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

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76

Abstract

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

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

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

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

Introduction

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

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

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

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

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

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

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

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

Patients and Methods

Patients

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

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

Data collection

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

DNA extraction

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

ARMC5 sequencing

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

Variant nomenclature and interpretation

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

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

Predictive criteria for ARMC5 pathogenic variants

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

Statistical analysis

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

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

Results

Patient characteristics

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

ARMC5 genotyping

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

Demographic and anthropometric data

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

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

Clinical presentation

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

Hormonal investigations

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

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

Adrenal morphology

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

Correlation between adrenal size and levels of cortisol excess

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

Clinical consequences of hypercortisolism

ARMC5-mutated patients had more frequently hypertension requiring antihypertensive medication than wild-type patients (87.8 vs 70.7%, respectively, OR=2.97 [1.22-7.23], p=0.016). Diabetes mellitus was more frequent as well in *ARMC5*-mutated than in wild-type patients (52.1 vs 35.3%, respectively, OR=2.00 [1.08-3.69], p=0.027). Frequencies of osteoporosis and venous thrombo-embolism did not differ between the two 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 [0.49-3.73], p=0.567, respectively).

Treatment

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

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

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

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

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

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

Type of variants

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

Identification of predictive criteria for ARMC5 pathogenic variant

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

Until recently, *ARMC5* was the only known genetic cause for isolated PBMAH. *ARMC5* has never been associated with a rare presentation of PBMAH characterized by the illegitimate expression of the GIP receptor (GIPR) in the adrenocortical cells, leading to a cortisol secretion consecutive to food intake. In late 2021, almost thirty years after the first description of the food-dependent Cushing syndrome (30,31), two groups – including ours – discovered 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

Disclosure

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Legends of Table and Figures

Table 1. Clinical, biological, and imaging data of *ARMC5*-mutated and wild-type patients. Descriptive statistics are presented as n (%) or mean [standard deviation]. Odds ratio and mean differences are presented with 95% confidence intervals between brackets. Abbreviations: 95% CI, 95% confidence interval; F, female; M, male; y, years; BMI, body mass index; UFC, urinary free cortisol; ULN, upper limit of normal; IGT, impaired glucose tolerance; OD, odds ratio; MD, mean difference.

Table 2. Sensitivity, Specificity, Positive and Negative Predictive Values for the prediction of *ARMC5* variants of clinical, biological, imaging and combined parameters. Sensitivity, Specificity, Positive and Negative Predictive Values are provided with 95% confidence intervals between brackets. Abbreviations: 95% CI, 95% confidence interval; 24-hour UFC, 24-hour urinary free cortisol; ULN, upper limit of normal; LDDST, low dose dexamethasone suppression test.

Figure 1. Adrenal CT-scans of one *ARMC5*-mutated patient harboring numerous nodules on each adrenal (A); and one *ARMC5*-wild type patient with unique nodule on each adrenal (B). Adrenals are surrounded by white dotted lines.

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