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CLINICAL REPORT

New pathogenic variants in *ARMC5* gene in a series of Italian patients affected by primary bilateral macronodular adrenocortical hyperplasia (PBMAH)

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Abstract

Background: To perform genetic screening for *ARMC5* gene germline pathogenic variants in patients with primary bilateral macronodular adrenal hyperplasia (PBMAH).

Subjects and Methods: In a group of 10 PBMAH patients, we performed complete sequencing of the coding region of the *ARMC5* gene and MLPA analysis for large deletion detection. In subjects with the *ARMC5* variant, we searched *ARMC5* gene somatic variants on tumor samples.

Results: Among 10 PBMAH patients, we identified four *ARMC5* germline variants (40%). One variant, c:174dupC p.Glu59Argfs*44, was already known; one variant p.Gly323Asp, was already reported and classified as likely disease-causing VUS (class 3–4); two variants p.Leu596Arg and p.Arg811Pro, were never reported before. For p.Gly323Asp and p.Arg811Pro, we identified second deleterious variants at the somatic level, enforcing the possible pathogenic effect of germline variants.

Conclusions: Our results underscore the importance of performing genetic testing also in sporadic PBMAH patients and broaden the spectrum of molecular variants involved in PBMAH syndrome.

KEYWORDS

ARMC5-gene, Cushing syndrome, meningioma, PBMAH

1 | INTRODUCTION

Primary bilateral macronodular adrenal hyperplasia (PBMAH) is a rare cause of ACTH-independent cortisol excess, characterized by macronodular enlargement of adrenal glands. PBMAH has not yet established diagnostic criteria, however pathognomonic radiological features for diagnosis are an enlargement of the adrenal glands, with multiple nodules, by definition >1 cm in diameter, but which can be 5 cm or larger, giving rise to very heterogeneous radiological images. From the point of view of the secretory aspect, overt Cushing Syndrome (CS) is

a rare presentation of PBMAH, which is responsible for less than 2% of all cases of CS (De Venanzi et al., 2014; Hsiao et al., 2009), even if in Chinese population Zhou observed that up to 6.20% of CS develop in patients with PBMAH (Zhou et al., 2019). More frequently patients with PBMAH have Subclinical Cushing Syndrome (SCS): we do not know the real frequency of SCS in PBMAH, but we know that 35–40% of bilateral adrenal adenomas are associated with SCS, and a part of them are certainly attributable to PBMAH (Espiard et al., 2018). Most patients have only mild symptoms, as hypercortisolism develops very gradually according to slow nodular growth; the diagnosis is often delayed, usually between the fifth and sixth decade of life, and lastly achieved after abdominal imaging performed for other reasons. PBMAH considered a rare disease in the past, is actually more and more recognized in clinical practice as a heterogeneous clinical entity characterized by a wide spectrum of severity regarding both functional aspects and morphological features. (Bouys et al., 2022; Chevalier et al., 2021; He et al., 2021; Yan et al., 2022).

Pathophysiology of PBMAH is not attributable to a single pathogenic mechanism: aberrant membrane G protein-coupled receptors expression, abnormality in activation of the cAMP/PKA and wnt/b-catenin signaling pathway, inappropriate autocrine ACTH secretion, all have been identified as possible mechanisms involved both in nodules development and hormonal dysregulation (Drougat et al., 2015; He et al., 2021). The bilateral involvement of the adrenal glands as well as the presence of familial forms have suggested that genetic factors may have a relevant etiopathogenic role. However, in adult PBMAH subjects, no germline pathologic variant has been identified in genes already known as involved in adrenal hyperplasia in complex hereditary syndrome such as *MEN1* (*OMIN, 613733; HGNC, 7010*), *APC* (*OMIN, 611731; HGNC, 583*), *FH* (*OMIN, 136850; HGNC, 3700*), *GNAS1* (*OMIN, 139320; HGNC, 4392*) (Espiard et al., 2015; Espiard & Bertherat, 2015). Loss of function pathologic variants in phosphodiesterase [*PDE11A (OMIN, 604961; HGNC, 8773*)] gene or activating variants in ACTH receptor [*MC2R, (OMIN, 607397; HGNC, 6930*)] gene have been other reliable candidates: in fact, rare pathologic variant of *MC2R* gene (Swords et al., 2004) and variants of *PDE11A* (Libé et al., 2008; Vezzosi et al., 2012), already identified in micro-nodular adrenal hyperplasia, has been found also in PBMAH. However, most PBMAH patients were until recently "genetically unresolved". Thanks to a whole genomic study, the recent identification of germline variants in the tumor suppressor gene Armadillo Repeat-Containing Protein 5 [*ARMC5*, 16p11.2, NM_001105247.2 (OMIN, 615549; HGNC, 25781)], (Assié et al., 2013) has opened up a new way to understand the disease pathophysiology and to improve the clinical management. Little is known about *ARMC5*: it is a cytoplasmic protein and it probably acts as a tumor suppressor gene. *ARMC5* mutations are involved in about 25–50% of PBMAH and it seems that genetic forms are characterized by a more severe clinical course (Bouys et al., 2022; Cavalcante et al., 2022; Faucz et al., 2014; Ferreira et al., 2020; Vena et al., 2022).

2 | AIM OF THE STUDY

The genetic characterization of PBMAH patients, in addition to providing an early diagnostic and therapeutic opportunity to the family members of the probands, allows a better approach to the treatment of the index case. It is in this spirit that we approached the study of the *ARMC5* gene in our series of patients.

3 | SUBJECTS AND METHODS

3.1 | Patients

Ten unrelated patients presenting clinical features of PBMAH have been observed at the outpatient clinic, wards, and laboratory of our institution. Diagnosis of PBMAH was based on CT or MRI abdominal imaging. Table 1 are summarized the relevant clinical features of the subjects. Genetic counseling was offered to all patients, and all of them accepted genetic screening. When possible, for probands carrying a possible damaging *ARMC5* variant we extended genetic counseling and analysis to first-degree relatives.

3.2 | DNA amplification and sequence analysis of the ARMC5 gene

After obtaining informed written consent, the genomic DNA was isolated from peripheral blood (Maxwell-16, Promega, GmbH), and the entire *ARMC5* coding region (NM_001105247.2) was amplified with specific intronic primers, (for PCR conditions and primers see supplementary material). The amplicons were purified with Microcon 50 (Millipore, MA) and then on Performa DTR Gel Filtration Cartridges columns (EdgeBio, CA). The analysis of the coding regions was performed with direct sequencing in Sanger, (*310-DNA-Analyzer*, Life Technology-ThermoFisher Scientific MA). Multiplex ligation-dependent probe amplification (MLPA) analysis was performed to exclude large deletions using kit Probemix P481-A1 *PRKAR1A-ARMC5* (MRC-Holland)

TABLE 1 Biochemical, instrumental features, and follow-up of PBMAH patients

Case	Cortisol (ug/dl)	UFC 24 h (ug/24 h)	DHEAS (ug/dl)	ACTH (pg/ml)	ODST (ug/dl)	Aldosterone (pg/ml)	Renine active (uUI/ml)	Metanephrine (mg/24 h)	Normetanephrine (mg/24 h)	Adrenal imaging (MRI/CT)	Follow-up years
1	17	472	0.52	3	12.7	61	16	0.577	0.818	Multiple bilateral nodular lesions the largest 3.4 × 6cm right, 5 × 3.2 cm left	2
2	11	70.3	0.22	9	8	205	0.4	0.068	0.294	Multiple bilateral nodular lesions the largest 4.3 × 2.3 cm right, 4.5 × 4.4 cm left	11
3	18.2	70.5	n.a.$^{+}$	9	10.3	85	21	0.158	0.243	Multiple bilateral nodular lesions the largest 7.3 × 3.5 cm right, 7.4 × 5.4 cm left	3
4	22.13	463.5	0.15	9.78	5.08	n.a.$^{+}$	n.a.$^{+}$	0.117	0.247	Bilateral hyperplasia with nodular lesions, the largest 3.5 × 3.5 cm right, 4.1 × 2.9 cm left	7
5	12.67	262.7	n.a.$^{+}$	10.2	1.5	132	33.7	0.135	0.374	Bilateral hyperplasia with nodular lesions, the largest 2.4 × 2.1 cm right, 2.2 × 1.7 cm left	4
6	18	88	n.a.$^{+}$	14	2	37	14	0.273	0.735	Multiple bilateral nodular lesions the largest 1.2 cm right, 1.9 cm left	2
7	12	80	0.36	10.7	1.5	278	63	0.074	0.499	Multiple bilateral nodular lesions the largest 3.4 cm right, 1.8 cm left	4
8	15.4	60	2.22	16	2	n.a.$^{+}$	n.a.$^{+}$	0.304	0.254	Bilateral hyperplasia with nodular lesions the largest 1.4 × 7cm right, 1.1 × 4left	2
9	153	n.a.$^{+}$	n.a.$^{+}$	3	9.9	65.4	5.5	n.a.$^{+}$	n.a.$^{+}$	Multiple bilateral nodular lesions the largest 3.0 cm right, 1.5 cm left	3
10	188	195	64	3	5.7	73	6.3	n.a.$^{+}$	n.a.$^{+}$	Bilateral hyperplasia with nodular lesions, the largest 6 cm right, 8 cm left	2

Note: The table shows biochemical, instrumental features, and follow-up of PBMAH patients.

Abbreviations: ODST, Overnight dexamethasone suppression test; n.a. Not available; UFC, Urinary free Cortisol.

TABLE 2 Classification of germline and somatic variants found in the ARMC5 gene

		Variant type		Database		
c.DNA	Protein	Germline variants	Somatic variant	HGMD	Exact	Varsome
c.968G > A	p.Gly323Asp	missense		likely disease-causing mutation (DM?)	not reported	VUS: PM2[b], PP3[c]
c.1787T > G	p.Leu596Arg	missense		not reported	not reported	not reported
c.2432G > C	p.Arg811Pro	missense		not reported	not reported	not reported
c.174*dup*C	p.Glu59Arg*fs*44*	frameshift		desease causing (DM)	not reported	not reported
c:283_286*del*TCGG	p.Ser95Pro*fs**41		frameshift			not reported
c.2025_2025*del*T	p.Leu676Trp*fs**13		frameshift			not reported

[a]Variants reclassified on the basis of the frequency in the population obtained by consulting the reference databases, on the basis of the co-occurrence of certainly pathogenic variants in the same gene, on the basis of the results of the in silico prediction programs, on the basis of the phenotypic characteristics of the patient and his family members (as suggested by the Italian Society of Medical Genetics SIGU).

[b]PM2: absent from controls, all variants were checked in the Exome Aggregation Consortium Database (Exac).

[c]PP3: Multiple lines of computational evidence support a deleterious effect on the gene or gene product (Annex Table 2, Mariani et al., 2020).

(*PRKAR1A*: OMIN, 188830; HGNC, 9388) according to manufacturer's instructions. The MLPA fragments were analyzed on a *310-DNA-Analyzer*. The nomenclature of the new variants was attributed to the directives of the Human Genome Variation Society (HGVS). Four computational tools were used to predict the possible effects of the amino acid substitutions on protein function: MutationTaster (http://mutationmaster.org) and UMD-Predictor (http://umd-predictor.eu/). Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (https://sift.bii.a-star.edu.sg/). See Table 2 for in silico evaluation of the pathological effect of the *ARMC5* variants.

All variants have been submitted to ClinVar database

c.968G > A, p.Gly323Asp, URL: SUB11026311.
c.1787T > G, p.Leu596Arg, URL: SUB11026475.
c.2432G > C, p.Arg811Pro, URL: SUB11026482.
c.174dupC, p.Glu59Argfs*44, URL: SUB11026499.
c.283_286delTCGG, p.Ser95Profs*41, URL: SUB11026520.
c.2025_2025delT, p.Leu676Trpfs*13, URL: SUB11026593.

4 | RESULTS

All patients in this series (6 males – 4 females, age range 48–73, mean 59.7) satisfied conventional criteria for the diagnosis of PBMAH. Seven among them had anomalies in cortisol production: five subjects had overt CS (median age 58.0 years), two SCS (median age 56.0 years), and three had normal cortisol levels (median age 65.0 years).

None of them had a family history of PBMAH, of note *patient-4* had familial antecedents for meningioma, and *patient-9* had familial antecedents for meningioma (sister) and adrenal adenoma (father).

Genetic screening identified *ARMC5* gene variants in four patients, in two subjects 4 and 9, we identified two known pathogenic or likely-pathogenic variants: p.Glu59Argfs*44 (E59R*fs**44) and p.Gly323Asp (G323D); in two subjects 2 and 3, sequencing detected new *ARMC5* variants: p. Leu596Arg (L596R) and p.Arg811Pro (R811P).

4.1 | Description of the clinical and genetic data of patients with ARMC5 pathologic variants

4.1.1 | Patient-2

The patient was a 59 years-old-man, diabetic in adequate glycaemic control with metformin, dyslipidemic, and hypertensive for a long time. Arterial blood pressure was in quite good control with ACE inhibitors, beta-blockers, and calcium-antagonist. In the basal workup for the suspicion of secondary hypertension abdominal ultrasound examination was performed showing the presence of multiple nodular lesions in both adrenal glands. Subsequent CT scan confirmed the presence of multiple adenomas: the bigger ones measuring 4.2 cm on the right gland and 4.5 cm on the left. The biochemical screening revealed the normal level of metanephrines, aldosterone, cortisol, and DHEAS. Insufficient cortisol suppression after overnight low-dose dexamethasone test and low plasmatic ACTH levels confirmed the diagnosis of subclinical hypercortisolism.

The follow-up reserved an unexpected surprise: at the age of 61, he was admitted to the Ophthalmology Department for ptosis, conjunctival and periorbital

		In silico tools prediction programs					
ClinVar	**LOVD**	**Mutation taster**	**PolyPhen**	**UMD predictor**	**SIFT**	**CADD**	**Variant reclassification[a]**
not reported	not reported	disease causing	benign	Pathogenic	damaging	not reported	class IV (DM)
not reported	not reported	disease causing	probably damaging	Pathogenic	damaging	not reported	class III/IV
not reported	not reported	disease causing	probably damaging	Pathogenic	neutral	not reported	class III/IV
not reported	not reported	disease causing	probably damaging	Pathogenic	damaging	not reported	
not reported	not reported	disease causing					
not reported	not reported	disease causing					

hyperemia, and decreased visual acuity. CT brain scan and MRI images showed the presence of diffuse dural meningeal enhancement and thickening, brain parenchyma was normal suggesting the diagnosis of hypertrophic pachymeningitis. This rare disorder may occur with other immunologic diseases, infective disease, and cancer. IgG4 plasmatic levels were elevated supporting the diagnosis of IgG4-related pachymeningitis and episcleritis even in absence of other organ involvement. No report until now describes the co-occurrence of hypertrophic pachymeningitis with PBMAH. The patient had a good response to high-dose systemic steroid treatment and methotrexate, after 6 years of rheumatologic follow-up, he is still in good remission for this immunologic disorder. Actually, at the age of 66 years, nodular lesions in adrenal glands are only slightly increased.

Genetic screening of *ARMC5* detected the novel germline variant c.1787T > G resulting in a substitution of a Leucine in Arginine at codon 596; this variant has not been reported before (Table 3).

4.1.2 | Patient-3

The patient was a 66 years-old-man, affected by difficult-to-control hypertension, diabetes type-2 on insulin therapy for six years, complicated by autonomic neuropathy (orthostatic hypotension, excessing sweating, diarrhea). In the diagnostic work-up for chronic diarrhea, lasting for 15 years, slightly elevated levels of plasmatic chromogranin-A were suspected for neuroendocrine tumor: the Gallium68-PET-TC revealed a bilateral enlargement of adrenal glands, confirmed by MRI. Subsequent CT scan confirmed the presence of multiple adenomas: the bigger ones measuring 7.3 × 3.5 cm on the right gland and 7.4 × 5.4 cm on the left. Biochemical characterization was consistent with the diagnosis of SCS. After 2 years follow-up urinary cortisol levels allowed the diagnosis of overt CS. Adrenal venous sampling showed a prevalence in left adrenal secretion, the patient was submitted to left adrenalectomy, the site of the larger nodular lesion.

After surgery, the patient showed progressive improvement in glycaemic and blood pressure control, however, after a few months plasma ACTH levels (6 pg/dl) and lack of complete serum cortisol inhibition after a low dose overnight dexamethasone suppression test (6mcg/dl), was suggestive for a persistent condition of autonomous cortisol secretion.

The genetic analysis detected the novel germline variant of *ARMC5* gene c.2432G > C, p.Arg811Pro, resulting in a substitution of Arginine in Proline at codon 811, this variant has not been reported before. (Table 3). For this patient it was possible to search for the second hit at the somatic level: genetic analysis of the tumor tissue showed the presence of a deleterious frameshift variant: c.283_286*delTCGG p*.Ser95Pro*fs**41.

Genetic screening was extended to his offspring: one carried the *ARMC5* father's variant. Subsequent phenotyping revealed the presence of an adrenal adenoma and impaired cortisol suppression after an overnight low-dose dexamethasone test.

4.1.3 | Patient-4

The patient, a 56 years-old-man, was hospitalized due to a car accident, he was submitted to whole-body TC with incidental evidence of bilateral enlarged adrenal glands for the presence of multiple adenomas, the bigger on the left gland measuring 4.1 cm and 3.5 cm on the right gland.

TABLE 3 Clinical phenotype and genetic analysis in PBMAH patients

Case	Age (Y)[a]	Sex	CS/SCS	Familial PMAH	Germline mutations	Somatic mutations	Adrenalectomy	Other diseases	Family history
1	48	M	CS	no	neg		left adrenalectomy	thyroid nodules	
2	54	M	SCS	no	p.Leu596Arg		no	thyroid nodules, IgG4-related pachymeningitis	
3	62	M	CS	no	p.Arg811Pro	c:283_286*del*TCGG, p.Ser95Pro*fs**41	left adrenalectomy	prostate adenocarcinoma	
4	54	M	CS	no	p.Glu59Arg *ins*Cfs*44		no	left parietal meningioma	*father*: meningioma and metabolic syndrome
5	66	F		no	neg		no	thyroid nodules	
6	58	M	SCS	no	neg		no	thyroid nodules, prostate adenoma	
7	73	F		no	neg		no		
8	56	M		no	neg		no		
9	64	F	CS	no	p.Gly323Asp	c.2025_2025*del*T, p.Leu676Trp*fs**13	right adrenalectomy	right frontal meningioma, thyroid nodules patients	*father*: parathyroid adenoma and adrenal nodule; *sister*:cerebral meningioma
10	62	F	CS		neg		left adrenalectomy		
FAMILY MEMBERS									
case 3	38	M		yes	neg				
	46	F	SCS	yes	p.Arg811Pro			adrenal adenoma, PRL secreting pituitary microadenoma	
case 9	41	M		yes	p.Gly323Asp				
	32	M		yes	neg				

Note: Family patients are family members of case3 and case9. The table shows the age of ascertainment of the pathology, sex, presence or absence of clinical or subclinical CS, familiarity with PBMAH, and other diseases.

The patient was affected by hypertension, impaired fasting glycemia, and dyslipidemia. Laboratory tests were consistent with CS: insufficient cortisol suppression after overnight low-dose dexamethasone test, low plasmatic ACTH levels, twofold increase in urinary cortisol. At the age of 63, he was admitted to the Neurological Ward due to transient dysarthria and paresthesias involving the left side of the body: brain TC scan revealed the presence of a left parietal meningioma measuring 3.4 × 2.1 × 3.4 cm. The patient was submitted to meningioma surgical resection.

Considering that the secretory aspect was stable over time, the metabolic complications of the disease were mild and well managed by therapy, in accordance with the patient's will, it was decided not to proceed with surgery. After 7 years of follow-up, the patient feels good, and nodular lesions in adrenal glands are stable. The patient reported a family history of metabolic syndrome and the detection of a meningioma in the father. Genetic screening detected a known frameshift variant: duplication of Cytosine in position c.174*dup*C p.Glu59Argfs*44 (Albiger et al., 2017) (Table 3).

4.1.4 | Patient-9

The patient was a 64 years-old-woman, she came to our attention complaining of weight gain (6 kg in two years) hypertension, vertebral, radial, and femoral osteoporosis. The patient also presented with a non-secreting microadenoma of the left part of the pituitary gland, a frontal meningioma, and a non-hyperfunctioning multinodular goiter with normal calcitonin. The patient had undergone an hysteroannessiectomy in 2000 for uterine cervix carcinoma. Familial anamnesis revealed a parathyroid adenoma and an apparently non-secreting adrenal adenoma in the father, dead of cardiac insufficiency, and a cerebral meningioma in the sister, previously treated with gamma knife radiosurgery. The laboratory workup revealed a normal free cortisol and cortisone urinary excretion, low plasma ACTH levels (3 pg/ml), and lack of inhibition of serum cortisol levels after low-dose overnight dexamethasone suppression test (9.9 mcg/dl), indicating a condition of autonomous glucocorticoid secretion. Serum aldosterone and plasma renin concentration were normal, and a condition of impaired fasting glucose was evident. MRI of the abdomen revealed enlarged adrenal glands, both containing multiple nodules, the major in the right of 3 cm, while in the left the greatest was 1.5 cm in diameter. Due to the progressive increase in weight, worsening of glycaemic, and blood pressure control, the patient underwent laparoscopic right adrenal surgery. The histological exam revealed a 6.5 × 2.5 × 2.5 cm multinodular adrenal gland with a prevalent adenoma of 3 cm in diameter. After surgery, the patient showed progressive improvement in glycaemic and blood pressure control and a sense of well-being. Laboratory scenario confirmed normal free cortisol and cortisone urinary excretion, low plasma ACTH levels, and lack of complete serum cortisol inhibition after low dose overnight dexamethasone suppression test (8.4 mcg/dl), consistent with a persistent condition of autonomous cortisol secretion. The patient had already submitted in the past to genetic screening for the *MEN1* variant, with negative results.

The genetic analysis detected the know germline variant of *ARMc5* gene c.968G > A, resulting in a substitution of a Glycine in Aspartate at codon 323 this variant has been reported by Mariani and has been classified as a variant of uncertain significance (Mariani et al., 2020) (Table 3). For this patient, it was possible to search for the second hit at the somatic level: genetic analysis of the tumor tissue showed the presence of a deleterious frameshift variant: p.Leu676Trp*fs*13*.

Genetic screening was extended to the offspring: one 42 years old man carried the *ARMC5* mother's variant. Subsequent phenotyping excluded the presence of autonomous cortisol secretion (normal basal cortisol 15.7 mcg/dl, normal ACTH 22 pg/ml, suppression after overnight low-dose dexamethasone test <1 mcg/dl).

5 | DISCUSSION

Retrospective analyses carried out on PBMAH patients and their relatives with CS have shown that *ARMC5* pathologic variants are the most frequent cause of hereditary forms and are identified in more than 80% of patients with familial presentation (Chevalier et al., 2021; Gagliardi et al., 2014); however, *ARMC5* pathologic variants are involved also in apparently sporadic disease (Albiger et al., 2017; Bouys et al., 2022; Cavalcante et al., 2022; Espiard et al., 2015; Yu et al., 2018), and when considering all PBMAH cases, they are identified in about 20–25% of cases. Results from the study by Bouys and Espiard (Bouys et al., 2022; Espiard et al., 2015) on a large cohort of unrelated PBMAH confirms the widespread opinion that *ARMC5* pathologic variants are associated with a more severe disease phenotype: *ARMC5*-mutated patients show an overt CS more frequently, compared to wild-type patients, adrenals are bigger with a higher number of nodules and are more frequently treated with surgery than non-mutated patients. Results from genetic screening in our little sample confirm the common involvement of *ARMC5*; among our patients with the sporadic presentation, we found an unexpectedly high frequency of *ARMC5* genetic variants: 4 out of 10 subjects (40%). We also observed that all subjects carrying *ARMC5* variants had CS

(3 subjects) or SCS (1 subject), while only three subjects among six patients without *ARMC5* variants had CS or SCS, confirming that the presence of an *ARMC5* variant is predictive of a more severe form of the disease.

Kyo (Kyo et al., 2019) observed that 50% of patients with pathogenic variants in *ARMC5* also had extra-adrenal tumors (breast, thyroid, parathyroid) suggesting an active role of the gene in non-adrenal oncogenesis. However, neither LOH nor the presence of a second hit could be demonstrated: the role of *ARMC5* pathologic variants in non-adrenal neoplasms, therefore, has yet to be investigated. In contrast, the possible role in predisposition to meningioma has stronger evidence: until now nine cases have been described of patients with PBMAH and meningioma (Alencar et al., 2014; Elbelt et al., 2015; Ferreira et al., 2020; Jojima et al., 2020), among them seven with a pathogenic germline variant in *ARMC5* gene: Alencarreported co-occurrence of meningiomas in 3 out of 7 members (43%) of a large Brazilian *ARMC5* mutated family; Elbelt identified the presence of *ARMC5* somatic pathologic variant p.*Arg502fs* in meningioma tissue from a subject carrying the *ARMC5* germline pathologic variant c.323_324*ins*C, p.*Ala110fs*9*, Jojima showed LOH for the *ARMC5* locus in meningioma tissue from a subject carrying the *ARMC5* germline pathologic variant c.799C > T, p.Arg267*. We also could support the hypothesis that *ARMC5* could be involved in a complex hereditary neoplastic syndrome as two of four patients with *ARMC5* pathologic variants had meningioma and one of them had a family history of meningioma (Ferreira et al., 2020). In light of these data, according to the previous suggestion (Jojima et al., 2020) we propose that for all patients carrying *ARMC5* gene variants together with endocrinological screening, brain CT should be suggested at diagnosis and follow-up. It is known that the most common genetic alterations associated with the development of meningiomas are found on the *NF2* gene (OMIN, 607379; HGNC, 7773) with a frequency of 58% (including the loss of the long arm of Chr22, carrying NF2); the loss of the short arm of Chr16, home of *ARMC5*, is present only in 3% of cases, however, more frequently (20%) was observed a gain on 16p, suggesting that instability in 16p could play a relevant role in meningioma development. *ARMC5* could then become a new gene responsible for familial *NF2*-negative meningiomas (Alencar et al., 2014).

The pathogenic role of variants found during a genetic screening is not always easy to decode, especially for the novel missense variants, such new variants we found in our patients are p.L596R and p.R811P. The position of the pathologic variants along the gene has an important role in evaluating the possible deleterious effect; the *ARMC5* gene has two most conserved regions throughout evolution involved in protein interaction and dimerization: the *ARM-repeat domain* in the N-terminus (Armadillo/beta-catenin-like repeat superfamily, a tandemly repeated sequence motif with approximately 40 amino acid long), and the BTB/POZ domain in the C- terminal (Broad-complex, *Tramtrack bric-a-brac/Pox virus, and zinc-finger complex*). More than 60% of the pathologic variants described in the literature are within or near the two domains. (Zhang et al., 2018).

The p.Leu596Arg variant is not included in the ARM and BTB domains but many other deleterious variants had been found in the same region, as shown in Figure 1, where are reported most of the known pathogenic variants, with the two new variants we found. The presence of arginine (polar amino acid, positively charged due to the presence of NH3 group, basic) in place of Leucine (non-polar amino acid) changes the ionic charge, with a possible effect on protein function. Unfortunately, we could not identify the "second hit" at the somatic level, even if we performed on tissue specimens both NGS and Sanger sequencing: however, we need to underly the technical limitation of both techniques which may miss mutation in somatic mosaicism when the mutated allele is lower than 20%.

The p.***Arg811Pro*** variant is within the BTB/POZ domain, Arginine is an amino acid that can undergo methylation processes, and the transition to Proline, an amino acid with the ability to form hinges and interrupt the linearity of the polypeptide, supports the idea that the variant introduces a major disturbance in protein synthesis. For the p.Arg811Pro variant we could identify the second hit at the somatic level, confirming a possible deleterious effect of the germline mutation.

The p.Gly323Asp variant is located within the ARM-repeat domain. The replacement of a Glycine (a sterically simple non-polar amino acid) with an Aspartic Acid (polar amino acid negatively charged due to the presence of a $-CH_2COO^-$) which is a bulky amino acid could lead to a substantial alteration of the structure of the protein. The p.Gly323Asp variant reported by Mariani (Mariani et al., 2020) was classified as likely disease-causing pathologic variant (VUS class III), the identification of a deleterious variant in tumor sample allows the reclassification of the variant to a pathogenetic class (VUS class IV-V). The bioinformatic tools, that we used for the evaluation of the potential pathogenicity of variants, give consistent results, all conferring a possible damaging effect on the two new variants, however, in silico analysis could be considered a preliminary approach. Even if the unequivocal attribution of pathological effect could only come from in vitro functional tests or linkage studies, identification of the "second hit" in histological specimen offers an important contribution to the characterization of new genetic variants and should be encouraged also in clinical practice: by this way we could reclassify two class 3 VUS in class 4.

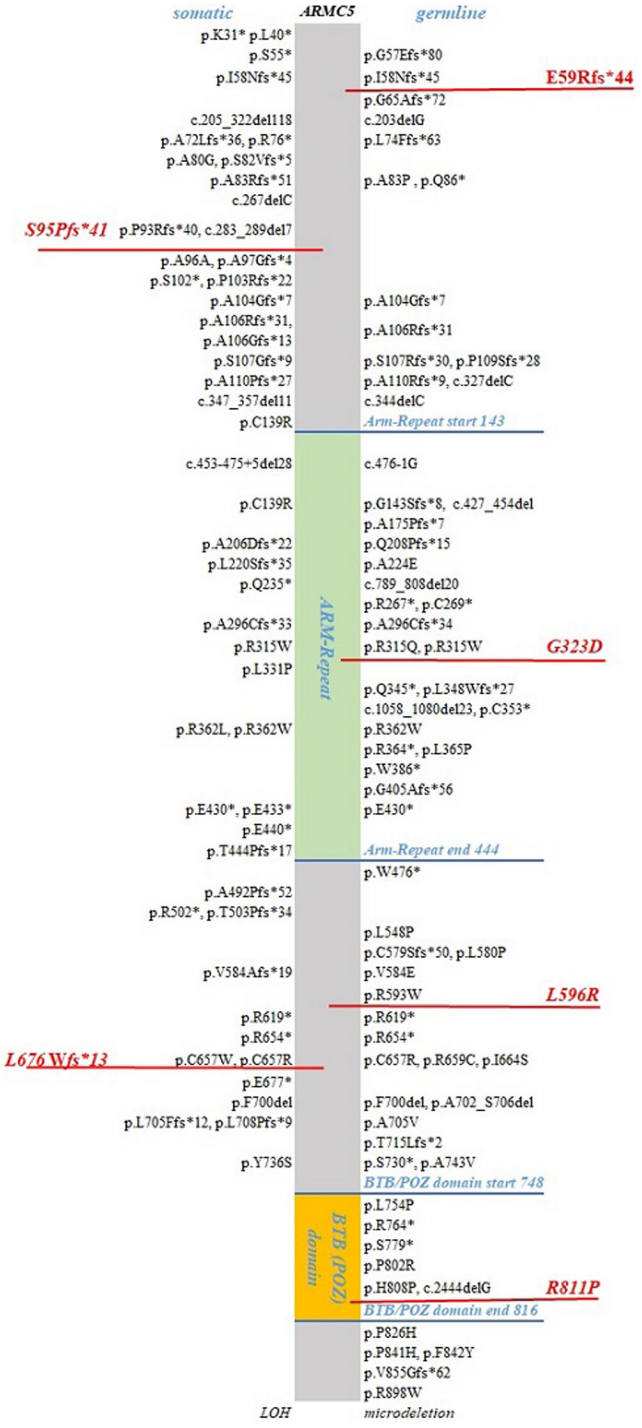

FIGURE 1 Distribution along the gene of known pathogenic variants; in red variants found in our patients, on the left side somatic variants, and the right germinal variants

In most laboratory, functional tests are not available and linkage studies are very difficult to perform as we often deal with late-onset disease, very little kindred, and incomplete penetrance; so we do not have another way to better define the clinical relevance of molecular variants of unknown significance. In addition, the current expansion of cancer molecular characterization techniques has made molecular analysis on tissue specimens more and more suitable, and targeted genetic sequencing of pathologic tissue could give useful information.

Recently has been identified the new causative gene *KDM1A* associated with food-dependent Cushing syndrome observed in PBMAH (Chasseloup et al., 2021; Vaczlavik et al., 2022); in this hereditary syndrome cortisol excess is driven by aberrant adrenal expression of the glucose-dependent insulinotropic polypeptide (GIP) receptor. Until now, no KDM1A causative gene variants have been found in non-food-dependent PBMAH, but it would be interesting to extend the analysis to a larger number of PBMAH.

Clinical features along with pathology and molecular biology of these patients enforce the hypothesis that PBMAH is a very heterogeneous disease, also from a genetic point of view, and if deeper phenotyping should be advisable before genetic analysis, from another point of view it could be intriguing to apply in the clinical setting a multigene NGS analysis comprehensive of genes associated to adrenal hyperplasia: *ARMC5, KDM1A, MEN1, APC, GNAS1, PDE8, PRKAR1A, PRKACA, PDE11*, and *MC2R*.

6 | CONCLUSIONS

Results of our little experience strongly support the indication to perform genetic screening in all patients with PBMAH, also with the sporadic presentation, unfortunately, the hereditary nature of the disease is still underestimated due to the phenotypic variability of the patients, and in the absence of genetic screening, a lot of SCS in PBMAH are still underdiagnosed.

The attribution of a pathogenic role of new genetic variants is one of the greatest challenges for clinical genetics, however, the desired application of genetic screening to an increasing number of pathologies will lead us to face this question more and more frequently. Data sharing and publication, along with the extension of genetic testing to family members and tailored follow-up of genetic variant carriers will help us to give a clear clinical substance to molecular results.

AUTHOR CONTRIBUTIONS

Conception and design: M. G., A. P., L. M., and M. C. Development of methodology: M. G., A. P., and L. M. Acquisition of data: M. G., A. P., P. L. P., R. L., and M. S. L. Writing, review, and revision: M. G., A. P., L.M., and R. L.

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CONFLICT OF INTERESTS

The authors declare that there is no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICAL COMPLIANCE

The authors declare that all details that could reveal the identity of patients have been omitted from the text. Patients provided informed consent for the publication. The clinical study protocol was approved by the institutional review board of ASST-Spedali Civili of Brescia.

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