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► **To cite this version:**

Anna Vaczlavik, Lucas Bouys, Florian Violon, Gaetan Giannone, Anne Jouinot, et al.. KDM1A inactivation causes hereditary food-dependent Cushing syndrome. *Genetics in Medicine*, 2022, 24 (2), pp.374-383. 10.1016/j.gim.2021.09.018 . hal-03604178

**HAL Id: hal-03604178**

**<https://hal.science/hal-03604178>**

Submitted on 22 Jul 2024

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## ***KDM1A* inactivation causes hereditary food-dependent Cushing's syndrome**

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## **CONFLICT OF INTEREST**

The authors declare no conflict of interest.

## ABSTRACT

**Purpose:** To investigate the genetic cause of Food-dependent Cushing's syndrome (FDCS) observed in patients with primary bilateral macronodular adrenal hyperplasia (PBMAH) and adrenal ectopic expression of the GIP receptor (GIPR). Germline *ARMC5* alterations have been reported in about 25% of PBMAH index cases but are absent in patients with FDCS.

**Methods:** A multi-omics analysis of PBMAH tissues from 36 patients treated by adrenalectomy was performed (RNAseq, SNP array, methylome, miRNome, exome sequencing).

**Results:** The integrative analysis revealed three molecular groups with different clinical features: G1, 16 patients with *ARMC5* inactivating variants; G2, 6 FDCS patients with GIPR ectopic expression; and G3, 14 patients with a less severe phenotype. Exome sequencing revealed germline truncating variants of *KDM1A* in 5 G2 patients, constantly associated with a somatic loss of the *KDM1A* wild-type allele on 1p, leading to a loss of *KDM1A* expression both at mRNA and protein levels ( $p=1.2 \times 10^{-12}$  and  $p < 0.01$ , respectively). Subsequently, *KDM1A* pathogenic variants were identified in 4/4 additional index cases with FDCS.

**Conclusion:** *KDM1A* inactivation explains about 90 % of FDCS PBMAH. Genetic screening for *ARMC5* and *KDM1A* can be offered now for the majority of PBMAH operated patients and their families, opening the way to earlier diagnosis and improved management.

**KEY WORDS:** Adrenal, adrenocortical tumors, *ARMC5*, *LSD1*, *KDM1A*, Tumor Suppressor gene, Cushing's syndrome, Cortisol, GIPR

## INTRODUCTION

Primary bilateral macronodular adrenal hyperplasia (PBMAH) is an adrenal cause of cortisol excess (Cushing's syndrome) due to bilateral benign adrenocortical tumors<sup>1,2</sup>. Tumor growth is slow and the disease is usually diagnosed between the age of 40 to 60 years old<sup>3</sup>. PBMAH can be diagnosed after the investigation of clinical signs of cortisol excess or in patients with adrenal incidentaloma. Indeed, about 15% of adrenal incidentalomas are bilateral and PBMAH represents a significant proportion of them<sup>4</sup>. Considering that adrenal incidentaloma is observed in 4 to 7% of the population, true incidence of PBMAH is probably underestimated. Nevertheless, the diagnosis is nowadays much more frequent. This increased frequency led to the clinical observation that PBMAH is a heterogeneous disease with variable severity of cortisol excess and adrenal enlargement<sup>5</sup>, challenging diagnostic and management recommendations. It is likely that the diagnosis is made years after the start of the disease, because of the slow growth of the adrenal nodules and the progressive cortisol dysregulation. Surgery is the most common treatment with either bilateral or unilateral adrenalectomy. Indication for surgery is based on the level of cortisol excess (considering its long-term clinical consequences) and on adrenal imaging.

Progress has been made to understand the genetic basis of PBMAH with the aim to offer earlier diagnosis by familial screening. Indeed, in accordance with the bilateral presentation, germline predisposing pathogenic variants have been identified for a subset of PBMAH patients. The most common genetic alterations are germline inactivating variants of the tumor suppressor gene armadillo repeat containing 5 (*ARMC5*), found in 20 to 25% of apparently sporadic PBMAH patients, 50% of operated patients and 80% of patients with evident familial presentation<sup>6-10</sup>. Familial screening for *ARMC5* variants shows that the penetrance of the disease is high<sup>7,11</sup>. Indeed, relatives with *ARMC5* pathogenic variant commonly present undiagnosed PBMAH. Investigations of these relatives usually reveal mild cortisol excess with its long term complications, or even overt Cushing's

syndrome that has not been diagnosed before, as often for rare disorders. Beyond *ARMC5* pathogenic variants, PBMAH patients may occasionally present alterations in genes responsible for a multiple tumor syndrome, including multiple endocrine neoplasia type 1 (*MEN1*), adenomatous polyposis coli (*APC*) and fumarate hydratase (*FH*) genes<sup>12</sup>. However, the majority of apparently sporadic PBMAH patients do not present any pathogenic variant in known genes.

In PBMAH patients, clinical investigations have almost constantly shown abnormal cortisol secretion due to illegitimate membrane receptors expression<sup>3,13</sup>. Among these receptors, the best example is adrenal ectopic expression of the glucose-dependent insulintropic polypeptide receptor (GIPR) that has been demonstrated in PBMAH patients with food-dependent Cushing's syndrome<sup>14,15</sup>. In food-dependent Cushing's syndrome, an abnormal stimulation of the adrenal cortex by the glucose-dependent insulintropic polypeptide secreted after food ingestion leads to cortisol excess.

Systematic screening for food-dependent Cushing's syndrome has been reported positive in 10 to 30% of the PBMAH patients<sup>13,16</sup>, being more frequent in patients with overt Cushing's syndrome than subclinical Cushing's syndrome. In PBMAH patients with *ARMC5* pathogenic variants, screening for illegitimate membrane receptor expression has shown the frequent occurrence of cortisol response to posture test<sup>9,11</sup>. By contrast, food-dependent Cushing's syndrome in PBMAH patients due to *ARMC5* germline pathogenic variants has never been reported, suggesting a different genetic cause. Considering the clinical heterogeneity of PBMAH, it is tempting to speculate that different molecular alterations, driven by different germline genetic predisposition factors, might explain phenotypic variance. Hence, elucidating this heterogeneity by identifying the underlying genetic predispositions would help to better define PBMAH subtypes. Knowledge about the germline predisposition is essential for improving PBMAH management, for both patients and their relatives. Based on this hypothesis the present study used an integrated genomic approach to explore the PBMAH tumors structure and correlate this structure with clinical and pathological features to identify new genetic causes.

## **PATIENTS AND METHODS**

### ***Patients***

36 adult patients who had undergone surgery for PBMAH were included (23 females and 13 males) in the genomic study. All were diagnosed with various levels of adrenocorticotrophic hormone (ACTH)-independent hypercortisolism and underwent computed tomography imaging. Food-dependent Cushing syndrome was diagnosed by a 50 % increased of plasma cortisol during a specific stimulation test as previously reported<sup>13</sup> or by a 50 % lower 8 a.m. fasting plasma cortisol level compared to midnight level. The diagnosis of PBMAH were histologically confirmed in all cases after adrenalectomy. Detailed clinical description is provided in table 1 and in table S1A. From these 36 patients, 52 adrenal tumor samples were collected. Tissue samples were snap-frozen in liquid nitrogen rapidly after surgery. These samples were prospectively collected in the CorticoMEDullosurrénale Tumeur Endocrine (COMETE) network (table S2). Subsequently 5 additional PBMAH patients with Food-dependent Cushing's syndrome from 4 families were investigated.

### ***DNA and RNA Extraction, sequencing, SNP array and methylome analysis***

RNA, miRNA and genomic DNA from PBMAH or leukocyte were isolated as previously described<sup>6</sup> (see also the supplemental methods). RNA and miRNA extracted (table S2) have been sequenced using Illumina technology on the Genomic platform of Cochin Institute (see for details the supplemental methods). DNA samples (table S2) were treated by bisulfite and hybridized to the Infinium MethylationEPIC BeadChip (Illumina, San Diego, CA, US; around 865000 sites). For SNP array analysis, DNA sample (table S2) have been analyzed using Illumina HumanCore BeadChip (306702 SNPs). Exome sequencing on DNA from 15 PBMAH and their paired leukocyte samples have been performed on the Genomic platform of Cochin Institute (see for details the supplemental methods). Sequence alignments, quantification of expression, copy-number analysis and unsupervised or



supervised analysis are described in detail in the supplemental methods. All data and supervised analysis are available on EMBL-EBI BioStudies site (data set S-BSST640) and for a part in tables S3-S7. Sanger sequencing of *KDM1A* is described in the supplemental methods.

### ***Pathological analysis and Immunostaining***

3 µm paraffin sections obtained from formalin-fixed tissue, were stained with hematoxylin, eosin and saffron using a Tissue-Tek Prisma Plus (Sakura Finetek Europe BV; Zoeterwoude, The Netherlands). Immunohistochemistry was performed on 3 µm paraffin sections in a Leica BOND-III System (Leica, Berlin, Germany) with HIER (Heat Induced Epitope Retrieval) and EDTA buffer (Bond Epitope Retrieval Solution 1, pH 6.0, Leica BOND-III System). Sections were stained for 20 minutes using antibody directed against KDM1A (Abcam, Cambridge, UK, ab-17721) diluted at 1:400. Based on the intensity of KDM1A staining evaluated on the whole slides, patients were classified into two categories either similar staining in tumoral and non-tumoral tissues or from weaker to no staining in tumoral tissues. All microscopic slides (H&E saffron and immunohistochemistry) were analyzed by two pathologists (F.V. and M.S.).

### ***Statistical Analysis***

Calculations were performed using R statistical software. The Bioconductor limma package was used to test for mRNA, miRNA differential expression or methylation as described in the supplemental methods. Plots were generated using pheatmap package. Comparisons between PBMAH patients were performed using Wilcoxon and Kruskal-Wallis tests for quantitative variables and using Fisher's test for qualitative variables. All p-values were two-sided, and the level of significance was set at  $p < 0.05$ .

### ***Code availability***

The R scripts used to process the data and conduct the analyses described herein are available upon request.

## RESULTS

### ***Integrated genomics identifies three groups of PBMAH***

Unsupervised transcriptome (figure 1A), chromosome alterations (figure 1B), miRNome (figure S1) and methylome (figure S2) classification of PBMAH tumors led to the identification of three distinct molecular groups (figure 2A): group G1, gathering the 16 *ARMC5*-mutated patients; group G2, gathering all the 6 patients diagnosed before surgery with a food-dependent Cushing's syndrome phenotype (table S1A); group G3, containing the remaining 14 PBMAH patients.

In group G2, *GIPR* expression was high, while almost not expressed in G1 and G3 tumors (mRNA  $\log_2(\text{fold change})=6.7$ ,  $p=2.1 \times 10^{-13}$ ; figure 1A, figure S3 and table S3). This clearly identified the G2 tumors as a group of PBMAH with ectopic expression of the *GIPR*, responsible for food-dependent Cushing's syndrome. Analysis of the differentially expressed genes between the three clusters showed that the most significantly down-regulated gene in G2 is lysine demethylase 1A (*KDM1A*;  $\log_2(\text{fold change})=-2.9$ ,  $p=1.2 \times 10^{-12}$ , figure 1A, figure S3 and table S3).

Somatic copy number alterations were very limited in PBMAH, a feature dramatically different from the very aggressive adrenocortical cancers<sup>17,18</sup>: half of the tumor samples did not show any chromosomal aberrations. Recurrent alterations were on chromosome 16p and 1 (figure 1B). The copy neutral loss of heterozygosity (LOH) at 16p was observed in tumors from 6 patients, all having a pathogenic *ARMC5* germline variant. Since *ARMC5* is located at 16p, the wild type allele is lost and, as expected from a tumor suppressor gene, a somatic *ARMC5* pathogenic variant was observed in all the tumors with no LOH (figure 2A and table S2). The other recurrent alteration was a loss at 1p36.33-11.2 observed in tumors from seven patients, including the 6 tumors of the G2 group.

### ***The three molecular groups of PBMAH present different clinical and pathological characteristics***

The correlation of the integrated genomics classification with the clinical and pathological analysis showed specific profiles (figure 2A). In G1 *ARMC5* group, sex distribution was balanced and 08:00

a.m. fasting plasma cortisol as well as the plasma cortisol after dexamethasone suppression (1 mg) were higher than in the G2 and G3 group (table 1 and figure 2A). In G2 group, all patients are females and their 08:00 a.m. fasting cortisol levels were lower than in G1 and G3 tumors, which is consistent with the diagnosis of food-dependent Cushing's syndrome. In G3 group, patients presented with a less severe disease, with lower urinary free cortisol, lower midnight cortisol levels and smaller adrenal size compared to G1 group, and lower midnight plasma cortisol levels compared to G2 group (table 1).

Pathological analysis revealed significant differences in the percentage of eosinophilic non-oncocyctic cells among the three groups (table 1, figure 2 and table S1A). These eosinophilic cells are a specific feature of G2 tumors, with >30% of eosinophilic cells in G2 while absent or rare (<5%) in G3 tumors, and detectable but low (between 5 and 20%) in G1.

#### ***KDM1A is the molecular cause of the G2 (GIPR) group of PBMAH***

Exome sequencing on paired blood leukocytes and tumors DNA from 5 G2 and 10 G3 PBMAH cases did not identify any recurrent somatic pathogenic variant (table S7). In the 5 G2 patients, exome sequencing revealed germline truncating variants in *KDM1A* gene (figure 3A), corresponding to the gene with the lowest expression in G2 (figure 1A). All these variants have not been reported previously in the general population and cause a premature stop codon (patient P24: NM\_015013.4:c.386delA, p.N129Tfs\*60; P18: NM\_015013.4:c.1774\_1775insG, p.Q592Rfs\*10; P6: NC\_000001.10:g.23405482\_23405495dupGGATGTGAAGTGAT, NM\_015013.4:c.1796-1\_1808dupGGATGTGAAGTGAT, p.I603Mfs\*5; P36: NM\_015013.4:c.1912C>T, p.Q638\* and P29: NM\_015013.4:c.2155delA, p.S719Vfs\*4). Interestingly, *KDM1A* gene is located at 1p36.12, a chromosomal region constantly lost in all the G2 tumors (figure 1B). All these *KDM1A* variants were confirmed by Sanger sequencing. For these heterozygous germline variants, allelic imbalance was observed in tumors, with a majority of mutated allele (figure 3A). It should be noted that in three patients from G2 for whom both adrenals could be studied (P18, P29 and P36), 1p LOH was observed

on both sides, with conservation of the germline mutated allele (table S6). However, although the sixth G2 tumor also presented 1p LOH (P8, leukocyte DNA not available for exome sequencing), no variant in the *KDM1A* coding sequence was identified by Sanger sequencing. In G2 tumors, no other gene showed significant recurrent damaging alterations. Exome sequencing of DNA of more than 70 patients with various types of other adrenocortical tumors (adenomas, cancers, and micronodular adrenocortical hyperplasia), previously performed in our laboratory, did not find any pathogenic *KDM1A* variants. Four additional *KDM1A* pathogenic variants (patient SP1:

NC\_000001.10:g.23356961G>A, NM\_015013.4:c.352-1G>A; SP2: NM\_015013.4:c.1771C>T, p.R591\*; SP3: NM\_015013.4:c.2391\_2393delCTT, p.F798del and SP4: NM\_015013.4:c.2117\_2125del, p.L706\_G709delinsR, figure 3A) were identified in the five PBMAH patients operated for food-dependent Cushing's syndrome (including two from the same family) not included in the initial cohort of the 36 patients of the genomics study (table S1B). As expected, SP1 patient and her affected daughter shared the same variant. In tumors from all additional index cases, the 1p LOH was also observed with conservation of the germline mutated allele (data not shown).

Analysis of *GIPR* and *KDM1A* expression by RT-PCR in all the 52 tumors from the 36 patients of this genomics study showed that loss of *KDM1A* expression was constant but also exclusive to the tumors expressing the *GIPR* (figure S4A). Moreover, an inverse correlation was observed between *GIPR* and *KDM1A* expression ( $r=-0.75$ ,  $p=1.9 \times 10^{-10}$ ; figure S4B). By immunohistochemistry, a loss of the nuclear staining of the *KDM1A* protein was observed in cells of the G2 tumors but not in non-tumoral adjacent adrenal, nor in tumors from the G1 and G3 groups ( $p<0.01$ , figure 3B). Finally, gene set enrichment analysis (GSEA) showed an enrichment in gene signature specific for *KDM1A* inactivation or inhibition previously described<sup>19,20</sup> in the G2 group compared to other groups (NES=2.14  $p<0.0001$ ; figure S5 and table S8). Altogether, these data support that the loss of *KDM1A* function drives the development of a subgroup of PBMAH (figure S6).

## DISCUSSION

PBMAH is a disease more often diagnosed today than in the past, presenting specific diagnostic and therapeutic challenges. The report of *ARMC5* as a gene frequently responsible for PBMAH opened the possibility for familial screening in a subset of patients. Attention gained with the increased frequency of PBMAH diagnosis led to the observation that it is a heterogeneous disease. This study explains this heterogeneity in operated patients by the identification of three different molecular groups with distinct clinical and pathological characteristics: one group caused by *ARMC5* pathogenic variants with a more severe phenotype, one group with *GIPR*-overexpressing tumors responsible for food-dependent Cushing's syndrome and caused by *KDM1A* pathogenic variants and a third group with a milder phenotype of cortisol excess. Interestingly, not only the molecular biology but also pathology differs among the three groups: for example, the eosinophilic cells represent a high proportion of cells in the G2 tumors but are rare in G3 tumors. This correlation further supports the hypothesis of different clinico-pathological groups of PBMAH as recent progress in other type of adrenal tumors have suggested<sup>21</sup>.

This study identified the lysine-specific demethylase 1 *KDM1A* (also known as Lysine-specific histone demethylase 1A *LSD1*) gene as a tumor suppressor gene responsible for the genetic predisposition to PBMAH associated with food-dependent Cushing's syndrome. A germline *KDM1A* variant was observed in 9/10 index cases of the patients with Food-dependent Cushing's syndrome investigated in this study (5/6 patients out of the 36 patients investigated in the integrated genomics study and 4/4 additional index patients subsequently investigated specifically for *KDM1A* alteration).

Furthermore, the affected daughter of the additional index patient SP1 harbored the same germline *KDM1A* variant than her mother. This suggests that *KDM1A* explains the vast majority of Food-dependent Cushing's syndrome PBMAH and clearly establishes that this is a genetic disease. Most of the patients reported with Food-dependent Cushing's syndrome in the literature are female<sup>3,13-16,22-</sup>

<sup>23</sup>. In keeping with this observation all the patients with *KDM1A* pathogenic variant of the present study are female while such increased prevalence of female is not observed in the patients with an *ARMC5* pathogenic variant. Although adrenal Cushing's syndrome is more frequent in female this suggests that females with *KDM1A* pathogenic variant are more prone to develop Cushing's syndrome than males, as previously reported for *PRKAR1A* germline alterations and adrenal Cushing's syndrome due to primary pigmented nodular adrenocortical dysplasia<sup>24</sup>. Allelic loss of the chromosome 1p that contains the *KDM1A* locus is constant in all the tumors with *KDM1A* pathogenic variant. This is in agreement with the Knudson model for a tumor suppressor gene. Interestingly, chromosome 1 LOH in food-dependent Cushing's syndrome PBMAH has been previously described<sup>25</sup>. The tumor from the single G2 group patient with no *KDM1A* identified variation nevertheless presents loss of *KDM1A* expression. Because the tumor from this patient presents a 1p LOH it is likely that a *KDM1A* alteration in the non-coding part of the gene or an epigenic change could be present. *KDM1A* is an epigenetic transcriptional repressor that primarily demethylates histone H3 on lysine 4 and plays an important role in genome homeostasis. *KDM1A* is ubiquitously expressed<sup>26</sup> and shows homogeneous expression in the normal adrenal cortex (data not shown). *KDM1A* has been initially identified as a regulator of embryonic stem cells<sup>27</sup>. It inhibits pluripotency and its inactivation results in aberrant activation of transcriptional enhancer in embryonic stem cells<sup>28</sup>. *KDM1A* has been shown to play an important role in the control of gene expression by the glucocorticoid receptor<sup>29</sup> as well in various physiological process and cancer<sup>30</sup>. Moreover, *KDM1A* has been shown to play a role in insulin cell differentiation<sup>31,32</sup>. Under normal circumstances, the GIPR is expressed in insulin-secreting cells and not in the adrenal cortex. PBMAH are tumors with a very slow progression and long term evolution; and some evidence suggests abnormal cellular differentiation as the mechanism of development of the disease<sup>33</sup>. For example, gonadal differentiation has been suggested for PBMAH following *ARMC5* inactivation<sup>34,35</sup>.

PBMAH may develop in the G2 group of patients as the result of abnormal differentiation caused by inactivation of the lysine-specific demethylase 1 (*KDM1A*) gene. Based on the enriched expression of

genes known to be regulated by KDM1A inhibition<sup>20</sup> associated with G2 group, we can hypothesize that the histone 3 on lysine 4 methylation marks altered by KDM1A loss of expression could also lead to abnormal transcription of genes involved in adrenocortical cell differentiation. This would result in ectopic GPR expression and tumor development, with a consecutive food-dependent steroid excess (figure S6).

*KDM1A* gene has been implicated in familial multiple myeloma<sup>36</sup>. Interestingly, among the six G2 patients, one with germline *KDM1A* pathogenic variant (P24) also had a monoclonal gammopathy of unknown significance (MGUS), a premalignant condition predisposing to multiple myeloma. Another G2 patient (P18), at the time of inclusion in the study had also a familial history of PBMAH due to food-dependent Cushing's syndrome and familial myeloma on her maternal side. Indeed, her mother harbors the same *KDM1A* heterozygous inactivating variant (data not shown) and was diagnosed with myeloma, no material was available as part of this research to study *KDM1A* in the hematological diseases of these patients. The two index cases (P18 and SP1) with a previously known familial history both presented a germline *KDM1A* inactivating variant. The six other index cases had no known familial history at the time of inclusion in this study. Therefore, *KDM1A* appears, as well as *ARMC5*, responsible for PBMAH that seems sporadic but also in a subset of patients with clear familial presentation. It should be noted that no food-dependent Cushing's syndrome has been reported so far in *ARMC5* mutated families, while the PBMAH patients with *KDM1A* pathogenic variant of the two families of the present study were all diagnosed with food-dependent Cushing's syndrome.

In conclusion, this study sets the ground for the description and understanding of the clinical and molecular heterogeneity of PBMAH. It identifies three different molecular groups with specific clinical and pathological characteristics, the genetic cause of two of these groups being now identified. For the third group (G3) the genetic mechanism is still to be identified, since no recurrent alteration could be demonstrated by this study. This could be due to genetic or epigenetic



alterations not detectable by the genomic methods used, or by the existence of different alterations among the patients of this group. After the report of *ARMC5* in 2013, this study now identifies a second genetic cause, *KDM1A*, that could explain the vast majority of PBMAH patients with Food-dependent Cushing's syndrome and about 15 to 20% of operated patients. This provides, along with *ARMC5* genetic analysis, a second genetic cause to be investigated in PBMAH patients. This further demonstrates that PBMAH is a genetic disorder. This finding has major impact for patient follow-up and family screening, and should also prompt investigation of susceptibility to myeloma in PBMAH patients identified with *KDM1A* variant. This progress in PBMAH classification will also help to improve patient's management, offering the possibility of familial screening for earlier diagnosis and management of adrenal Cushing's syndrome, helping to reduce the long-term morbidity of cortisol excess.

## **DATA AVAILABILITY**

Sequence, expression and supervised analysis data generated during this study are available at EMBL-EBI BioStudies site (data set S-BSST640): <https://www.ebi.ac.uk/biostudies/studies/S-BSST640?key=4c2c6ad2-d5d5-4412-8f93-4788887c20cf>.

## **ACKNOWLEDGEMENTS**

This study was supported in part by the Agence Nationale de la Recherche (ANR-2010-1136-02; 18-CE14-0008-01 and the joint program with FAPESP 2015/50192-9 for the MCF and JB teams), the Fondation pour la Recherche Médicale (FRM EQU201903007854), the Programme Hospitalier de Recherche Clinique Grants (AOM95201, AOM 14079, PRT-K 148663) to the COMETE Network, the Assistance Publique-Hôpitaux de Paris (Grant Genhyper P061006) and a grant to CAS by the intramural program of NICHD, NIH. LB is recipient of a fellowship from the CARPEM and the Fondation ARC. IC is recipient of research a fellowship from Fondation pour la Recherche Médicale (SPF201809007096). MR is supported by grants from the Else Kröner-Fresenius Stiftung (2012\_A103 and 2015\_A228) and the DFG in the context of the CRC/TRR 205/1.

MR and JB clinical departments are part the European Reference Network on Rare Endocrine Conditions (Endo-ERN) – Project ID No 739572.

We thank the biological resource center of Cochin hospital and the tumor bank of Cochin hospital, the biological resource center of CHU-Bordeaux for their help in samples collection. We thank the genomic platform of Cochin Institute for genomic studies and exome sequencing, the Integragen GeCo team for part of the bioinformatic analysis, all the members of the Genomics and Signaling of Endocrine Tumors team of Cochin Institute for their help in these studies. We also thank the patients and their families, and the physicians and staff involved in patient's management for their active participation.

## **AUTHOR CONTRIBUTIONS**

Conceptualization: MCF, CAS, RL, GA, BR, JB; Data curation: LB, GG, EL, BR; Formal analysis: AV, LB, GG, AJ, RA, EL, GA, BR, JB; Funding acquisition: MCF, CAS, MR, RL & JB; Investigations: AV, LB, FV, IPC, AB, PV, MB, FB, KP, CR, MS, MON, SE, PE, MH, IT, LG, LGr, BD, MR, EP, RL, GA, BR, JB; Methodology: GA, BR. Supervision: RL, GA, BR, JB; Validation: AV, LB, BR, JB; Writing original draft: AV, LB, FV, GA, BR, JB; Writing, review and editing: all the authors.

## **ETHICS**

The study was approved by the ethic committees of Cochin Hospital, Ile de France I and Ludwig-Maximilians-University, Munich. Written informed consent was obtained and archived from the patients. All the data were de-identified.

## **MATERIALS & CORRESPONDENCE**

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## LEGENDS OF FIGURES

### Figure 1. Transcriptome and chromosome alterations of PBMAH tumors

A. Unsupervised clustering of PBMAH tumors based on their transcriptome profile identified three expression clusters: the E1 cluster (blue) contained all the tumors with *ARMC5* pathogenic variants, the E2 cluster (red) included all tumors from patients diagnosed before surgery with a typical food-dependent Cushing's syndrome, and the E3 cluster (green) contained tumors from patients without germline *ARMC5* pathogenic variant or food-dependent Cushing's syndrome (tables S1A and S2). The middle panel shows the heatmap of the most differentially expressed mRNA between the groups (table S3). The bottom part shows the *GIPR* and *KDM1A* expressions in each tumor. See also figure S3.

B. Chromosome alterations of PBMAH tumors. Cumulative proportions of chromosomal gains and losses are provided by chromosomal location (top). Chromosomal gains and losses for each patient are indicated (bottom) (tables S2 and S6). Losses are represented in blue, gains in red, and copy-neutral losses of heterozygosity (cnLOH) in purple.

### Figure 2. Multi-omics, clinical and pathological data identifies three groups of patients

A. Associations between the omics groups (table S2), the most recurrent chromosome alterations (16p and 1p, tables S2 and S6), the pathological features and clinical annotations (table S1A) are provided (Fisher's exact test p value). The G1 group (blue) contains tumors from patients with *ARMC5* germline pathogenic variant, characterized by a more severe Cushing's syndrome and larger adrenals as shown in table 1, with constant somatic 16p copy-neutral loss of heterozygosity (a third of the tumors) or somatic *ARMC5* inactivating variant; the G2 group (red) contains tumors from six patients showing *GIPR* overexpression, with constant somatic loss of heterozygosity in 1p and a significantly lower morning plasma cortisol than the two other groups, due to a food dependent

Cushing's syndrome ; and the third more heterogeneous group (green) containing only tumors from *ARMC5*-wild type patients without *GIPR* overexpression.

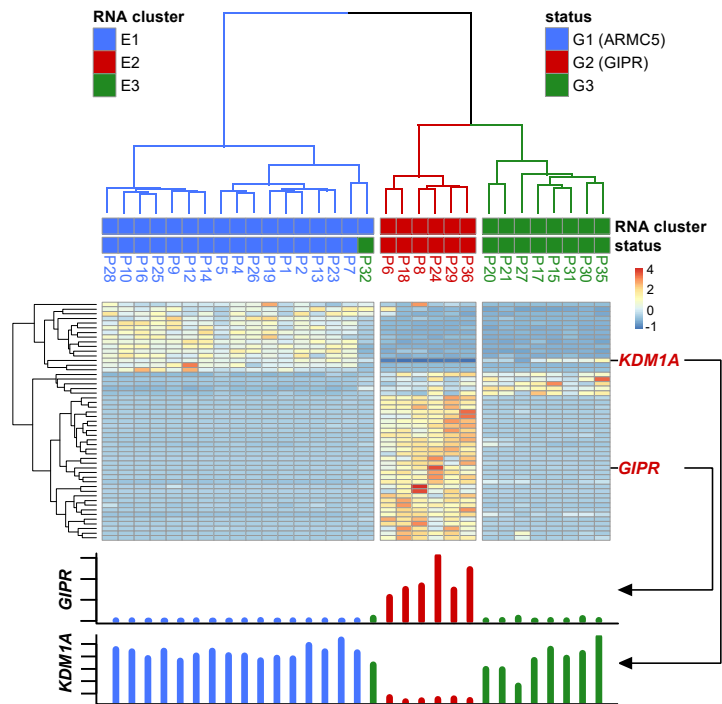
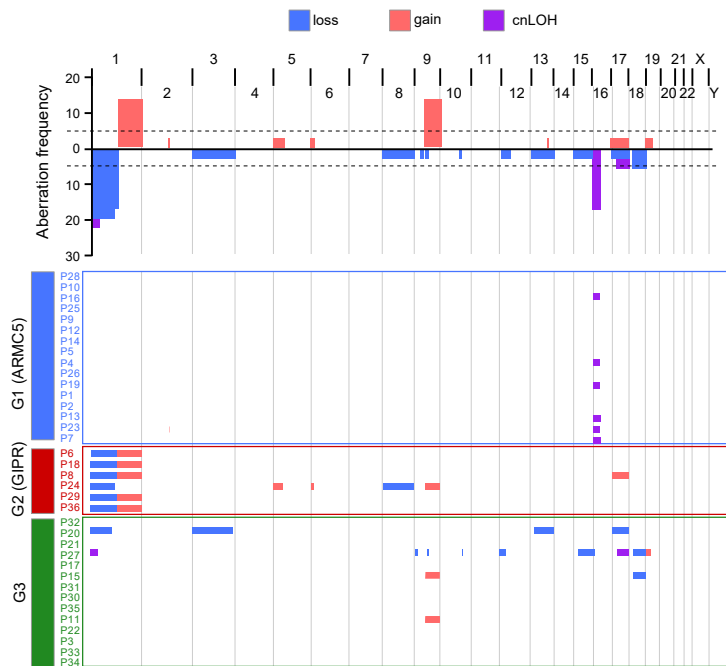
B. Microscopic images showing morphological variations between the 3 groups. Tumor of a patient with *ARMC5* pathogenic variant showing less than 20% of eosinophilic cells (left); tumor of a patient with *GIPR* overexpression showing more than 30% of eosinophilic cells (middle); tumor of a patient from the third group showing less than 5% of eosinophilic cells. H&E saffron, magnification x5. Scale bar=500  $\mu\text{m}$ .

### **Figure 3. *KDM1A* pathogenic variants and *KDM1A* immunohistochemistry**

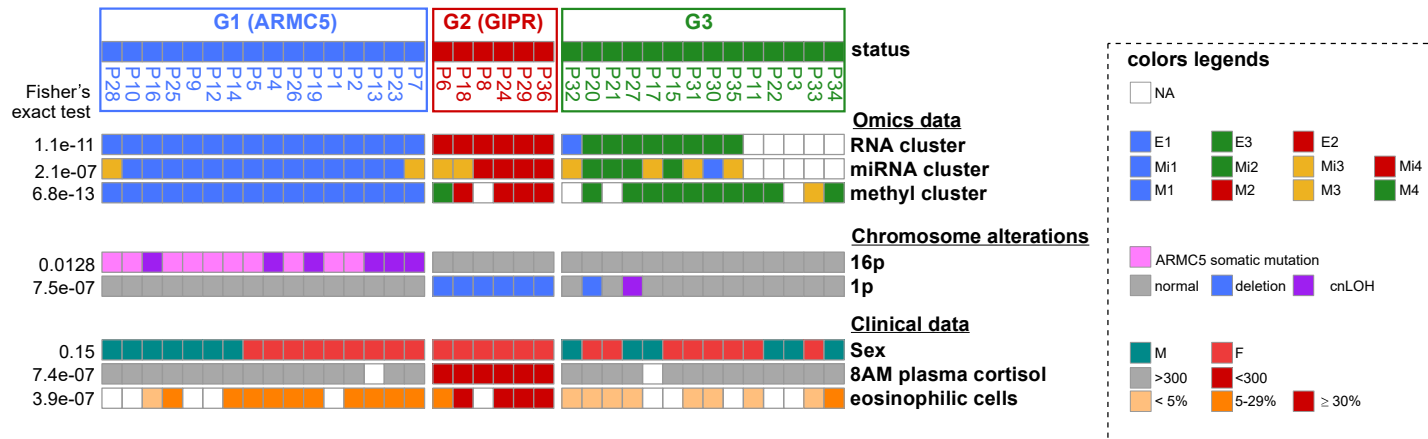
A. Location in the *KDM1A* protein and cDNA (NM\_015013) of the various genetic alterations found in patients. Pathogenic variants identified in the initial cohort are represented by a red star and variants identified in the four additional index cases are represented by a purple star. Yellow triangles represent the location of two supplementary short exons in the NM\_001009999 isoform (60 and 12 bp, respectively). None of these two exons were altered in this study. Electropherogram obtained by Sanger sequencing of exon 15 showing the variant identified in patient P36 at heterozygous state in leukocyte DNA and at hemizygous state in tumoral DNA secondary to the loss of the wild-type 1p allele.

B. Immunohistochemistry of *KDM1A* is presented for one patient per PBMAH group. The majority of cells showed weaker or no nuclear *KDM1A* staining in the tumors compared to the adjacent non-tumoral adrenal gland in the 4 patients tested from the *GIPR* group (G2). By contrast the *KDM1A* staining was observed in the tumor cells of the *ARMC5* (G1, n=4) and the third groups (G3, n=4). *KDM1A* immunohistochemistry, magnification x20. Scale bar=50  $\mu\text{m}$ .

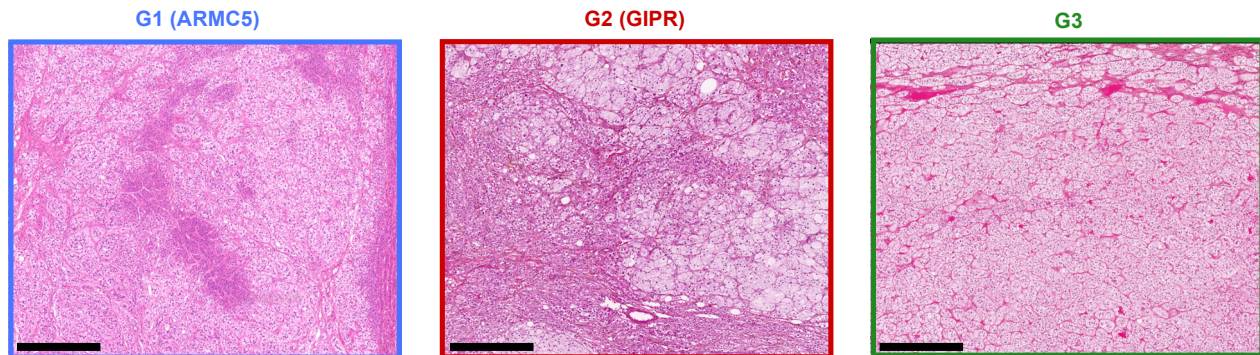


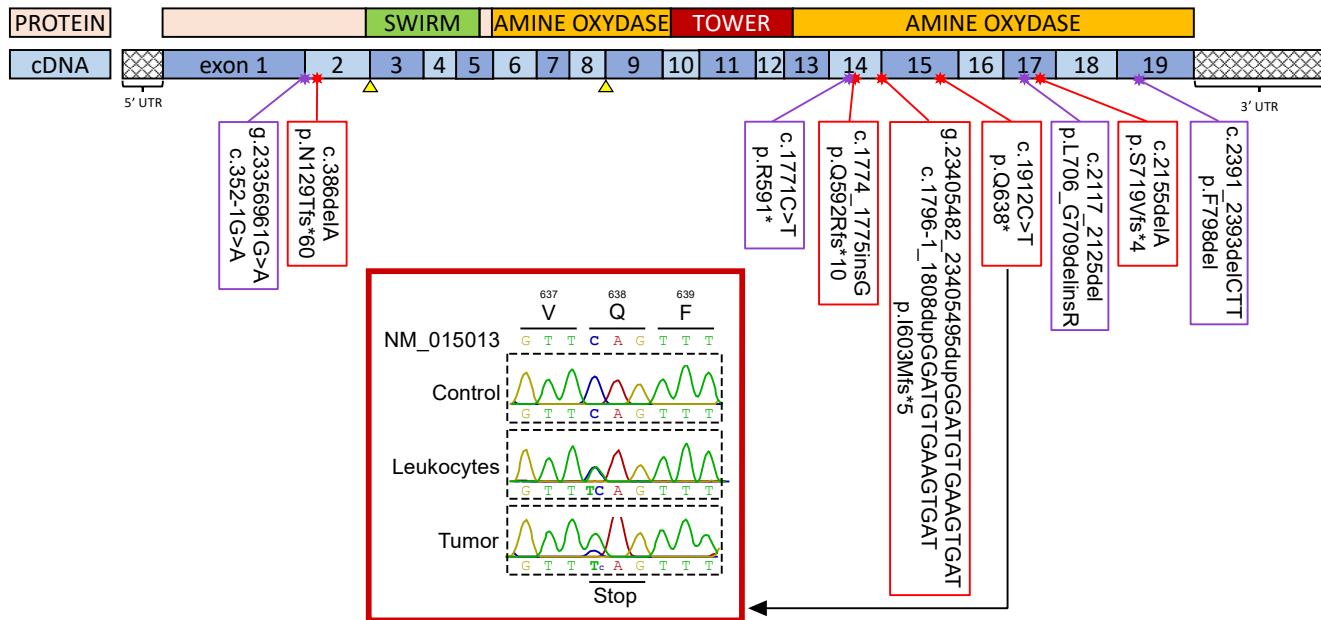
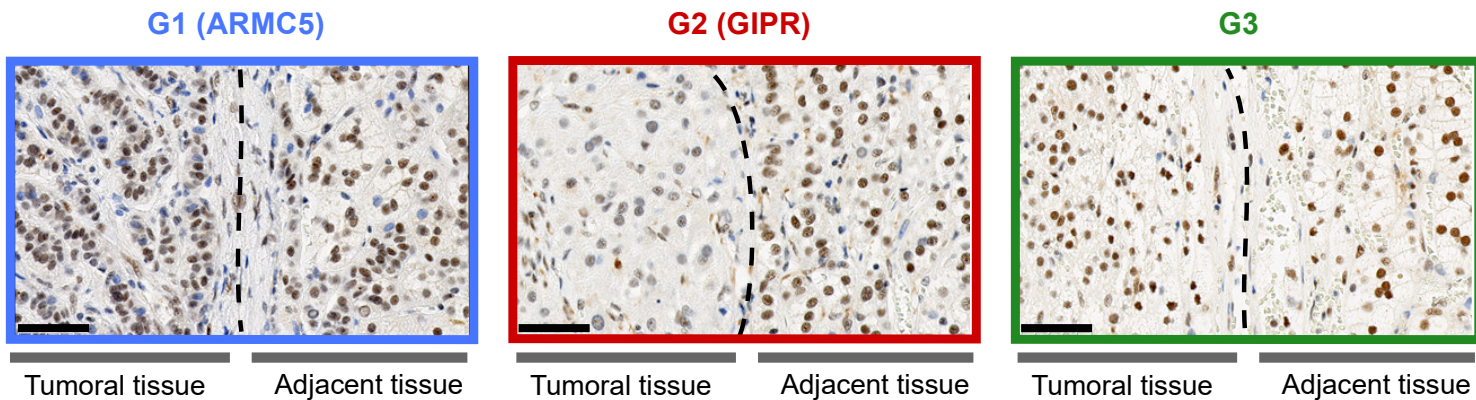
**A****B**

**A**



**B**



**A****B**

**Table 1. Clinical, Hormonal, Imaging and Pathological data of PBMAH patients.**

| Characteristics   | Total cohort         | G1 (ARMC5)        | G2 (GIPR)         | G3                | p value <sup>d</sup>  |
|---|----------------------|-------------------|-------------------|-------------------|-----------------------|
| Number of patients, n (%) <sup>b</sup>  | 36 (100)             | 16 (44.4)         | 6 (16.7)          | 14 (38.9)         | -                     |
| Sex (F/M), n (%) <sup>b</sup>   | 23 (63.8) /13 (36.1) | 9 (56.3)/7 (43.7) | 6 (100) /0 (0)    | 8 (57.1)/6 (42.9) | 0.16                  |
| Age at diagnosis, y <sup>a</sup>  | 50 [30-73]           | 49.5 [30-73]      | 43.5 [31-64]      | 52.5 [40-67]      | 0.4                   |
| 24h urinary free cortisol, nmol/24h <sup>a</sup><br>(normal range <248 nmol/24h)                      | 641.5 [38-2493]      | 791 [289-2338]    | 1076 [176-1909]   | 316 [38-2493]     | 0.056 <sup>#</sup>    |
| Morning plasma cortisol after 1 mg<br>dexamethasone, nmol/L <sup>a</sup><br>(normal range <50 nmol/L) | 400 [81-1140]        | 693 [294-1140]    | 174 [160-400]     | 194 [81-582]      | 0.0064 <sup>*#</sup>  |
| 08:00 AM plasma cortisol, nmol/L <sup>a</sup>   | 453.5 [57-1283]      | 676 [405-1283]    | 214 [57-290]      | 444 [372-817]     | <0.001 <sup>*#§</sup> |
| Midnight plasma cortisol, nmol/L <sup>a</sup>   | 437.5 [86-1178]      | 447.5 [232-1178]  | 607 [461-1156]    | 199 [86-766]      | 0.0096 <sup>*§</sup>  |
| Adrenal size on CT (left + right),<br>mm <sup>a</sup>   | 80 [28-200]          | 100 [60-200]      | 95 [57-115]       | 61 [28-150]       | 0.066 <sup>#</sup>    |
| Surgery (bilateral/unilateral), n (%) <sup>b</sup>  | 29 (80.6) /7 (19.4)  | 15 (93.8)/1 (6.2) | 5 (83.3)/1 (16.7) | 8 (57.1)/6 (42.9) | 0.13                  |
| Proportion of eosinophilic cells in<br>adrenal tumor, n (%) <sup>b,c</sup>                            |                      |                   |                   |                   | <0.001 <sup>*#§</sup> |
| <5%   | 9 (36.0)             | 1 (9.1)           | 0 (0.0)           | 8 (88.9)          |                       |
| 5-29%   | 12 (48.0)            | 10 (90.1)         | 1 (20.0)          | 1 (11.1)          |                       |
| >30%  | 4 (16.0)             | 0 (0.0)           | 4 (80.0)          | 0 (0.0)           |                       |
| Diabetes, n (%) <sup>b</sup>  | 13 (43.3)            | 7 (58.3)          | 1 (20.0)          | 5 (38.5)          | 0.33                  |
| Hypertension, n (%) <sup>b</sup>  | 26 (83.9)            | 13 (100)          | 4 (80.0)          | 9 (69.2)          | 0.11                  |

This table gives the results of the hormonal investigations, pre-operative adrenal imaging and pathological analysis of the 36 patients from the integrated genomics analysis cohort.

Abbreviations: F, female; M, male; y, years; CT, computed tomography.

<sup>a</sup>Quantitative variables are presented as medians [range].

<sup>b</sup>Qualitative variables are presented as absolute number of patients and relative counts percentage.

<sup>c</sup>Data about count of eosinophilic cells in adrenal tumors were available for 25 patients (11 G1 patients, 5 G2 patients, 9 G3 patients).

<sup>d</sup>p value is given for the three-groups comparison; p values for the two-groups comparisons are expressed as follows:

\*G1(ARMC5) patients significantly different from G2 (GIPR) patients (p<0.05);

#G1(ARMC5) patients significantly different from G3 patients (p<0.05);

§G2(GIPR) patients significantly different than G3 patients (p<0.05).

