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High expression of adrenal P450 aromatase (CYP19A1) in association with *ARMC5*-primary bilateral macronodular adrenocortical hyperplasia

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Abstract

Primary bilateral macronodular adrenocortical hyperplasia (PBMAH) is a rare cause of ACTH-independent Cushing syndrome (CS), which has been associated with ectopic G-protein coupled receptors (GPCRs) in the adrenal cortex. We recently studied a 51-year-old male with PBMAH who presented with severe CS and hyperestronemia, manifesting clinically with a cushingoid appearance, gynecomastia, telangiectasias and other signs of high estrogens. Hyperestronemia was further confirmed with aberrant responses to exogenous hCG. Analysis of adrenal tissues following bilateral adrenalectomy showed high expression of P450 aromatase (CYP19A1). The subject carried a germline non-sense pathogenic variant in *ARMC5* (p.R173*), with two independent somatic pathogenic variants identified in the right (p.S571*) and left (p.Q235*) adrenal tissues, respectively. The expression of ARMC5 was drastically decreased in the hyperplastic regions when compared to either the adjacent non-hyperplastic regions and samples from PBMAH without pathogenic variants in *ARMC5*. We found expression of CYP19A1 in other cases of PBMAH, although there were no differences in aromatase expression between *ARMC5*-mutant and *ARMC5*-non-mutant cases. We conclude that in select cases, PBMAH can be associated with aromatase expression resulting in elevated estrogens, irrespective of sex. Additionally, CYP19A1 expression may not depend on the *ARMC5* variant status.

Keywords

PBMAH; Cushing syndrome; CYP19; *ARMC5*

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I. Introduction

Primary bilateral macronodular adrenocortical hyperplasia (PBMAH) is a rare cause of ACTH- independent Cushing syndrome (CS), characterized by subclinical hypercortisolemia, bilateral macronodules and aberrant expression of G-protein coupled receptors (GPCRs) [1]. Approximately 20–40% of patients harbor a germline pathogenic variant in the Armadillo containing protein 5 (*ARMC5*) gene [1–5]. Consistent with the two-hit model of tumorigenesis, an additional somatic pathogenic variant in adrenal tissues is required for the development of adrenocortical hyperplasia, supporting the hypothesis that *ARMC5* is a tumor suppressor gene.

Deregulation of members of PKA pathway through either genomic rearrangements such as the loss of 17q22–24 locus containing the *PRKAR1A* gene, germline or somatic duplication of 19p13.1 and 19q13.32, respectively or genetic variants including *PDE11A* and somatic *GNAS1* have been previously reported [6–12]. Although rarely, variants of the corticotropin (ACTH) receptor (*MC2R*) may as well be involved [13]; typically, the autonomous secretion of steroids in PBMAH could be coupled to aberrant expression of several GPCRs including receptors for glucose- dependent insulinotropic peptide (GIPR), b-adrenergic (ADRB), vasopressin (AVPR2, AVPR3), serotonin (HTR7), glucagon (GCGR), angiotensin II (AT1R), vasopressin (AVPR1), luteinizing hormone/human chorionic gonadotropin (LHCGR), or serotonin (HTR4) [14–23]. Ectopic or eutopic GPCRs expression activates steroidogenesis by mimicking the intra-cellular events normally initiated by MC2R [24]. Production of other steroid hormones, such as aldosterone, testosterone, and estrogens may also occur [22, 25].

Aberrant expression of LHCGRs resulting in hypercortisolemia was first identified in a woman with transient CS during sequential pregnancies that developed clinical PBMAH after postmenopausal sustained LH increase and mitigated by leuprolide therapy [17]. Here, we present the first case of an adult male with *ARMC5*-mutated PBMAH manifesting clinically with severe CS and biochemically with hypercortisolemia and hyperestronemia. Analysis of adrenal tissues following bilateral adrenalectomy was negative for LH expression but showed high expression of the P450 aromatase (CYP19A1) enzyme.

II. Methods

II.1. Subject and data collection

The subject participated in research protocol 00-CH-0160 that was approved by the Institutional Review Boards of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (until 2010) and Diabetes and Digestive and Kidney Diseases (2010 to the present), at the National Institutes of Health. The PBMAH cohort used for western blot analysis of CYP19A1 expression has previously been published [26].

II.2. ARMC5 sequencing in blood and adrenal DNA samples

DNA extracted from blood leukocytes and adrenal nodules (following manufacturer protocol, Qiagen) was sequenced for the *ARMC5* gene as previously described [3]. Whole exome sequencing for this sample is ongoing.

II.3. Immunohistochemistry

Hematoxylin and eosin staining were performed by Histoserv (Germantown, MD, USA). After deparaffinization in Histo-Clear (HS-202; Nationals Diagnostics) and rehydratation, five microns sections of adrenal tissue were unmasking by 20 minutes of boiling in Vector Antigen Retrieval Solution (H3300; Vector Labs). After blocking, ARMC5 (NBP1–94024; Novus Biologicals), CYP11B1 (PA5–63290, ThermoFischer scientific), CYP11B2 (NBP2–13891, Novus biologicals), CYP19 (ab18995, Abcam), ER (6F11, Leica/Novocastra), LH (National Hormone and Peptide program), inhibin A or synaptophysine (SP11, Roche) primary antibody were incubated overnight at 4C and recognized by the appropriate secondary antibody (MP-7401; Vector Labs). The horseradish peroxidase activity was detected with 3'3-diaminobenzidine tetrahydrochloride (SK- 4105; Vector Labs).

II.4. Western blot

Forty nanograms of protein extracted from frozen adrenals tissues in RIPA buffer (50 mM Tris- HCl, pH7.4, 150 mM NaCl, 1% Triton X-100, 0.5% Na-deoxycholate, 1mM EDTA, 10 mM NaF, protease and phosphatase inhibitors (EMD Biosciences)), was separated in 10% SDS Page and transferred onto nitrocellulose membrane. Proteins were then incubated with anti-rabbit anti-P450 aromatase (ab18995, Abcam) and anti-mouse anti-GAPDH (sc-47724, Santa Cruz) primary antibodies overnight at 4C before recognition by the IRDye 800CW anti-rabbit (926–68073, LiCOR) and IRDye 680LT anti-mouse (926–32212, LiCOR) secondary antibody, respectively. Signals is detected by LiCOR CLx and CYP19 expression was quantified using Image StudioLite.

III. Results

III.1. Case report

A 51-year-old Caucasian male with type 1 diabetes mellitus was referred to the National Institutes of Health for evaluation of severe CS and worsening hyperglycemia. He presented with progressive facial fullness, central obesity, fatigability, inability to lose weight, erectile dysfunction and easy bruising. His physical examination demonstrated a Cushingoid appearance, facial redness with telangiectasia's, gynecomastia and decreased testicular size. Testing showed elevated 24-hr urinary free cortisol (UFC) and 17-hydroxysteroids (17OHS), undetectable ACTH, elevated HDL cholesterol (81 mg/dL; High >60mg/dL), increased HDL size (10.1 nm; 8.5–10.5 nm, normal particle number), hyperestronemia (80 pg/mL; 10–60 pg/mL), elevated inhibin A (5.5 pg/mL; <2 pg/mL) and central hypogonadism (Table 1). During the Liddle's test, there was no suppression of UFC, 17OHS, or estrogens (estrone and estradiol). Abdominal imaging revealed characteristics consistent with bilateral macronodular adrenocortical hyperplasia with internodular atrophy (Figure 1) suggesting PBMAH as the most likely diagnosis.

III.2. Provocative aberrant GPCR testing

After baseline biochemical testing, he underwent a modified provocative testing for aberrant GPCRs as previously described [22]. Given his type 1 diabetes, several tests were avoided, including the glucagon test. Testing was performed after an overnight fast, with the subject

in a supine position for at least one hour prior to testing. Briefly, during the postural test, blood was drawn with the subject supine at times −15 and 0 minutes, and after 30, 60, 90, and 120 minutes of ambulation. This was followed by the mixed meal test, where the subject returned to a supine posture for 60 minutes and blood samples were drawn before and 30, 60, 90, and 120 minutes after a standard mixed meal. Three hours after the meal, a 250 ug of ACTH 1–24 (Cosyntropin) was administered intravenously, and blood samples for cortisol was collected at 0, 30 and 60 minutes. On the following morning, the hCG (10,000 U i.m.) stimulation test was performed after an overnight fast, and blood samples for estrogens and cortisol were drawn at −15, 0, 30 60, 90, and 120 minutes

During the posture test, serum cortisol increased from 8.9 mcg/dL to 19 mcg/dL at 60 minutes; aldosterone values were flat throughout the test. His meal test was unremarkable. Following the ACTH stimulation test, cortisol increased from 11.9 mcg/dL to 31 mcg/dL at 60 minutes. Following the hCG administration, estrogens increased but not the cortisol levels (data not shown). The subject underwent bilateral adrenalectomy with resolution of hypercortisolemia, hyperestronemia and normalization of inhibin A.

III.3. Genetic testing of adrenal tissues

Since inactivating pathogenic variants in *ARMC5* are the most frequent genetic alterations in PBMAH, sequencing of *ARMC5* was prioritized. The synonymous variant, p.R146R (c.438G>A; rs201280100) and one nonsense pathogenic variant (c.517C>T, p.R173*; NM_024742) (Figure 1A) were identified in germline DNA; the latter was confirmed in adrenal tissue. Additionally, two additional somatic nonsense pathogenic variants in each adrenal tissues were detected; p.Q235* (c.703C>T; NM_024742) in the left adrenal and p.S571* (c.1712C>G; NM_024742) in the right adrenal (Figure 1A).

III.4. Immunohistochemistry

The adrenal cortex was hyperplastic and the overall histology was consistent with PBMAH (supplementary Figure 1). Immunohistochemistry for ARMC5 confirmed that the described above disease-causing variant caused a decrease of ARMC5 expression in the hyperplastic tissue when compared to either the non-hyperplastic areas or the affected adrenal tissue, without alterations in *ARMC5* gene (Figure 1B). Altogether, these results confirmed that the inactivation of *ARMC5* was responsible for the development of PBMAH.

To better understand the adrenal origins of the excess estrogens, we studied by immunohistochemistry the expression of ER and LHCGR which were negative in the hyperplastic areas; synaptophysin, a marker for other primary adrenocortical hyperplasias was also negative (supplementary Figure 1). Staining for inhibin A was patchy (supplementary Figure 2). While CYP11B1 and CYP11B2 expression was decreased in the hyperplastic area, CYP19A1 was increased when compared to the non-hyperplastic areas but also when compared to PBMAH tissues without a pathogenic variant in *ARMC5*. These results suggested that the estrogen excess was due, at least in part, to CYP19A1 expression.

III.5. CYP19A1 expression in PBMAH nodules

Given the increased expression of CYP19A1 in this subject, we sought to determine whether this was characteristic of other cases with PBMAH. Therefore, we analyzed CYP19A1 expression by immunohistochemistry in two other PBMAH cases, one with a nonsense variant, p.R364* (rs1386368908) and the other with a missense variant, p.I170V (rs35923277) (Figure 4A). These two PBMAH samples showed low CYP19A1 expression in the hyperplastic area. To compare quantitatively CYP19A1 expression between these two groups, we performed a western blot on 4 non-mutated and 7 mutated PBMAH tissues showing a 30 kDa band instead of the one expected at 53 kDa. This is most likely a cleaved form of CYP19A1 that may at least in part, explain why we were not able to detect aromatase activity in PBMAH lysate (Data not shown). However, this analysis confirms that CYP19A1 expression in PBMAH did not depend on the *ARMC5*-sequence status (Figure 4B).

IV. Discussion

We studied the case of an adult male subject with *ARMC5*-mutant PBMAH who presented with severe CS, gynecomastia, and hyperestronemia. Aberrant LHCGR expression resulting in hypercortisolism has been described before in female patients with PBMAH who were then treated with leuprolide [17–19]. Analysis of the adrenal tissue of our subject demonstrated high expression of P450 aromatase (CYP19A1) without a high expression of the LHCGR.

Abnormal steroidogenesis in PBMAH is coupled to aberrant GPCRs expression in adrenal tissues. To this day, the potential correlation between disease-causing *ARMC5* variants and aberrant GPCRs expression has not been well investigated in a large cohort of subjects with PBMAH. However, mice that are heterozygote for *Armc5* (*Armc5*$^{+/-}$) presented with a transient increase of three GPCRs, adrenergic receptor 2A (*Adra2a*), arginine vasopressin receptor 1A (*Avpr1a*) and 5- hydroxytryptamine receptor 1D (*Htr1d*) [26], suggesting that inactivation of *ARMC5* may be directly responsible for their overexpression. However, in these studies, the mouse receptor for LH was not significantly altered in *Armc5*$^{+/-}$ mice.

Recently, we published a similar case of PBMAH in a female with post-menopausal estrogen excess whose adrenal lesions showed LHCGR and aromatase presence that were both more intensely expressed in the hyperplastic areas [25]. Endogenous hypercortisolemia and hyperestrogenemia were treated by leuprolide acetate with good clinical outcome. Non-novel p.R52T (c.155G>C; rs77972073; NM_016953) damaging variants in *PDE11A*, two benign variants in *INHA* (rs371366906) and one benign variant in *ARMC5* (rs35923277) were found in hyperplastic adrenal tissues of this subject [25]. The pathogenicity of the *PDE11A* variant was confirmed by the drastic decrease of PDE11A expression in the hyperplastic tissue [25], which is consistent with previous reports confirming the link between phosphodiesterases and adrenal tumor formation [27]. This case is different from the one described herein in its unique genetic findings (*ARMC5* status), LHCGR expression, and response to leuprolide acetate therapy. However, we did not explore other genetic or epigenetic alterations that would have contributed to our findings.

Adrenocortical cells in PBMAH generally demonstrate inefficient steroidogenesis: this was confirmed recently both *in vitro* and *in vivo* (the latter in mouse studies) showing that *ARMC5*- deficient cells have an overall decline in their steroidogenic efficiency due to down-regulation of the expression of critical steroidogenic enzymes [1, 26]. Thus, the overexpression of CYP19A1 that was seen in the subject of this report was unexpected. We, then, explored the possibility that CYP19A1 was indeed differentially upregulated in *ARMC5*-mutant tissues. However, our studies in additional samples failed to demonstrate significant differences in CYP19A1 expression between *ARMC5*-mutant and *ARMC5*-non-mutant adrenocortical samples from subjects with PBMAH. We speculate that other secondary genetic and/or other molecular events may be responsible for the upregulation of CYP19A1 in the subject of this report.

In conclusion, we have described the first male subject with *ARMC5*-mutant PBMAH to present with estrogen excess due to increased aromatase (CYP19A1) expression in his hyperplastic adrenal glands. The report speaks volumes for the clinical and biochemical heterogeneity of PBMAH and the various hormonal syndromes associated with it.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations:

ACTH	Adrenocorticotropic hormone
CS	Cushing syndrome
PBMAH	Primary Bilateral Macronodular Adrenocortical Hyperplasia
ER	Estrogen Receptor
LH	Luteinizing Hormone
CYP11B1	cytochrome P450 family 11 subfamily B member 1
CYP11B2	cytochrome P450 family 11 subfamily B member 2
CYP19	cytochrome P450 family 19
GPCRs	G-protein coupled receptors

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Highlights

- First *ARMC5* mutated PBMAH case associated with Cushing syndrome and hyperestronemia
- Nodules are negative for ER, LHCGR by immunohistochemistry
- Drastic overexpression of CYP19A1 in the hyperplastic cells
- CYP19A1 expression is not differently associated with *ARMC5* mutational status

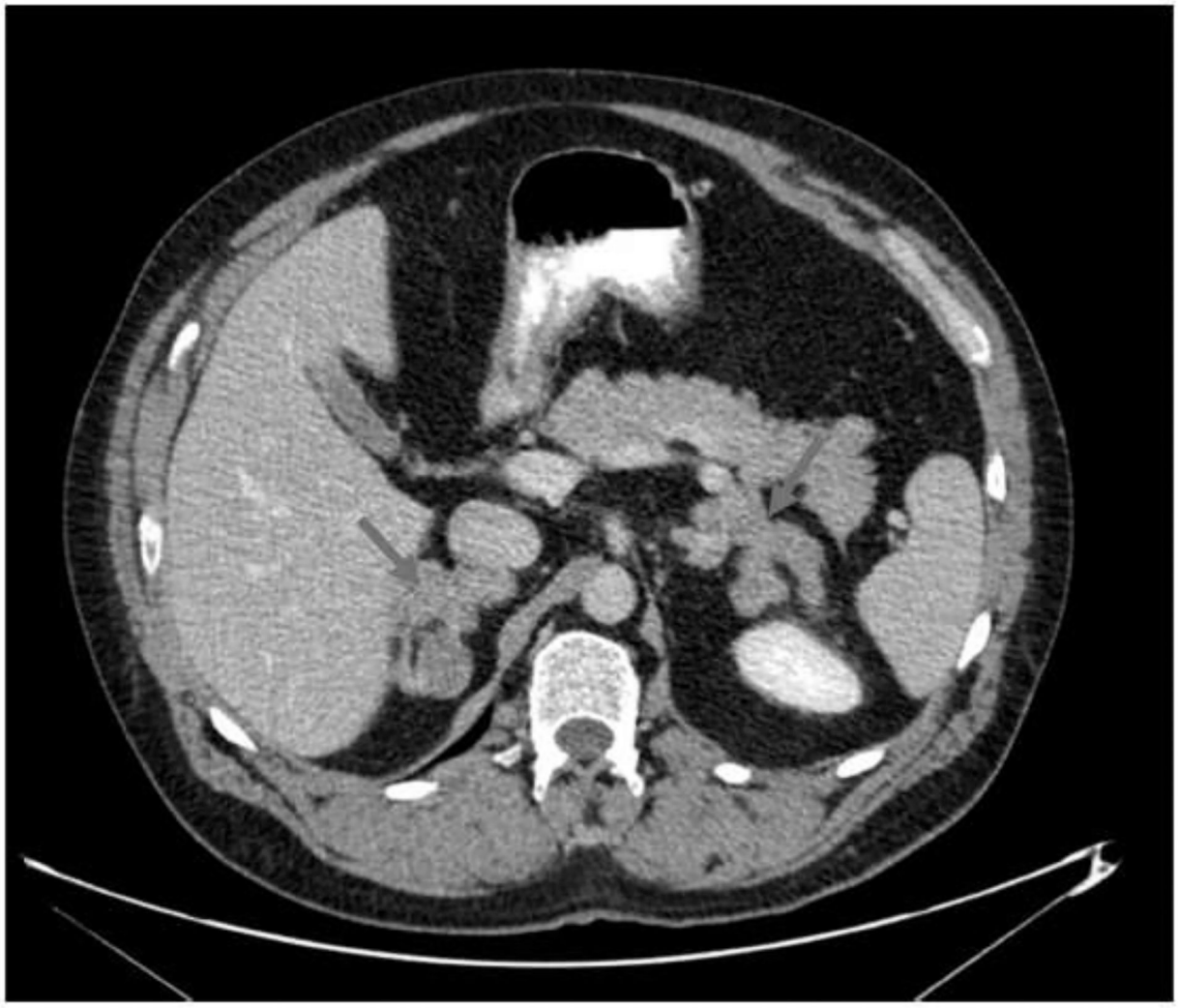

Figure 1:
The abdominal imaging showing characteristic features of PBMAH, with bilateral adrenal hyperplasia and internodular atrophy.

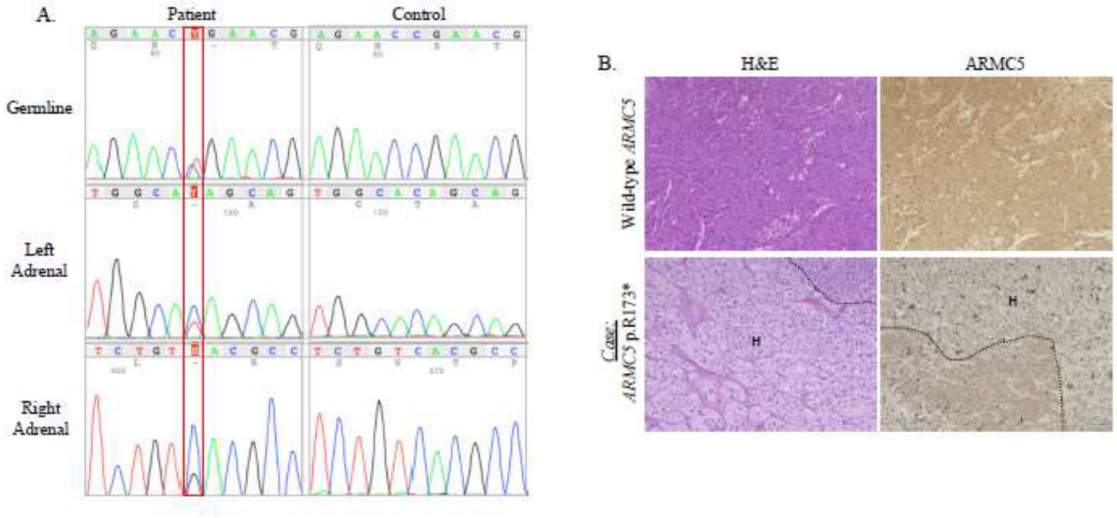

Figure 2:

ARMC5 status and expression in hyperplastic tissues. A. Chromatogram of *ARMC5* exon 2, 3 and 4 from a control (right panel) and the subject (left panel). The subject has three different non-sense pathogenic variants: one germline, p.R173* (c.517C>T), two somatic; one in the left, p.Q235* (c.703C>T) and one in the right adrenal tissue, p.S571* (c. 1712C>G). B. Histology and ARMC5 immunohistochemistry from our case, harboring *ARMC5* p.R173*, and from a control case with no variants in *ARMC5*. ARMC5 is less expressed in the nodules of the subject with the pathogenic variant in *ARMC5* when compared to the case used as control. H: Hyperplastic area.

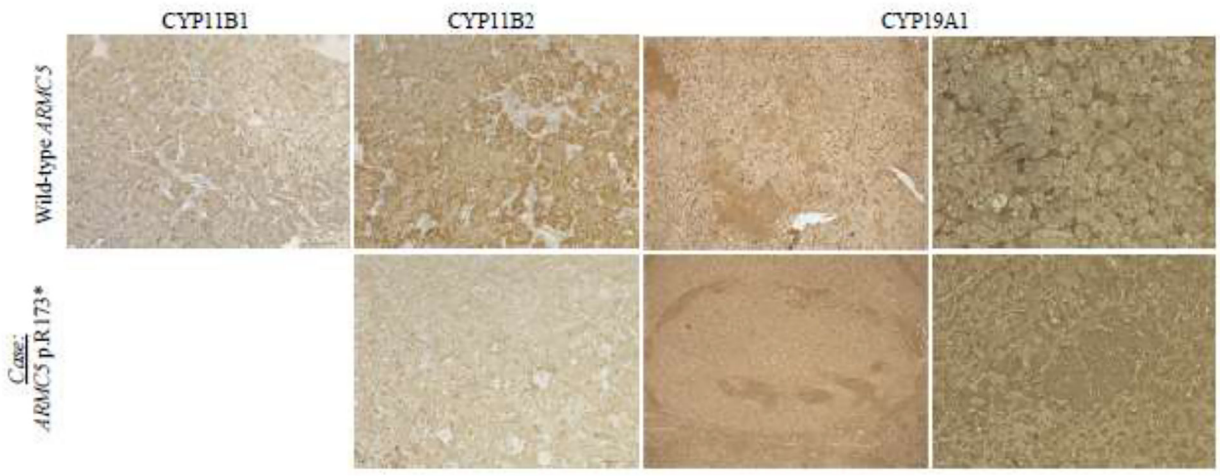

Figure 3:

Comparison of CYP11B1, CYP11B2 and P450 aromatase expression in our subject with estrogen excess and in PBMAH without a pathogenic variant in *ARMC5*. While CYP11B1 and CYP11B2 expression was decreased, P450 aromatase (CYP19A1) was drastically increased in our case.

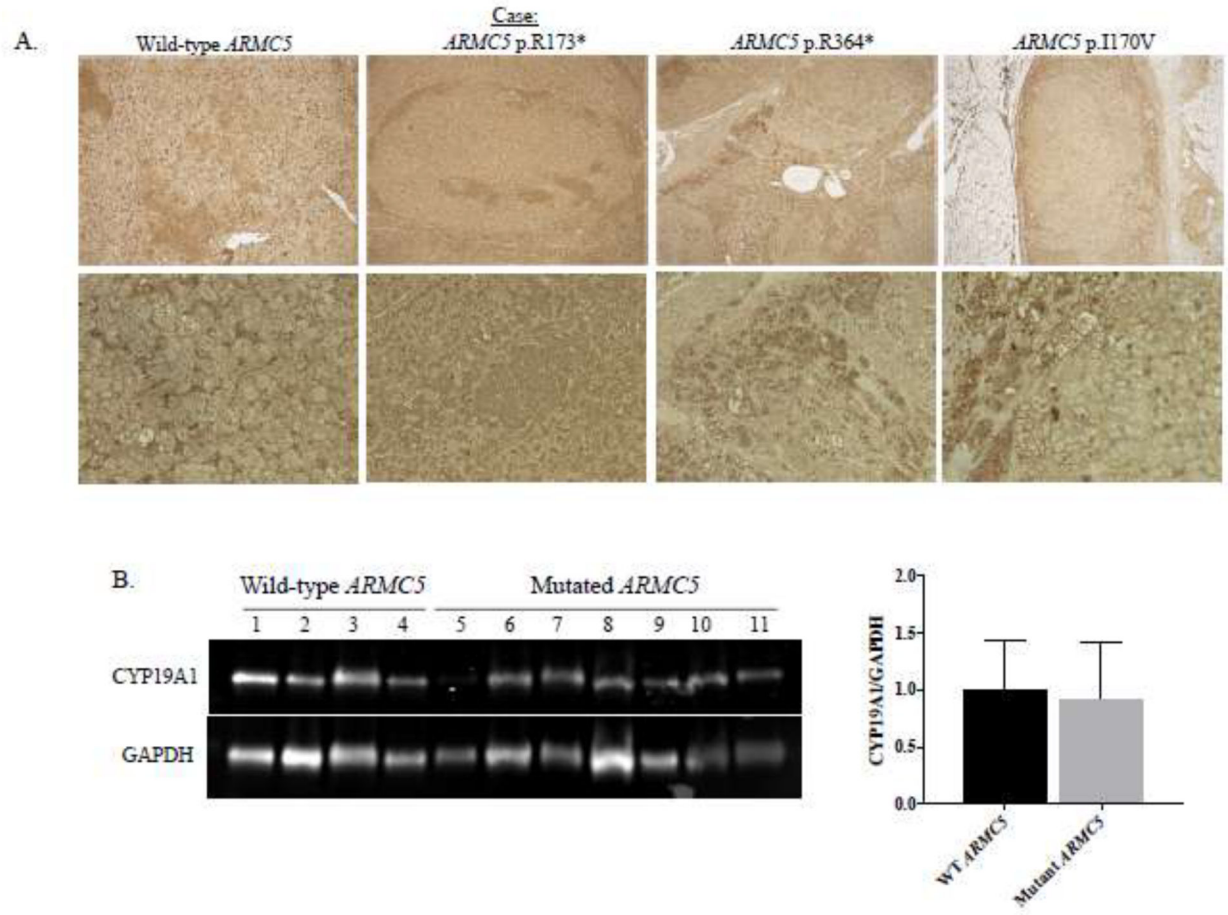

Figure 4:

Analysis of CYP19A1 expression in PBMAH grouped per *ARMC5*-mutation status. A. P450 aromatase is increased exclusively in the case of PBMAH with estrogen excess in the tissues analyzed by immunohistochemistry. B. Western blot for P450 aromatase showed no differences between the cohort of PBMAH with and without pathogenic variants in *ARMC5*.

Table 1.

Laboratory investigations pre-and post-bilateral adrenalectomy.

Laboratory investigation*	Pre-adrenalectomy	Post-bilateral adrenalectomy
11:30pm Serum cortisol(< 1.8 ug/dL)	**9.8 Ug/dL**	
ACTH (5–46 pg/mL)	**<5 pg/mL**	
Estradiol (10–40 pg/mL)[a]	**48 pg/mL**	<10 pg/mL
Fractionated estrogens, E1+E2 (10–60 pg/mL)[a]	**86 pg/mL**	<10 pg/mL
Estrone (10–60 pg/mL)	**122 pg/mL**	17 pg/mL
Estriol Unconjugated [<0.07 ng/mL]	<0.07 ng/mL	
Androstenedione (26–125 ng/dL)	36 ng/dL	
11-Deoxycortisol (<49.00 ng/dL)[b]	**200 ng/dL**	
Progesterone (0.2–1.4 ng/mL)	<0.1 ng/mL	
17-OH Progesterone (13–120 ng/dL)	46 ng/dL	
17-OH Pregnenolone (55–455 ng/dL)	27 ng/dL	
Dehydroepiandrosterone sulfate (0.80–5.60 mcg/mL)	**0.38 ug/ml**	
Compound S (0.0–89.9 ng/dL)	**345.2 ng/dL**	
LH (1–8 U/L)	**0.1 U/L**	**0.1 U/L**
FSH (22–153 U/L)	**0.2 U/L**	**0.2 U/L**
Total testosterone (181.0–758.0 ng/dL)	**132.0 ng/dL**	**166 ng/dL**
Free testosterone [7.4–22.6 ng/dL]	**3.3 ng/dL**	**3.6 ng/dL**
Sex Hormone Binding Globulin (13–71 nmol/L)	**19 nmol/L**	
Inhibin (<2 pg/mL)[c]	**5.5 pg/mL**	<1pg/mL
Human Chorionic Gonadotropin (0–3 IU/L)	<1 IU/L	
24-hr unrinary 17-Hydroxysteroids (2–6 mg/24 hr)	**14.7 mg/24 hr**	
24-hr urinary free cortisol (3.5–45.0 mcg/24h)	**63.8 mcg/24 hr**	
Aldosterone, 24h Urine (2.0–20 mcg/24 h)	4.3 mcg/24 hr	

Abbreviations: ACTH, adrenocorticotropic hormone; FSH, follicle-stimulating hormone; Hr, hours; LH, luteinizing hormone.

*All steroids were measured in the serum using CLIA-certified immunoassays unless specified and references represent that of an adult male.

[a]This testing is mass-spectrometry (MS/MS) based with its performance characteristics determined by Mayo Clinic in a manner consistent with CLIA requirements.

[b]HPLC-MS/MS at ARUP Laboratory

[c]This test was performed by Mayo Clinic in a manner consistent with CLIA requirements. The testing method is an immunoenzymatic assay manufactured by Beckman Coulter Inc. and performed on the UniCel DxI 800.

[d]The testing method is a chemiluminescence assay manufactured by Siemens Healthcare Diagnostics and performed on the Immulite 2500 analyzer.