We report the largest series of index patients investigated for *ARMC5* and
97 confirm that *ARMC5* pathogenic variants are associated with a more severe phenotype in most
98 cases. To minimize negative *ARMC5* screening, genotyping should be limited to clear
99 bilateral adrenal involvement and autonomous cortisol secretion, with an optimum sensitivity
100 for routine clinical practice. These findings will also help to better define PBMAH diagnostic
101 criteria.

103 **Introduction**

104

105 Primary Bilateral Macronodular Adrenal Hyperplasia (PBMAH) is a rare (1) and highly
106 heterogeneous disease, its definition is equivocal but can be summarized by the existence of
107 bilateral benign adrenal nodules, measuring >1 cm, which can be causative of an adrenal
108 Cushing syndrome (2). Taking into account the small proportion of patients showing overt
109 hypercortisolism, PBMAH is mostly incidentally diagnosed on abdominal imaging. Thus, its
110 frequency is probably underestimated.

111 PBMAH can be isolated or associated with other disorders in dominantly inherited diseases
112 such as multiple endocrine neoplasia type 1 (3), familial adenomatous polyposis (4) and
113 hereditary leiomyomatosis and renal cell cancer (5), consecutive to germline inactivating
114 pathogenic variants of *MEN1*, *APC* and *FH* genes, respectively; or in McCune-Albright
115 syndrome (6) due to post-zygotic activating variants of *GNAS* gene.

116 In its isolated form, which is the most frequent presentation, the bilateral nature of adrenal
117 nodules and the description of familial cases suggest a genetic predisposition.

118 In 2013 frequent germline inactivating variants in the *armadillo repeat containing 5 (ARMC5)*
119 gene have been identified in 55% of 33 patients showing PBMAH that caused severe Cushing
120 syndrome treated by adrenalectomy (7). The inactivation model of *ARMC5* suggests that it
121 acts as a tumor suppressor gene: a first event inactivates one allele at the germline level, and a
122 second event happens at the somatic level, in the tumor DNA, leading to a bi-allelic
123 inactivation of *ARMC5* (7,8).

124 To date, more than eighty different *ARMC5* germline pathogenic variants have been
125 described, most of them are private, spread all over the exonic sequence, with only few
126 identified hotspots.

127 *ARMC5* protein function is not well characterized, but its inactivation seems to alter
128 apoptosis, leading to tumor growth (7,9,10).

129 According to the recent literature, the mutation rate of *ARMC5* is around 80% in case of a
130 clear familial presentation of PBMAH (11,12), 50% in patients clinically considered to
131 require surgery (7), and around 20 to 25% in research series with both operated and non-
132 operated patients (9,13–15). In these series, *ARMC5* pathogenic variants are associated with a
133 more distinct phenotype in terms of intensity of cortisol excess, adrenal size and number of
134 nodules (9,13).

135 The aim of this study is to improve the clinical characterization of these patients and to
136 identify clinical, biochemical or imaging criteria predictive for *ARMC5* pathogenic variant,
137 which could be helpful to establish a better clinical definition of PBMAH.

138

139

140 **Patients and Methods**

141

142 *Patients*

143 Since 2013, leukocyte DNA of 498 French and European index case patients have been sent
144 to Cochin hospital for *ARMC5* genotyping. In order to facilitate data centralization, we
145 selected all the European centers from which leukocyte DNA of at least five adult index
146 patients were sent for *ARMC5* genotyping since 2013, regardless of their hormonal status and
147 adrenal morphology (unilateral adrenal involvement was not an exclusion criterion). Thus,
148 352 consecutive unrelated patients were investigated, from the Endocrinology departments of
149 ten French (Cochin, Bicêtre, Lille, Bordeaux, Lyon, Saint-Antoine, Pitié-Salpêtrière,
150 Ambroise Paré, Nice and Rouen) and two German (Munich and Würzburg) university
151 hospitals. The study was approved by the ethic committees of Ile de France I, Ludwig-

152 Maximilians-University, Munich and Medical Faculty, University of Würzburg, Würzburg.
153 Written informed consent for genetic analysis was obtained from the patients. All the data
154 were de-identified.

155

156 *Data collection*

157 The clinical, biological and radiological data were retrospectively collected from the recorded
158 files. Clinical data included sex, age at diagnosis, body mass index (BMI), presence or
159 absence of treated hypertension, diabetes or impaired glucose tolerance, osteoporosis, and
160 history of venous thrombo-embolism. The biological assessment for cortisol excess included
161 24-hour urinary free cortisol (UFC), 08:00 AM plasma cortisol after 1 mg dexamethasone
162 overnight suppression test (1 mg DST), midnight plasma cortisol and 08:00 AM ACTH. For
163 the radiological assessment, the measurement of adrenal size (summed size of the longest
164 diameter of the two adrenals) and the number of adrenal nodules were recorded on adrenal
165 computed tomography (CT) scan. CT-scans were centrally reviewed by a single examiner,
166 blinded for the genetic status, for the 255 patients with available data from Cochin, Bicêtre,
167 Lille, Bordeaux and Lyon hospitals. For the 58 patients from the seven remaining centers for
168 whom CT-scan was available, the adrenals' size was collected from the recorded files based
169 on adrenal imaging, without central reviewing. Bilateral adrenal involvement was defined by
170 the presence of at least one nodule >1 cm in diameter on each adrenal; unilateral involvement
171 was defined by the presence of one or several nodules >1 cm in diameter on one adrenal, with
172 an apparent normal contralateral gland. For the patients who did not undergo adrenal surgery
173 or anti-cortisolic medical treatment, the last biological and radiological evaluation was used
174 for this analysis. For those who have been treated (surgical or medical treatment), the last
175 evaluation before the first treatment was used for this analysis.

176

177 ***DNA extraction***

178 Leukocytes were isolated from whole peripheral blood and DNA was extracted using the
179 DNA Isolation Kit for Mammalian Blood (ref. 11667327001, Roche Diagnostics GmbH,
180 Germany) as recommended in the standard protocol.

181

182 ***ARMC5 sequencing***

183 *ARMC5* sequencing for French patients has been performed by Sanger's method as previously
184 described (7) until July 2014, and by next-generation sequencing since August 2014, using the
185 Ion Personal Genome Machine system (Ion Torrent, Thermo Fisher Scientific, USA) between
186 August 2014 and March 2018, and a NextSeq 500 sequencer (Illumina) since April 2018.
187 Sanger's method has been used for all German patients. The generated sequences have been
188 aligned on the reference genome GRCh37/hg19 (Genome Reference Consortium / University
189 of California, Santa Cruz).

190

191 ***Variant nomenclature and interpretation***

192 Variants in the *ARMC5* gene are reported according to the reference sequence
193 NM_001105247.1 (canonical: ENST00000268314.9). Exon numbering is indicated according
194 to the NCBI nomenclature. Variants were named at the coding DNA and protein levels
195 according to the Human Genome Variation Society (HGVS) recommendations with the online
196 Mutalyzer software (Version 2.0.34; <https://mutalyzer.nl/name-checker>). An assessment of
197 variants' pathogenicity was performed according to the American College of Medical
198 Genetics and Genomics and the Association for Molecular Pathology (ACMG-AMP)
199 guidelines (16). Variants were classified in one out of the five classes given below: class 1:
200 benign variant, class 2: likely benign variant, class 3: variant of uncertain significance (VUS),
201 class 4: likely pathogenic variant, class 5: pathogenic variant. Assessment of variant

202 implication was mainly performed based on population databases (gnomAD,
203 <https://gnomad.broadinstitute.org>), variant databases (ClinVar,
204 <https://www.ncbi.nlm.nih.gov/clinvar/>), and prediction softwares. *In silico* predictions of the
205 effect of the variants were performed with Polyphen-2
206 (<http://genetics.bwh.harvard.edu/pph2/>) and SIFT version 2
207 (http://sift.jcvi.org/www/SIFT_enst_submit.html).

208

209 ***Predictive criteria for ARMC5 pathogenic variants***

210 In order to identify clinical predictive criteria for *ARMC5* pathogenic variants, sensitivity,
211 specificity, negative and positive predictive values were calculated for the following
212 parameters, isolated or combined: sex, hypertension, diabetes, need for treatment of cortisol
213 excess (anticortisolic medication or adrenal surgery), 24-hour UFC, plasma cortisol after 1 mg
214 dexamethasone suppression test, plasma ACTH, bilateral adrenal involvement, number of
215 adrenal nodules. The four patients harboring three different class 3 *ARMC5* variants (variants
216 of uncertain significance) were excluded from this analysis.

217

218 ***Statistical analysis***

219 Calculations were performed using R statistical software (v4.1.1.). Univariate logistic
220 regression models were used to test the association of clinical and biological variables with
221 *ARMC5* status. Quantitative variables are presented as mean values with standard deviation
222 (SD) and mean difference (MD) with 95% confidence intervals. Qualitative variables are
223 presented as percentage and odds ratio (OR) with 95% confidence intervals. Comparisons
224 between carriers of different types of *ARMC5* pathogenic variants (truncating vs non-
225 truncating) were performed using Mann-Whitney Wilcoxon test for quantitative variables and

226 Fisher's exact test for qualitative variables. All p-values were two-sided, and the level of
227 significance was set at $p < 0.05$.

228

229

230 **Results**

231

232 *Patient characteristics*

233 The clinical, hormonal and imaging characteristics of the 352 index case patients are
234 summarized in Table 1 and detailed in Supplementary Tables 1 and 2.

235

236 *ARMC5 genotyping*

237 Patients without *ARMC5* variation or harboring benign or likely benign missense or
238 synonymous *ARMC5* variants were considered as wild-type patients, as previously described
239 (9). Among the 352 analyzed patients, 52 (14.8%) showed 37 different *ARMC5* variants of
240 class 3, 4 or 5 (ACMG-AMP guidelines) and 300 had no *ARMC5* pathogenic variant. *ARMC5*
241 pathogenic variants were spread all over the coding sequence, most of them were private and
242 only few clear hotspots were identified: c.2290C>T p.(Arg764*), c.799C>T p.(Arg267*) and
243 c.2692C>T p.(Arg898Trp) in five, five and three patients, respectively (NM_001105247.1).
244 Missense and non-sense variants were the most frequent types of variants and were equally
245 distributed (36.5% for each), and 25% of variants were frameshift insertions or deletions and
246 2.0% of splice site pathogenic variants. All *ARMC5* variants of class 3, 4 and 5 are reported in
247 Supplementary Table 4.

248

249 *Demographic and anthropometric data*

250 In the total cohort, 230 patients (65.3%) were females and 122 (34.7%) were males, with a
251 more balanced repartition in the *ARMC5*-mutated group (57.7 and 42.3%, respectively) than
252 in the wild-type group (66.7 and 33.3%, respectively) but with no statistically significant
253 difference (OR=1.47 [0.80-2.67], p=0.211).

254 The mean age at diagnosis was 53.40 in *ARMC5*-mutated and 55.22 in wild-type patients
255 (MD=-1.82 [-5.19-1.55], p=0.272).

256

257 ***Clinical presentation***

258 In the total cohort, patients were more often investigated for adrenal incidentalomas identified
259 on abdominal imaging performed for another condition than for overt Cushing syndrome
260 (72.6 vs 27.4%). However, overt Cushing syndrome was a more frequent initial cause of
261 investigation for patients with *ARMC5* pathogenic variant compared to the wild-type patients
262 (43.1 vs 24.7%, respectively, OR=2.32 [1.25-4.28], p=0.007).

263

264 ***Hormonal investigations***

265 Patients harboring an *ARMC5* germline pathogenic variant had a more severe
266 hypercortisolism than wild-type patients, in terms of 24-hour urinary free cortisol (24-hour
267 UFC) (2.13 vs 1.15-fold ULN, respectively, MD=0.98 [0.28-1.67], p<0.001). An increased
268 24-hour UFC was observed in 58.8% of *ARMC5*-mutated patients and 31.1% of wild-type
269 patients (OR=3.17 [1.72-5.85], p<0.001). Morning plasma cortisol after 1 mg dexamethasone
270 overnight suppression test was significantly higher in *ARMC5*-mutated patients than wild-type
271 (340.45 vs 142.63 nmol/L, respectively, MD=197.82 [111.21-284.43], p<0.001).
272 Interestingly, 100% of *ARMC5*-mutated patients had a value of cortisol after 1 mg
273 dexamethasone suppression test above 50 nmol/L versus 79.7% of wild-type patients (non-
274 estimable OR), which is the threshold considered by the current ESE/ENSAT guidelines on

275 the management of incidentalomas to rule out autonomous cortisol secretion (17). Midnight
276 plasma cortisol was also significantly higher (313.01 vs 183.72 nmol/L, respectively,
277 MD=129.29 [46.33-212.26], $p<0.001$) and ACTH more suppressed (below 2 pmol/L in 86.0
278 vs 58.2%, respectively, OR=4.41 [1.92-10.15], $p<0.001$) in *ARMC5*-mutated patients than in
279 wild-type patients.

280

281 ***Adrenal morphology***

282 Bilateral adrenal macronodules (proven by pre-therapeutic CT-scan or post-operative
283 histology) were present in 100% of *ARMC5*-mutated patients and in 92.0% of wild-type
284 patients. Indeed, 24 wild-type patients harbored one (15 patients) or several nodules (9
285 patients) on one adrenal with an apparently normal contralateral gland. A pre-therapeutic
286 adrenal CT-scan was available for 313/352 patients, showing larger adrenals in *ARMC5*-
287 mutated patients than in wild-type patients (summed size of the longest diameter of the two
288 adrenals: 104.33 vs 82.84 mm, respectively, MD=21.49 [10.24-32.75], $p<0.001$) with more
289 numerous nodules (9.76 vs 3.18 nodules, respectively, MD=6.59 [4.01-9.16], $p<0.001$)
290 (Figure 1).

291

292 ***Correlation between adrenal size and levels of cortisol excess***

293 For *ARMC5*-mutated patients, adrenal size is significantly correlated with 24-hour UFC value
294 ($r=0.39$, $p=0.01$), and even more with plasma cortisol after 1 mg dexamethasone suppression
295 test ($r=0.66$, $p<0.001$). For wild-type patients, there is a weak correlation between adrenal size
296 and plasma cortisol after dexamethasone suppression ($r=0.16$, $p=0.017$), but no correlation
297 with 24-hour UFC ($r=-0.06$, $p=0.36$).

298

299 ***Clinical consequences of hypercortisolism***

300 *ARMC5*-mutated patients had more frequently hypertension requiring antihypertensive
301 medication than wild-type patients (87.8 vs 70.7%, respectively, OR=2.97 [1.22-7.23],
302 p=0.016). Diabetes mellitus was more frequent as well in *ARMC5*-mutated than in wild-type
303 patients (52.1 vs 35.3%, respectively, OR=2.00 [1.08-3.69], p=0.027).
304 Frequencies of osteoporosis and venous thrombo-embolism did not differ between the two
305 groups (24.2 vs 27.5%, OR=0.84 [0.36-1.99], p=0.696; and 10.9 vs 8.3%, OR=1.35 [0.49-
306 3.73], p=0.567, respectively).

307

308 ***Treatment***

309 In first intention, *ARMC5*-mutated patients have been more often treated in order to control
310 Cushing syndrome: 35 (67.3%) *ARMC5*-mutated patients were treated by surgery or cortisol
311 lowering drugs, vs only 112 (37.3%) wild-type patients (OR=3.46 [1.85-6.46], p<0.001). Both
312 surgery and cortisol lowering treatment were significantly more frequently applied in
313 *ARMC5*-mutated patients (48.1 vs 29.3%, OR=3.14 [1.61-6.12], p<0.001; and 19.2 vs 8.0%,
314 OR=4.61 [1.89-11.21], p<0.001, respectively).

315 Among operated patients, adrenalectomy was more often bilateral in *ARMC5*-mutated patients
316 than in wild-type patients: 13/25 (52.0%) vs 21/88 (23.9%) patients, respectively (OR=3.46
317 [1.37-8.72], p=0.007).

318 During the follow-up of the 34 patients initially treated by anticortisolic drugs, a subsequent
319 adrenalectomy was performed in 4 *ARMC5*-mutated patients (2 bilateral and 2 unilateral
320 adrenalectomies) and 6 wild type patients (2 bilateral and 4 unilateral adrenalectomies).

321 Among the 79 patients initially treated by unilateral adrenalectomy, a contralateral
322 adrenalectomy was performed in 9 patients (1 *ARMC5*-mutated and 8 wild type patients).

323 This led to a total number of 123 operated patients: 29 (55.8%) *ARMC5*-mutated patients
324 and 94 (31.3%) wild-type patients (OR=2.76 [1.52-5.02], p<0.001). In total, 47 patients were

325 treated by bilateral adrenalectomy: 16/29 (55.2%) *ARMC5*-mutated patients and to 31/94
326 (33.0%) wild type patients (OR=2.50 [1.07-5.85], p=0.032).

327

328 *Type of variants*

329 To test the hypothesis that truncating *ARMC5* variants (including non-sense and frameshift
330 variants) would be more deleterious than missense variants in terms of Cushing syndrome or
331 adrenal alteration, we compared different characteristics between patients harboring
332 truncating *ARMC5* variants and those with non-truncating *ARMC5* variants. No significant
333 difference was found regarding clinical, morphological and hormonal data, except for
334 midnight plasma cortisol, which was significantly higher in patients with truncating variants
335 (297 nmol/L) vs those with non-truncating *ARMC5* variants (138 nmol/L, p=0.002) (see
336 Supplementary Table 3). Interestingly, patients with truncating *ARMC5* variants presented
337 more frequently with overt clinical Cushing syndrome than patients with non-truncating
338 *ARMC5* variants (56 vs 21%, respectively, p=0.019). A similar analysis was performed to
339 compare *ARMC5* variants located in BTB and armadillo domains to the other variants and no
340 significant difference was demonstrated (data not shown).

341

342 *Identification of predictive criteria for ARMC5 pathogenic variant*

343 In order to identify predictive criteria for *ARMC5* pathogenic variants, clinical, biological and
344 imaging parameters were analyzed, both isolated or combined, and their respective sensitivity,
345 specificity, negative and positive predictive values for *ARMC5* variation were calculated, with
346 95% confidence intervals (95% CI). These values are reported in Table 2. The objective of
347 this analysis was to identify a criterion or an association of criteria allowing an increase of
348 *ARMC5* mutation rate, while keeping a 100% sensitivity and negative predictive value to
349 ensure that no *ARMC5* variant carrier would be missed. The better association of criteria is the

350 presence of a clear bilateral adrenal involvement and a plasma cortisol after 1 mg
351 dexamethasone suppression test above 50 nmol/L, holding a 100% sensitivity and negative
352 predictive value (95% CI 92-100% and 95-100%, respectively), a 27% specificity (95% CI
353 21-33%) and a 19% positive predictive value (95% CI 14-24%) in the 296 patients for whom
354 all the data was available. Indeed, this association allowed to increase the mutation rate (i.e.,
355 positive predictive value) from 14.8 to 18.3% without missing any mutated patient. 64 wild-
356 type patients did not meet these two criteria, meaning that a negative *ARMC5* genotyping
357 could have been avoided in 21.7% of patients.

358 Since *ARMC5*-mutated patients present larger adrenals with more numerous nodules than
359 wild-type patients, it could be hypothesized that a threshold number of adrenal nodules could
360 be used to discriminate *ARMC5*-mutated from wild type patients. The specificity and positive
361 predictive value for *ARMC5* pathogenic variant increase with the number of adrenal nodules
362 (Figure 2). However, a threshold number of adrenal nodules could not be established without
363 losing some *ARMC5*-mutated patients, since a few of them presented only two nodules. The
364 number of four nodules seems to be the better compromise with a 70% sensitivity and
365 specificity.

366 Similarly, even if *ARMC5*-mutated patients present higher values of 24-hour UFC than wild-
367 type patients, only 58.8% of them have a UFC value above ULN, and no threshold for UFC
368 value allows to discriminate *ARMC5*-mutated from wild-type patients without lowering
369 sensitivity and negative predictive value.

370

371

372 **Discussion**

373

374 PBMAH is a heterogeneous and challenging disease in terms of diagnosis and treatment. Its
375 molecular origins are only partially elucidated since the first description of *ARMC5*
376 pathogenic variants in 2013. In this study, we report the largest series of index patients to
377 date, with both clinical and genetic characterization and confirm that *ARMC5* variants are
378 associated with a more distinct phenotype, with more severe cortisol excess, larger adrenals,
379 and more frequent complications. According to our results, level of cortisol excess
380 significantly increases with adrenal size in *ARMC5*-mutated patients.

381 Patients harboring truncating *ARMC5* variants have an increased midnight plasma cortisol and
382 they are more often primarily investigated for clinical signs of Cushing syndrome rather than
383 adrenal incidentaloma compared to the patients harboring other types of variants, but there is
384 no significant difference between the two groups regarding other clinical, biological and
385 morphological parameters, maybe due to the limited number of *ARMC5*-mutated patients.

386 Bilateral adrenalectomy was historically the main treatment of PBMAH associated with
387 Cushing syndrome, but to avoid the morbidity consecutive to adrenal insufficiency, the
388 therapeutic management tends nowadays to be more conservative and unilateral
389 adrenalectomy (18–22) as well as anticortisolic drugs are more often considered today.
390 Recently, adrenal-sparing surgery, defined by a total adrenalectomy of the largest adrenal and
391 a partial adrenalectomy of the contralateral adrenal showed good results in the management of
392 cortisol excess related to PBMAH, while limiting the complications associated with a life-
393 long adrenal insufficiency (23). However, bilateral adrenalectomy remains the most frequent
394 treatment in *ARMC5*-mutated patients, contrarily to wild-type patients, likely because
395 Cushing syndrome is more severe.

396 The size of the cohort is the main strength of our work, and its principal limitation is its
397 retrospective nature: we were not able to analyze the expression of illegitimate receptors since
398 only few patients had exhaustive investigations. Similarly, cerebral imaging was available for

399 a very limited number of patients and thus did not allow to study the potential role of *ARMC5*
400 in meningioma as it has been previously described (24–26). As well, aldosterone secretion
401 was not assessed, due to the frequent prescription of drugs affecting the renin-angiotensin-
402 aldosterone system, in this widely hypertensive population; thus, some patients with bilateral
403 adrenal masses and no evidence for Cushing syndrome could have been misdiagnosed with
404 PBMAH instead of idiopathic primary aldosteronism.

405 In order to optimize *ARMC5* mutation yield, we suggest that the genetic screening should be
406 conditional to the presence of a clear bilateral adrenal involvement associated with a morning
407 plasma cortisol after 1 mg dexamethasone suppression above 50 nmol/L. These associated
408 criteria have a 100% sensitivity and negative predictive value, leading to a better
409 discrimination without missing any mutated patient. Nowadays, PBMAH is more frequently
410 diagnosed due to incidental findings on abdominal imaging performed for other conditions;
411 with these criteria a useless genotyping could be avoided for 21.6% of patients (64 out of 296
412 patients in this series), and this would reduce the burden of genetic laboratories and allow
413 faster genetic results with an improved cost-effectiveness of genetic screening. Despite a
414 better yield with these criteria, the mutation rate would not increase above 20%. Indeed, no
415 criterion or association of criteria is able to predict more accurately the presence of an
416 *ARMC5* pathogenic variant while keeping a 100% sensitivity, since the clinical presentation is
417 heterogeneous, even with regard to *ARMC5*-mutated patients only. Despite the valuable frame
418 of the ACMG-AMP recommendations, the classification of missense genetic variants remains
419 sometimes difficult: in our cohort, four patients harbored three different *ARMC5* variants of
420 uncertain significance for which no pieces of experimental data allowed a better classification.
421 For the strict descriptive analysis, we have chosen to assess them with the mutated patients
422 but they have been excluded for the analysis leading to the identification of predictive criteria
423 for *ARMC5* pathogenic variant, to avoid pitfalls of interpretation. However, the four patients

424 displayed the two associated criteria listed above, and thus, would not have been missed
425 neither if these criteria have been applied for the indication of *ARMC5* genotyping in real life
426 conditions. Thus, the rate of *ARMC5* variants of the ACMG classes 3, 4 and 5 in patients
427 harboring bilateral adrenal involvement and a possible autonomous cortisol secretion is
428 19.7%.

429 At this point, we recommend that genotyping should be offered to all patients presenting with
430 bilateral adrenal nodules and evidence for autonomous cortisol secretion, and to the first-
431 degree relatives of index patients with an identified *ARMC5* pathogenic variant. Mutation
432 carriers with cortisol autonomous secretion would be managed according to the
433 recommendation of the ESE Clinical Practice Guideline for the management of bilateral
434 adrenal incidentalomas (17). In case no active surgical or anticortisolic treatment would be
435 decided initially, we would suggest closer follow-up in *ARMC5* mutation carriers than in non-
436 mutated patients since *ARMC5* mutations are associated with a more severe disease.

437 In this study, cortisol after dexamethasone suppression was missing in 56 out of the 352
438 patients; among them, 22 patients had normal UFC values and none of these 22 had an
439 *ARMC5* pathogenic variant. Since the sensitivity of dexamethasone test is better than UFC for
440 the screening of Cushing syndrome, we can hypothesize that a cortisol after dexamethasone
441 suppression lower than 50 nmol/L is sufficient to obviate *ARMC5* genotyping for patients
442 presenting bilateral adrenal incidentaloma. On the other side, a normal 24-hour UFC value is
443 not sufficient to exempt from *ARMC5* screening. Indeed, more than 40% of *ARMC5*-mutated
444 patients had a normal UFC. This demonstrates that PBMAH driven by *ARMC5* inactivation
445 can be evoked in front of bilateral adrenal incidentaloma with no clinical patent Cushing
446 syndrome, as it has already been reported in some cases (27,28). This is supported by our data
447 since *ARMC5*-mutated patients are primarily investigated more often in front of adrenal
448 incidentaloma (56.9%) than overt Cushing syndrome (43.1%), even it is more balanced than

449 for wild-type patients (75.3 and 24.7%, respectively). Besides, low ACTH levels can be
450 helpful in questionable subclinical Cushing syndrome, as plasma cortisol or even UFC can be
451 faulted in many situations, as confounding treatments or renal insufficiency. However, its
452 accuracy remains limited since ACTH has been shown to be locally produced by a
453 subpopulation of steroidogenic cells in some cases of PBMAH, leading to an apparent ACTH-
454 dependent Cushing syndrome (29).

455 Here we present a deliberately non-selected population of patients, only based on the *ARMC5*
456 genotyping prescription, regardless of the adrenal morphology or the biological status: indeed,
457 some of them had even unilateral adrenal masses and many had no evidence for Cushing
458 syndrome. Among the 352 patients, 24 had only a unilateral adrenal involvement, including 9
459 with multiple nodules on one adrenal and 15 with a unique macronodule, and none of them
460 showed *ARMC5* pathogenic variant, which is supported by the absence of *ARMC5* mutation
461 observed in a recent series of unilateral adrenal incidentalomas (27). This underlines the need
462 to work for a better definition of PBMAH. Hence, we hypothesize that the proper definition of
463 PBMAH should include the presence of bilateral adrenal nodules, associated with at least a
464 possible autonomous cortisol secretion, defined by a morning plasma cortisol after 1 mg
465 dexamethasone suppression above 50 nmol/L. When taking into account these aspects, the
466 mutation rate of *ARMC5* in PBMAH would increase to close to 20%, similar to the range
467 observed in patients investigated in the setting of research programs (9,13–15).

468 Until recently, *ARMC5* was the only known genetic cause for isolated PBMAH. *ARMC5* has
469 never been associated with a rare presentation of PBMAH characterized by the illegitimate
470 expression of the GIP receptor (GIPR) in the adrenocortical cells, leading to a cortisol
471 secretion consecutive to food intake. In late 2021, almost thirty years after the first description
472 of the food-dependent Cushing syndrome (30,31), two groups – including ours – discovered
473 its genetic predisposition factor, explaining nearly 100% of the cases: germline inactivating

474 heterozygous variants of *KDM1A*, associated with a complete loss-of-heterozygosity of
475 chromosome 1p in the adrenal tumors, leading to a bi-allelic inactivation of the gene and a
476 complete loss of the expression of the protein, suggesting a role of a tumor suppressor gene,
477 similarly to *ARMC5* (32,33). The experimental data suggest that the loss of *KDM1A*, by
478 combined siRNA and pharmaceutical inhibition, leads to the *GIPR* overexpression in
479 adrenocortical cell line H295R (32). *KDM1A* is a histone demethylase well known in both
480 physiology and pathology, particularly in multiple myeloma (34). Interestingly, the two teams
481 report familial and apparently sporadic cases of multiple myeloma associated with food-
482 dependent Cushing syndrome. Vaczlavik *et al* did not identify any pathogenic variant of
483 *KDM1A* in non-food-dependent PBMAH, in both wild-type and *ARMC5*-mutated patients,
484 suggesting that *KDM1A* inactivation is only responsible for this very peculiar form of
485 PBMAH (33), but it would need to be confirmed in larger series.

486 Despite these recent major discoveries, the pathogenesis of this challenging disease remains
487 largely unresolved: considering that *ARMC5* is responsible for a fifth of apparently sporadic
488 PBMAH cases, and *KDM1A* for a very limited number of patients, other genetic
489 predisposition factors may be implicated in a subset of patients, but remain unknown at
490 present. Besides, in familial histories, index cases and relatives harboring the same germline
491 *ARMC5* variant often present with different levels of cortisol excess and adrenal size;
492 phenotype-modulating genetic factors could underlie this clinical heterogeneity and could be
493 elucidated by further familial investigations.

494 In conclusion, we present the largest series of index-case patients investigated for *ARMC5*
495 pathogenic variants with clinical characterization and confirm the more pronounced condition
496 of most of *ARMC5*-mutated patients compared to non-mutated patients with PBMAH in terms
497 of cortisol excess, adrenal morphology and metabolic complications. These results allow to
498 propose a new and unique definition of PBMAH, associating a clear bilateral adrenal

499 involvement on imaging with at least a possible cortisol autonomous secretion defined by a
500 cortisol after dexamethasone suppression test above 50 nmol/L. From now on, *ARMC5*
501 genotyping should be offered only to the index patients presenting with these two simple
502 criteria, and to all the first-degree relatives of index patients with an identified *ARMC5*
503 pathogenic variants, in order to reduce negative screenings, allow earlier genetic results and
504 thus optimize routine clinical practice.

505

506 **Disclosure**

507

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510 of the research reported.

511

512

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514

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521

522

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524

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641 **Legends of Table and Figures**

642

643 **Table 1. Clinical, biological, and imaging data of *ARMC5*-mutated and wild-type**

644 **patients.** Descriptive statistics are presented as n (%) or mean [standard deviation]. Odds ratio

645 and mean differences are presented with 95% confidence intervals between brackets.

646 Abbreviations: 95% CI, 95% confidence interval; F, female; M, male; y, years; BMI, body

647 mass index; UFC, urinary free cortisol; ULN, upper limit of normal; IGT, impaired glucose

648 tolerance; OD, odds ratio; MD, mean difference.

649

650 **Table 2. Sensitivity, Specificity, Positive and Negative Predictive Values for the**
651 **prediction of *ARMC5* variants of clinical, biological, imaging and combined parameters.**

652 Sensitivity, Specificity, Positive and Negative Predictive Values are provided with 95%

653 confidence intervals between brackets. Abbreviations: 95% CI, 95% confidence interval; 24-

654 hour UFC, 24-hour urinary free cortisol; ULN, upper limit of normal; LDDST, low dose

655 dexamethasone suppression test.

656

657 **Figure 1. Adrenal CT-scans** of one *ARMC5*-mutated patient harboring numerous nodules on
658 each adrenal (A); and one *ARMC5*-wild type patient with unique nodule on each adrenal (B).

659 Adrenals are surrounded by white dotted lines.

660

661 **Figure 2. Graphical representation of the sensitivity and specificity of the total number**
662 **of adrenal nodules for *ARMC5* pathogenic variant.** The grey line represents specificity; the

663 black line represents sensitivity.