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Phosphodiesterase 2A and *3B* variants are associated with primary aldosteronism

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CONFLICT(S) OF INTEREST/DISCLOSURE(S)

C.A.S. holds patents on the function of the *PRKAR1A*, *PDE11A*, and *GPR101* genes and related issues; his laboratory has also received research funding on GPR101, abnormal growth hormone secretion and its treatment by Pfizer, Inc; F.R.F. holds patent on the GPR101 gene and/or its function. The other authors have nothing to disclose.

Abstract

Familial primary aldosteronism (PA) is rare and mostly diagnosed in early-onset hypertension (HT). However, “sporadic” bilateral adrenal hyperplasia (BAH) is the most frequent cause of PA and remains without genetic etiology in most cases. Our aim was to investigate new genetic defects associated with BAH and PA. We performed whole-exome sequencing (paired blood and adrenal tissue) in 6 patients with PA caused by BAH that underwent unilateral adrenalectomy. Additionally, we conducted functional studies in adrenal hyperplastic tissue and transfected cells to confirm the pathogenicity of the identified genetic variants. Rare germline variants in *phosphodiesterase 2A* (*PDE2A*) and *3B* (*PDE3B*) genes were identified in 3 patients. The *PDE2A* heterozygous variant (p.Ile629Val) was identified in a patient with BAH and early-onset HT at 13 yrs of age. Two *PDE3B* heterozygous variants (p.Arg217Gln and p.Gly392Val) were identified in patients with BAH and HT diagnosed at 18 and 33 yrs of age, respectively. A strong PDE2A staining was found in all cases of BAH in zona glomerulosa and/or micronodules (that were also positive for CYP11B2). PKA activity in frozen tissue was significantly higher in BAH from patients harboring *PDE2A* and *PDE3B* variants. *PDE2A* and *PDE3B* variants significantly reduced protein expression in mutant transfected cells compared to WT. Interestingly, *PDE2A* and *PDE3B* variants increased SGK1 and SCNN1G/ENaCg at mRNA or protein levels. In conclusion, *PDE2A* and *PDE3B* variants were associated with PA caused by BAH. These novel genetic findings expand the spectrum of genetic etiologies of PA.

Keywords

primary aldosteronism; adrenal hyperplasia; phosphodiesterases

INTRODUCTION

Primary aldosteronism (PA) is characterized by an autonomous aldosterone production, leading to increased sodium reabsorption, hypertension (HT), renin suppression and variable degrees of hypokalemia (FunderCareyManteroMuradReinckeShibataStowasser and Young, 2016). PA prevalence is around 10% in referred patients with HT and 15–20% in those with resistant hypertension (Hannemann and Wallaschofski, 2012, DoumaPetidisDoumasPapaefthimiouTriantafyllouKartaliPapadopoulosVogiatzis and Zamboulis, 2008). The most common causes of PA are aldosterone-producing adenomas (APAs) and bilateral adrenal hyperplasia (BAH), also called idiopathic hyperaldosteronism (FunderCareyManteroMuradReinckeShibataStowasser and Young, 2016). Recently, aldosterone-producing cell clusters (APCCs) have been suggested as a precursor lesion for APAs and possibly other histologic forms of PA (Nishimoto et al., 2015, OmataTomlins and Rainey, 2017).

BAH can be diagnosed in the context of familial forms of PA, which account for <5% of cases and are transmitted as an autosomal dominant trait (Fernandes-RosaBoulkroun and Zennaro, 2017). Familial hyperaldosteronism type I (FH-I) or glucocorticoid-remediable aldosteronism is caused by a chimeric gene with the *CYP11B1* promoter and *CYP11B2* coding sequences (SutherlandRuse and Laidlaw, 1966, LiftonDluhyPowersRichCookUlick and Lalouel, 1992). FH-III is caused by germline mutations in the *KCNJ5* gene, encoding

the potassium channel Kir 3.4. Most of FH-III cases have early-onset HT and macronodular BAH (Scholl et al., 2012, GellerZhangWisgerhofShackletonKashgarian and Lifton, 2008). FH-IV was identified in several unrelated individuals with PA and HT (before 10 yrs of age) harboring *CACNA1H* germline mutations (Scholl et al., 2015). Additionally, *de novo* germline *CACNA1D* mutations have been described in two children with PA and neuromuscular abnormalities (Scholl et al., 2013).

FH-II is clinically and biochemically indistinguishable from sporadic forms of PA. The prevalence of FH-II was reported to be 6% in a large PA cohort (PallaufSchirpenbachZwermannFischerMorakHolinski-FederHofbauerBeuschlein and Reincke, 2012). FH-II is diagnosed when at least two first-degree members of the same family have confirmed PA (APA or BAH). A linkage analysis was established with the chromosomal region 7p22 in some kindreds with FH-II (LaffertyTorpyStowasserTaymansLinHuggardGordon and Stratakis, 2000). However, no genetic defects were found in genes located in this locus (ElphinstoneGordonSoJeskeStratakis and Stowasser, 2004, JeskeSoKelemenSukorWillysBulmerGordonDuffy and Stowasser, 2008). Recently, germline mutations in the *CLCN2* gene, encoding the ubiquitously expressed inwardly rectifying chloride channel CLC-2, were identified in FH-II kindreds and as *de novo* mutations in early-onset PA (Fernandes-Rosa et al., 2018, Scholl et al., 2018). Moreover, the age of HT and PA diagnosis in individuals carrying *CLCN2* mutations varied from very early ages (< 10 yrs) to young adults (Scholl et al., 2018).

Besides familial cases, germline defects have been also described in sporadic PA. Three different germline *KCNJ5* mutations were identified in individuals with sporadic PA (Daniil et al., 2016, MurthyXuMassimoWolleyGordonStowasser and O'Shaughnessy, 2014). In addition, a germline *CACNA1H* mutation was described in a patient with PA without familial history and HT diagnosed at age of 48 yrs (Daniil et al., 2016). Interestingly, *ARMC5* variants, predicted to be damaged *in silico*, were identified in African Americans with sporadic PA (Zilbermint et al., 2015). This link between *ARMC5* and PA increased the spectrum of molecular alterations of PA in a specific population. Based on this finding, we can speculate that genetic defects associated with other forms of BAH can be a clue to define new etiologies for PA.

A high prevalence of somatic mutations in the *CACNA1D* were identified in APCCs from BAH (Omata et al., 2018). A *KCNJ5* somatic mutation was identified in only one APCC (Omata et al., 2018). However, since BAH affects both adrenals, it is reasonable to speculate the presence of germline susceptibility defects in what has been considered so far as "sporadic BAH causing PA". Therefore, we analyzed here a cohort of PA patients with BAH by exome sequencing to investigate new genetic defects associated with bilateral aldosterone excess.

MATERIALS AND METHODS

The study was approved by the Ethics Committees of the Hospital das Clínicas, University of São Paulo and informed written consent was obtained from all patients. Since we were

interested in performing paired (blood and adrenal tissue) exome sequencing analysis, we selected six patients (4 females and 2 males) with PA caused by BAH that underwent unilateral adrenalectomy (Table 1). PA screening and confirmatory testing followed the 2016 Endocrine Society Guideline for PA management (FunderCareyManteroMuradReinckeShibataStowasser and Young, 2016). In 5 patients, unilateral adrenalectomy was guided by computed tomography (CT) findings. None of 6 patients had PA biochemical cure neither HT remission after unilateral adrenalectomy. BAH was defined by the absence of PA cure after unilateral adrenalectomy and by the findings of adrenal hyperplasia on histopathology analysis with a positive CYP11B2 staining in all cases (Table 1). Except for case 3, the nodules observed on CT imaging (with at least 1 cm diameter) were not identified in the histopathological analysis.

DNA and RNA extraction

Genomic DNA was extracted from peripheral blood leukocytes by the proteinase K–SDS salting out method (MillerDykes and Polesky, 1988). After surgical resection, representative areas of tumor or hyperplastic tissue were macrodissected by a pathologist. Tumor fragments were immediately frozen in liquid nitrogen and stored at −80ºC until total RNA and DNA extraction using the AllPrep DNA/RNA Mini Kit (*Qiagen, Courtaboeuf Cedex, France*). Before tumor RNA and DNA extraction, tumor fragment was cut in a cryostat and slides stained by hematoxylin and eosin to confirm the representativeness of hyperplastic tissue.

Whole exome sequencing

Exons were captured with a SureSelect Human All Exon V6 Kit (*Agilent Technology, Santa Clara, CA*) and sequenced on a HiSeq2500 system (*Illumina, San Diego, CA*). Alignment of raw data and variant calling were performed according to steps previously described (da Silva et al., 2019). Briefly, the FASTQ files were aligned to human reference genome GRCh37/hg19 with a Burrows-Wheeler alignment tool (Li and Durbin, 2010). Variant calling was performed with Freebayes (germline) and Lancet (somatic) in all BAM files, and the variants were annotated with ANNOVAR (WangLi and Hakonarson, 2010).

The exome and the targeted panel sequencing data were screened for rare variants with minor allele frequency <0.1% in the public databases: Genome Aggregation Database (gnomAD) (Brownstein et al., 2014), 1000 Genomes (Genomes Project et al., 2015), and the Brazilian population database (ABraOM) (Naslavsky et al., 2017). Then, we selected rare variants located in exonic and consensus splice-site regions. Subsequently, the filtration pipeline prioritized potentially pathogenic candidate variants (loss-of-function variants and variants classified as pathogenic by multiple *in silico* programs). The allelic variants were classified according to The American College of Medical Genetics and Genomics classification (Richards et al., 2015).

Sanger sequencing was used to confirm the potentially pathogenic variants identified by massively parallel sequencing and for segregation analysis. PCR products were sequenced with a BigDye® Terminator version 3.1 cycle sequencing kit followed by automated sequencing on an ABI PRISM® 3130xl genetic analyzer (*Applied Biosystems, Carlsbad,*

CA). *KCNJ5* somatic mutation was confirmed by Sanger sequencing as previously described (Vilela et al., 2019).

Immunohistochemistry

An immunoperoxidase immunohistochemical modified method with humid heat antigen retrieval was used as previously described (ShiKey and Kalra, 1991). Staining with anti-PDE2A rat monoclonal antibody (sc-271394, *Santa Cruz Biotechnology, Dallas, TX*) and anti-PDE3B rat monoclonal antibody (sc-376823, *Santa Cruz Biotechnology*) were performed in 13 adrenal lesions (6 APAs and 7 BHs). CYP11B2 (rat monoclonal, clone 41–17B, *Merck MABS125, Temecula, CA*) staining was also analyzed as previously described (Gomez-Sanchez et al., 2014).

Immunohistochemistry was evaluated by an experienced pathologist (M.C.N. Zerbini). Staining was evaluated according intensity as negative (0), low (1), medium (2), or strong (3). The percentage of positive tumor cells was visually scored as follow: 0 if 0% of tumor cells were positive; 1 if 1–25%; 2 if 26–50%, 3 if 51–75% and 4 if 76–100% (de Sousa et al., 2015). Protein expression was evaluated in zona glomerulosa, hyperplasia, micronodules and adenomas.

Quantitative real-time RT-PCR (qRT-PCR)

cDNA was generated from 1 μg of total RNA using the commercial kit Superscript III First Strand S (*Invitrogen, Carlsbad, CA*). Quantitative real-time PCR was performed in the ABI Prism 7000 sequence detector using TaqMan gene expression assays according to the manufacturer's instructions (*Applied Biosystems*). The PCR cycling conditions were as follows: 2 min at 95 °C, 40 cycles of 95 °C for 15 sec and 60 °C for 30 sec, and a final step at 72 °C for 30 sec. The assays for target genes were *SGK1* (Hs00178612_m_1), β-actin (*ACTB*, 4310881E) and β-glucoronidase (*GUSB*, 4310888E). The relative expression levels were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). A commercial pool of normal human adrenal cortex from autopsies was used as reference sample (*Clontech, Palo Alto, CA*).

PKA enzymatic activity in frozen tissue

PKA activity was analyzed in 7 frozen tissues from 2 APAs and 2 BAHs (case 1 and 3 from Table 1) and one normal adrenal (obtained from a normotensive individual that underwent radical nephrectomy). PepTag Assay for non-radioactive detection c-AMP-Dependent Protein Kinase *(Promega, Madison, Wisconsin, USA*) was employed following the manufacturer's protocol to measure PKA activity in frozen tissues. All experiments were performed in triplicate.

Mutagenesis

The human *PDE2A* WT (NM_002599.5) coding sequence was cloned into the pCMV6-AC-GFP vector (RG207219, *Origene, Rockville, MD, USA*). The human *PDE3B* WT (NM_000922.4) coding sequence was cloned into the pCMV6-Entry vector with C-terminal Myc-DDK tag (PS100001, *Origene*). The p.Ile629Val variant was introduced into the human *PDE2A* WT template and the p.Arg217Gln or p.Gly392Val variants were introduced into the

human *PDE3B* WT using the QuikChange Lightning Site-directed Mutagenesis Kit (210518–5, *Agilent Technologies, Santa Clara, CA*), following the manufacturer's protocol. The following mutagenic primers were used: PDE2A_Ile629Val_MUT _F: CAGTCAATTTTGTAGTTGTTGACGAAATTCA TGTCCTGCAGCATG and PDE2A_Ile629Val_MUT_R: CATGCTGCAGGACATGA ATTTCGTCAACAACTACAAAATTGACTG; PDE3B_Arg217Gln_MUT_F: AGAA CGCAGTGCTGGAGCCGCAGCG and PDE3B_Arg217Gln_MUT_R: CGCTGCGGC TCCAGCACTGCGTTCT; PDE3B_Gly392Val_MUT_F: TCTTTGGCCTACAGGAA ACTGAGAAAGCACCCATT and PDE3B_Gly392Val_MUT_R: AATGGGTGCTTT CTCAGTTTCCTGTAGGCCAAAGA.

Cell culture

Human Embryonic Kidney 293T cells (HEK 293T) were grown in Dulbecco's modified Eagle's medium (DMEM, high glucose, pyruvate, no glutamine; 10313, Gibco) supplemented with 10% fetal bovine serum (100–106, *Gemini Bio Products, West Sacramento, CA*) and 1% antibiotic (Penicillin-Streptomycin – 15140–148, Gibco) in a humidified atmosphere at 37°C with 5% CO2.

Analysis of protein expression

HEK 293T cells were seeded into 6-well plates at a density of 3×105 cells per well. After 24h of incubation, cells were transfected with Lipofectamine 2000 (11668030, Invitrogen) according to the manufacturer's protocol, using Opti-MEM I Reduced Serum Medium (31985–070, Gibco) and 500ng of each vector (human WT *PDE2A*, p.Ile629Val *PDE2A,* human WT *PDE3B*, p.Arg217Gln *PDE3B* and p.Gly392Val *PDE3B*) alone. The empty pCMV6-Entry vector was used as a negative control. After 24 hours of transfection, cells were washed with PBS and resuspended in 50µl of ice-cold lysis buffer (Tris-HCl 10 mM, pH 7,5, NaCl 150 mM, EDTA 1 mM, EGTA 1 mM, SDS 0.1%, Nonidet P-40 1%) containing a cocktail of protease and phosphatase inhibitors (*Sigma-Aldrich PPC1010*). The collected cells were incubated for 30 minutes on ice and then centrifuged for 15 minutes at 4 °C, 13000 rpm. Total protein concentration of supernatant was determined by Pierce™ BCA Protein Assay (23227, *Thermo Scientific*), following the manufacturer's protocol. After quantification of protein extracts, 50µg of total proteins of each sample were separated by electrophoresis in 10% polyacrylamide gel under denaturing conditions (SDS-PAGE). Proteins were then transferred to a nitrocellulose membrane (BioRad) and Western Blot was performed using antibodies against DDK (TA 50011–100, Origene) diluted 1:1000, GFP (TA 15004, Origene) diluted 1:1000 and GAPDH (SC-32233, *Santa Cruz Biotechnology*) diluted 1:2000. Fluorescent secondary antibodies (827–08364 IRDye 800CW Goat anti-Mouse and 926–68073 IRDye 680RD Donkey anti Rabbit, *LI-COR Biosciences, Lincoln, NE*) diluted 1:20000 and Odyssey CLx Imaging System (*LI-COR Biosciences*) were used to acquire the signal of the bands.

Primary antibodies against SGK (cat# sc-28338, *Santa Cruz Biotechnology*; mouse, 1:500), SCNN1G (13943–1-AP*, Proteintech*; coelho, 1:1000) and GAPDH (cat# sc-25778, *Santa Cruz Biotechnology*; rabbit, 1:1000) as a loading control were used. Membranes were then incubated in monkey anti-rabbit or donkey anti-mouse secondary antibodies for 1 hour at

room temperature before visualizing the membranes with SuperSignal West Pico chemiluminescent substrate (*Thermo Scientific*). Ratio of densitometry volumes for the proteins of interest to GAPDH were used for quantification (*Image Lab 5.2.1 software, Biorad*). All experiments were performed in triplicate.

PKA enzymatic activity assay

HEK 293T cells transfected with PDE2A and PDE3B variants as previously described were homogenized in ice-cold lysis buffer (20mM Tris (pH 7.5), 0.1mM sodium EDTA, 1mM dithiothreitol) with 1:100 protease inhibitor cocktail (*EMD Biosciences, La Jolla, CA*) and 0.5mM PMSF. Total protein was quantified by BCA assay.

PKA enzymatic activity was measured with a previously described method that utilizes P^{32}-labeled ATP and kemptide substrate with or without added cAMP (5μM) (NesterovaYokozakiMcDuffie and Cho-Chung, 1996). Each reaction (50 μL) was performed in duplicate and contained 10 μg total protein. Basal and total kinase activities were calculated as pmol kinase activity per minute per milligram of protein without or with a saturating concentration of cAMP (5μM), respectively. Activity values were adjusted by subtracting non-specific kinase activity that was assessed by performing replicate reactions in the presence of a specific PKA inhibitor (PKI, 5μM). All experiments were performed in triplicate.

Statistical analysis

Statistical analysis was performed using IBM SPSS Software (25.0; SPSS Inc., Chicago, IL) and GraphPad Prism (version 8.4.2; San Diego, CA). Data are expressed as mean ± SEM. Comparisons were carried out via unpaired two-tailed t test. P value <0.05 was considered significant.

RESULTS

Whole exome sequencing

All cases underwent unilateral adrenalectomy guided by CT or because of inconclusive lateralization index at AVS. Then, bilateral aldosterone excess was defined by the absence of PA biochemical and clinical cure after surgery and by the presence of adrenal hyperplasia with positive CYP11B2 areas on histopathology analysis (Table 1). Since BAH with PA are rarely treated surgically, our study brings a unique opportunity to study germline variants in patients with available hyperplastic tissue for staining and molecular analysis using frozen adrenal tissue.

Regarding population frequency, we excluded all variants present with a minor allele frequency >0.1% in the 1000 Genomes, Genome Aggregation Database (gnomAD), 1000 Genomes, and the Brazilian population database (ABraOM). Of these, only missense, nonsense, frameshift variants in coding regions, and splice sites were included. Next, we searched for rare germline variants, predicted to be deleterious in at least 3 *in silico* tools, in genes encoding ion channels or in genes previously associated with forms of adrenal hyperplasias (Table 2).

In 3 subjects with BAH associated with PA, we identified rare germline variants in *phosphodiesterase 2A* (*PDE2A*) and *3B* (*PDE3B*) genes. The *PDE2A* heterozygous variant (p.Ile629Val) was identified in a patient with PA and early-onset HT diagnosed at 13 yrs of age (**Case 1**, Table 1). Genetic analysis and subsequent clinical investigation showed that her mother carried the *PDE2A* variant and has HT, but not PA (Fig. 1A). Alignment of amino acid residues encoded by *PDE2A* showing that the 629 residue is conserved across human, mouse, and rat.

The *PDE3B* heterozygous variant (p.Arg217Gln) was identified in a patient with PA caused by BAH and early-onset HT at 18 yrs of age (**Case 2**, Table 1). Familial segregation analysis was not possible due to lack of DNA from relatives (Fig. 1B). In addition, a second *PDE3B* heterozygous variant (p.Gly392Val) was identified in a patient with PA and BAH with HT diagnosed at 33 yrs of age (**Case 3**, Table 1). Parents' DNA samples were not available for segregation (Fig. 1C). Alignment of residues encoded by *PDE3B* showing that the 217 and 392 residues are conserved across human, mouse, and rat. All *PDE2A* and *PDE3B* germline variants were classified as variants of uncertain significance according The American College of Medical Genetics and Genomics classification (Table 2).

A somatic *KCNJ5* mutation (p.Gly151Arg) was found in the hyperplastic tissue from case 3, harboring the *PDE3B* p.Gly392Val germline heterozygous variant. In the hyperplastic adrenal tissue of the patients with *PDE2A* and *PDE3B* variants, there was no evidence of loss of heterozygosity. Among all adrenal hyperplastic tissues, we did not identify any somatic rare and *in silico* deleterious variant (loss- or gain-of-function) in driver genes related to hyperplasia.

Autonomous cortisol secretion was investigated in 4 out of 6 patients. Case 1 (harboring the *PDE2A* p.Ile629Val variant) and case 2 (harboring the *PDE3B* p.Arg217Gln) had a negative hormonal screening for hypercortisolism. On the other hand, the patient with *PDE3B* p.Gly392Val had an abnormal cortisol levels after an overnight 1 mg dexamethasone suppression test (varying from 3.5 to 5.6 μg/dL in different occasions). ACTH, urinary and midnight salivary cortisol were normal.

Functional analysis with hyperplastic tissue from bilateral adrenal hyperplasias

Next, we investigated PDE2A and PDE3B staining in hyperplastic tissue from BAH associated with PA (Table 3). Normal adrenal gland tissue displayed a strong PDE2A expression in zona glomerulosa (Fig 2A). Interestingly, PDE2A immunoreactivity was present in zona glomerulosa and hyperplastic areas (Fig 2B–D). A strong positive PDE2A staining was detected in all 6 cases of BAH in subcapsular hyperplasia and/or micronodules (positive for CYP11B2 staining) (Table 3) (Fig. 2C and D). Additionally, we analyzed PDE2A staining in 5 APAs. PDE2A protein expression was strong and homogeneous in 4 APAs. In one APA, PDE2A staining was strong but only in the peripheral tumor area. PDE3B expression was mainly diffuse in the hyperplastic adrenal with a moderate/strong immunoreactivity in 3 out of 6 cases of BAH (Table 3).

PKA activity was investigated in frozen tissue from a normal adrenal, two APAs and 2 BAHs (from case 1 with germline *PDE2A* and case 3 with *PDE3B* p.Gly392Val variants).

Frozen tissue from the BAH patient harboring *PDE3B* p.Arg217Gln was no longer available for this study. PKA activity in frozen tissue was significantly higher in BAH harboring the germline *PDE2A* p.Ile629Val and *PDE3B* p.Gly392Val variants when compared to APAs and normal adrenal (Fig. 3A). Furthermore, we investigated expression of the *serum/glucocorticoid-regulated kinase 1* (*SGK1*) gene. Aldosterone is the most important regulator of SGK1, which in turns regulates ENaC (Pearce, 2003). *SGK1* gene expression was significantly higher in BAH from PA patients with *PDE2A (*p.Ile629Val) and *PDE3B* (p.Arg217Gln and p.Gly392Val) variants than in APAs without PDEs variants (Fig. 3B).

In vitro functional studies

PDE2A and *PDE3B* variants were generated by site-directed PCR mutagenesis and their protein expression levels were studied in HEK 293T cells after transfection with wild type or mutants. We found a significant decrease in the expression of PDE2A in cells transfected with the *PDE2A* p.Ile629Val mutant compared to cells transfected with wild type *PDE2A* (Fig. 4A). Similarly, *PDE3B* mutants (p.Arg217Gln and p.Gly392Val) lead to a significant reduction in PDE3B expression when compared to wild type PDE3B in transfected cells (Fig. 4B). Although *PDE2A* and *PDE3B* variants lead to a decrease in protein expression, these variants did not increase cAMP stimulated PKA activity in HEK 293T cells (Fig. 4C and D).

Next, we analyzed expression of SGK1 and sodium channel epithelial 1 subunit gama (SCNN1G or ENaCg) proteins; the latter is the final aldosterone target to increase renal sodium reabsorption. The *PDE2A* p.Ile629Val variant did not change SGK1 expression in HEK 293T transfected cells, whereas the *PDE3B* p.Arg217Gln variant significantly increased SGK1 expression. The *PDE3B* p.Gly392Val variant also increased SGK1 expression but did not reach statistical significance (Fig. 5A and B).

The *PDE2A* p.Ile629Val variant did not significantly increase SCNN1G expression, although we cannot rule out a biological effect based on mRNA expression data (Fig. 5C). The *PDE3B* p.Arg217Gln variant significantly increased SCNN1G expression. On the other hand, the *PDE3B* p.Gly392Val variant also increased SCNN1G expression but did not reach statistical significance (Fig. 5D).

DISCUSSION

In this study, we demonstrate that loss of function *PDE2A* and *PDE3B* variants might be associated with PA caused by BAH. PDEs constitute a large and complex superfamily that contains 11 PDE gene families comprising 21 genes; PDEs are critical regulators of the intracellular concentrations of cAMP and cGMP as well as of their signaling pathways and downstream biological effects (AzevedoFauczBimpakiHorvathLevyde AlexandreAhmadManganiello and Stratakis, 2014). Germline inactivating *PDE11A* and *PDE8B* sequencing defects were identified by genome-wide studies in patients with isolated micronodular adrenocortical disease (iMAD) and Cushing syndrome (Horvath et al., 2006, HorvathMericq and Stratakis, 2008). *PDE11A* defects may also predispose to primary macronodular adrenocortical hyperplasia, a disorder mostly associated with hypercortisolism (Vezzosi et al., 2012). In addition, *PDE8B* and *PDE11A* variants might contribute to

predisposition of adult and pediatric adrenocortical tumors (Rothenbuhler et al., 2012, Libe et al., 2008, Pinto et al., 2020). Besides cortisol-producing adrenocortical hyperplasias and tumors, inactivating *PDE11A* variants were also associated with testicular germ cell tumors (Azevedo et al., 2013). To date, pathogenic variants in *PDEs* have not been associated with BAH and PA.

PDE2A has higher affinity for cGMP than cAMP and its catalytic activity is allosterically stimulated by cGMP binding to the PDE2 GAF-B domain (ErneuxCouchieDumontBaraniakStecAbbadPetridis and Jastorff, 1981). Activation of PDE2A decreases the cAMP level and thereby inhibits ACTH-stimulated aldosterone secretion in mice (SpiessbergerBernhardHerrmannFeilWernerLuppa and Hofmann, 2009). Atrial natriuretic peptide up-regulates PDE2A activity via cGMP levels and decreases aldosterone secretion (NikolaevGambaryanEngelhardtWalter and Lohse, 2005). Interestingly, stabilization of ß-Catenin in zona glomerulosa cells resulted in expansion of the zona glomerulosa by directly stimulating the expression of *Pde2a* (Pignatti et al., 2020). Here, we identified a rare *PDE2A* variant in a patient with PA caused by BAH and very early-onset HT. The *PDE2A* p.Ile629Val variant lead to an important decrease in PDE2A expression. In addition, PKA activity was markedly increased in the adrenal hyperplastic tissue from this patient, but not in APAs.

PDE2A is expressed in zona glomerulosa of the murine adrenal cortex (SpiessbergerBernhardHerrmannFeilWernerLuppa and Hofmann, 2009). PDE2A expression was also found to be prominent in zona glomerulosa of rats, mice, cynomolgus, monkeys, dogs, and humans (StephensonCoskranWilhelmsAdamowiczO'DonnellMuravnickMennitiKleiman and Morton, 2009). In our study, we demonstrated that PDEA2 was strongly expressed in normal zona glomerulosa of adrenal cortex, subcapsular hyperplasia and micronodules from BAH associated with PA. Some micronodules from BAH were positive for both PDE2A and CYP11B2. Therefore, PDE2A represents a marker for zona glomerulosa and aldosterone-producing lesions.

PDE3A and *PDE3B* constitute PDE3B family and display high structural homology. PDE3A and B affinity for cAMP is higher than for cGMP (AzevedoFauczBimpakiHorvathLevyde AlexandreAhmadManganiello and Stratakis, 2014). In the current study, we identified two rare germline variants in *PDE3B* (p.Arg217Gln and p.Gly392Val) gene in two patients with BAH and PA. A significant reduction in PDE3B expression was evidenced in transfected cells with *PDE3B* mutants (p.Arg217Gln and p.Gly392Val). Furthermore, adrenal hyperplasia from one of these patients exhibited a significant increase in PKA activity when compared to normal adrenal and APAs. Although *PDE3B* defects have not been previously associated with any form of HT, germline *PDE3A* missense mutations were associated with autosomal dominant HT and brachydactyly type E (Maass et al., 2015). This particular genetic syndrome includes beyond brachydactyly type E, severe salt-independent HT, neurovascular contact at the rostral-ventrolateral medulla, altered baroreflex blood pressure regulation and stroke before age 50 years if untreated HT (Schuster et al., 1998). Very recently, a germline nonsense variant (p.Arg783*) in *PDE3B* gene was identified in a child with adrenocortical tumor without *TP53* mutation (Pinto et al., 2020).

Aldosterone increases the transcription of the basolateral Na^+/K^+-ATPase and the apical ENaC (ValinskyTouyz and Shrier, 2018). After binding to the cytosolic mineralocorticoid receptor, aldosterone promotes the transcription of aldosterone-regulated genes, including SGK1. SGK1 increases ENaC activity by reducing its ubiquitination and receptor internalization (StaubDhoHenryCorreaIshikawaMcGlade and Rotin, 1996). In our study, *PDE2A* and *PDE3B* variants increased the expression of SGK1 and/or SCNN1G (ENaCg) at mRNA or protein level. Our findings connect PDE2A and PDE3B with aldosterone signaling thorough SGK1 and ENaC regulation.

Besides sodium reabsorption, an increase in SGK1 activity exacerbates diet-induced obesity, metabolic syndrome and HT (Sierra-RamosVelazquez-GarciaVastola-MascoloHernandezFaresse and Alvarez de la Rosa, 2020). In addition, SGK1 activation stimulates hypercoagulability, fibrosis and inflammation (Lang and Voelkl, 2013). Aldosterone promotes fibrosis and inflammation via activation of SGK1 and NF-κB, which were inhibited by eplerenone (TeradaKuwanaKobayashiOkadoSuzukiYoshimotoHirata and Sasaki, 2008). Interestingly, a SGK1 inhibitor reversed the increase of blood pressure caused by hyperinsulinism and salt excess in mice (AckermannBoiniBeierScholzFuchss and Lang, 2011).

Defects in PDEs (*PDE11A* and *PDE8B*) have been associated with cortisol-producing micro- and macronodular adrenal hyperplasias, but not with PA (Hannah-Shmouni and Stratakis, 2020). Among the 3 patients with germline *PDE2A* or *PDE3B* germline variants, only one harboring the *PDE3B* p.Gly392Val variant had autonomous cortisol secretion. A steroid metabolome analysis revealed that glucocorticoid excess is frequent in PA (Arlt et al., 2017). However, glucocorticoid metabolite excretion was not different between APAs and BAHs.

The lack of AVS confirming bilateral aldosterone excess in our patients is a limitation of this study. Bilateral adrenal hyperplasia was defined by the absence of PA cure after unilateral adrenalectomy and by the findings of adrenal hyperplasia on histopathology analysis with a positive CYP11B2 staining in the resected adrenal. Despite the fact that 3 cases had unilateral disease based on imaging, the persistence of PA after adrenalectomy strongly indicates the presence bilateral disease. In hyperplastic adrenal lesions, we identified only one *KCNJ5* somatic mutation. In a previous study, Omata *et al.* identified *CACNA1D* somatic mutations in 58% of micronodules positive for CYP11B2 from BAH (Omata et al., 2018). The absence of *CACNA1D* somatic mutations in our study can be explained by the fact that genetic analysis was not guided by CYPB112 staining in adrenal lesions.

Most of the cases of BAH associated with PA are considered “sporadic” and remain without a genetic diagnosis. Aldosterone-producing BAH are treated with aldosterone antagonists in the great majority of the cases. Our cohort of 6 patients with PA and BAH treated surgically represent a unique cohort where we could investigate germline defects and conduct functional studies in tissues from adrenal hyperplasia. In this report, we add two new genes to the roster of molecules that may be involved in the pathogenesis of aldosterone production in BAH.

In conclusion, we demonstrated that *PDE2A* and *PDE3B* variants are associated with the pathogenesis of bilateral PA. PKA activity was higher in adrenal hyperplastic tissue from those patients. Additionally, *PDE2A* and *PDE3B* variants increased gene or protein expression of SGK1 and SCNN1G/ENaCg, downstream mediators of aldosterone signaling. This evidence suggests the potential pathogenicity of *PDE2A* and *PDE3B* variants and expand the spectrum of genetic etiologies for PA and FH-II, a genetically heterogeneous disorder (LaffertyTorpyStowasserTaymansLinHuggardGordon and Stratakis, 2000, ElphinstoneGordonSoJeskeStratakis and Stowasser, 2004).

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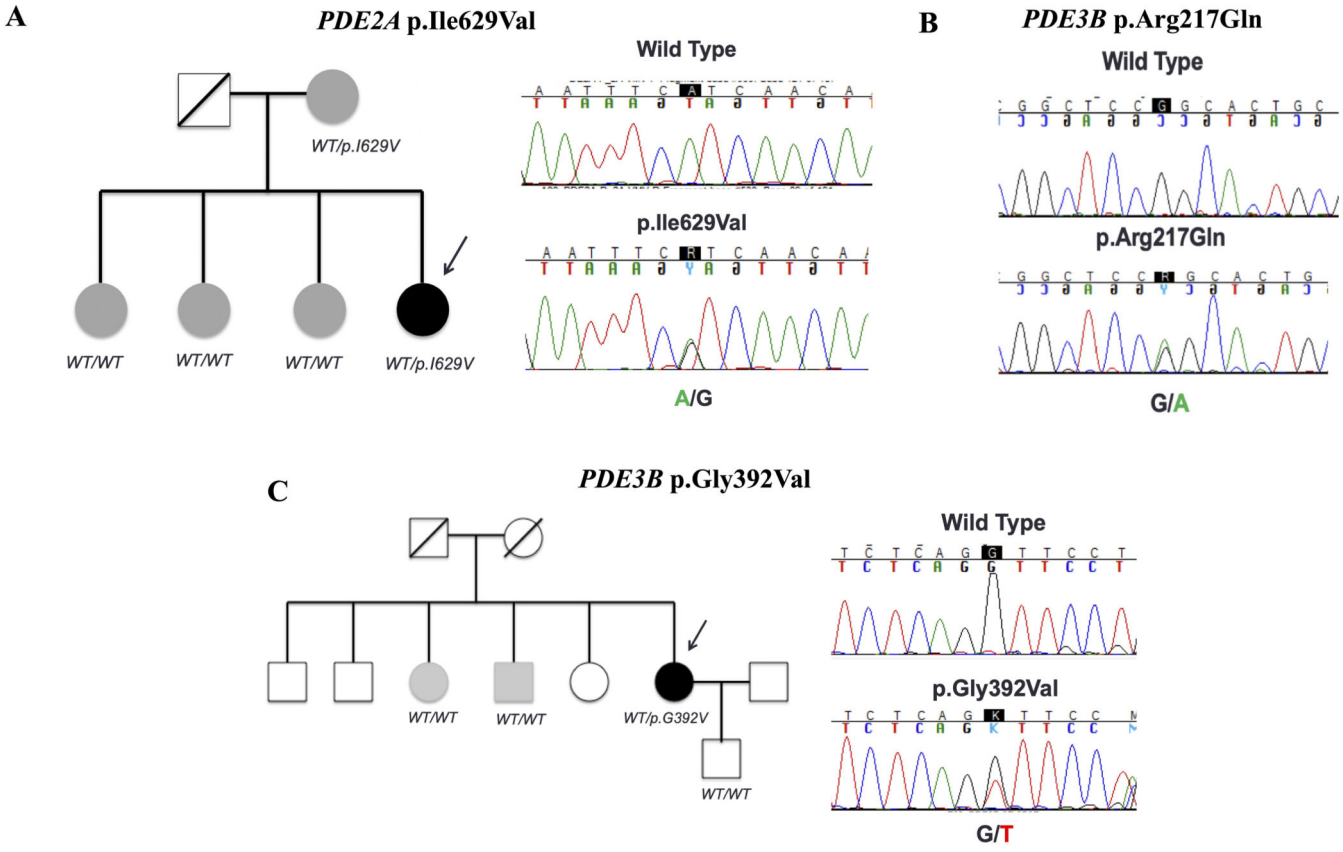

Figure 1.

A, A *PDE2A* heterozygous variant (p.Ile629Val) was identified in a patient with primary aldosteronism (PA) and early-onset hypertension (HT). Pedigree showed that her mother carries the *PDE2A* variant and has HT, but not PA. Sanger sequencing chromatograms showing the wild-type *PDE2A* sequence of an unaffected sister and the *PDE2A* p.Ile629Val variant of the index case. **B,** The *PDE3B* heterozygous variant (p.Arg217Gln) was identified in a patient with PA and early-onset HT. Familial segregation was not possible due to lack of DNA from relatives. Sanger sequencing chromatograms showing the *PDE3B* p.Arg217Gln variant of the index case. **C,** A *PDE3B* heterozygous variant (p.Gly392Val) was identified in a patient with PA. Pedigree showed that only the index case harbored the *PDE3B* variant (Parent's DNAs not available). Subjects with PA are shown with a black-filled symbol, those with HT (without PA) are represented by gray-filled symbol and non-affected subjects are shown with unfilled symbols. Index case is shown by an arrow.

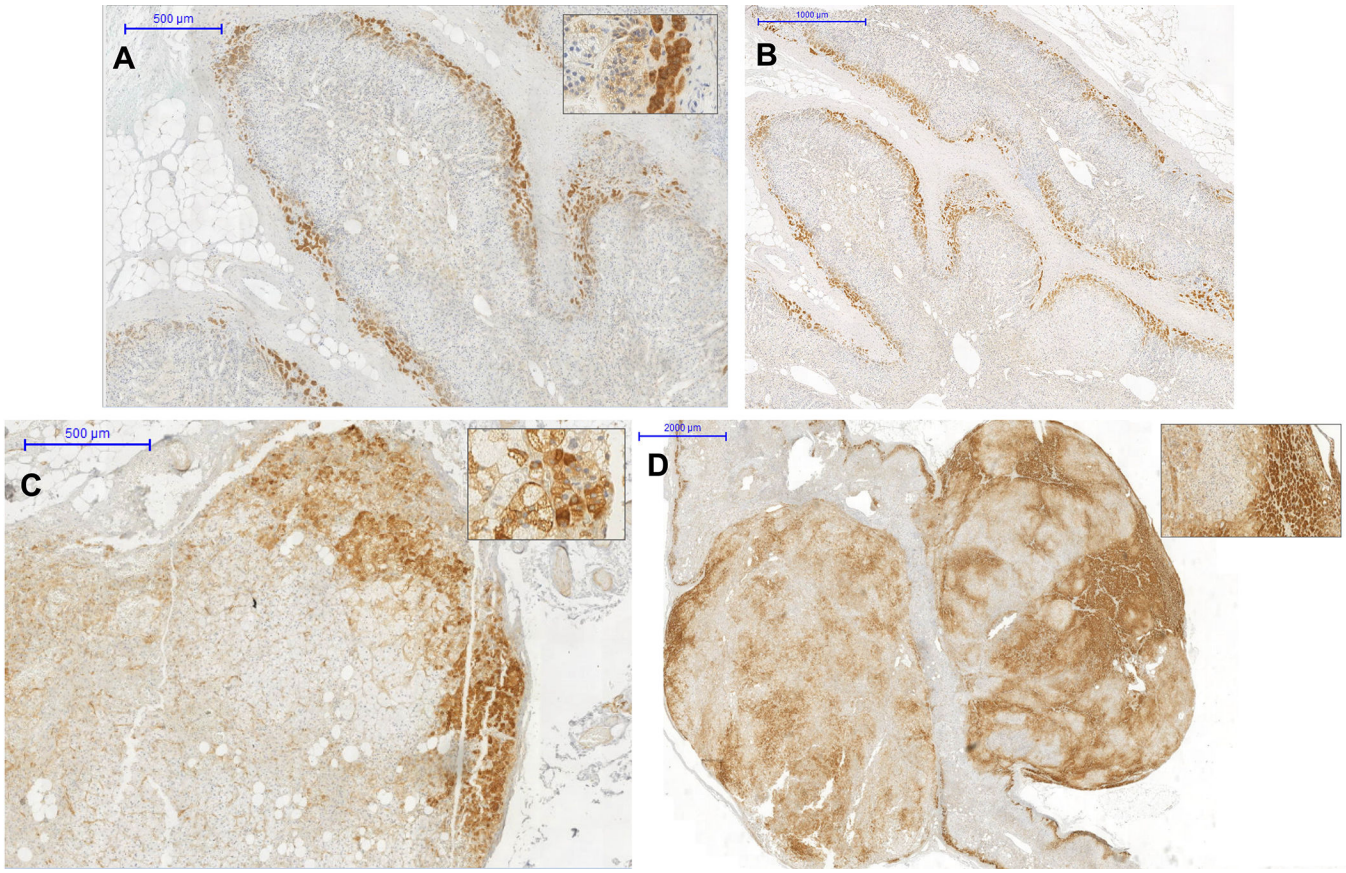

Figure 2.

A, Normal adrenal displaying a strong PDE2A expression in the zona glomerulosa. PDE2A immunoreactivity in zona glomerulosa from a BAH (**B**). **C and D,** A strong PDE2A staining was found in hyperplastic areas of BAH associated with PA. BAH, bilateral adrenal hyperplasia; PA, primary aldosteronism.

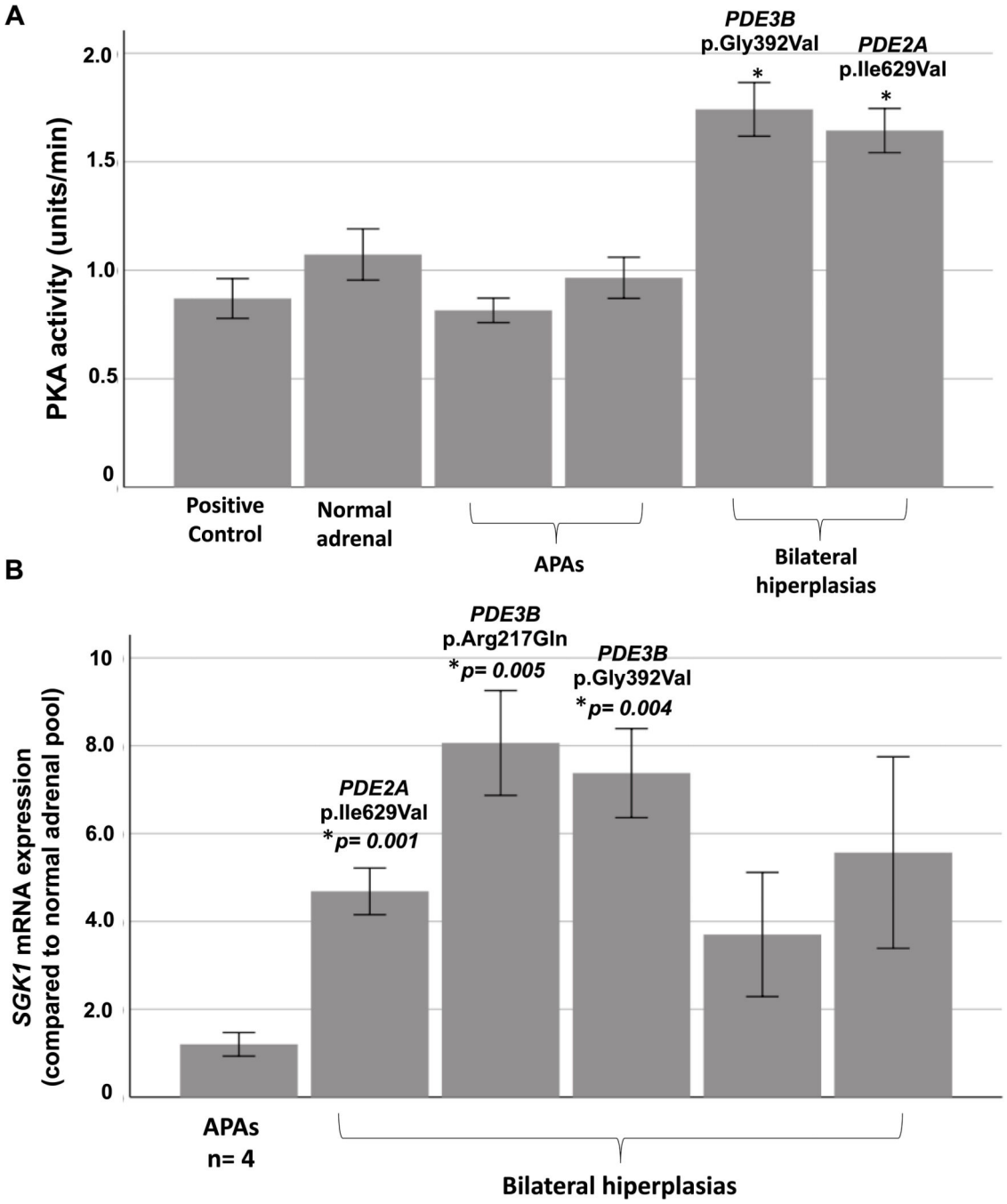

Figure 3.

A, PKA activity in frozen tissue was significantly higher in BAH from PA patients harboring the germline *PDE2A* p.Ile629Val and *PDE3B* p.Gly392Val variants when compared to APAs and normal adrenal. **B,** *SGK1* gene expression was significantly higher in BAH from PA patients with *PDE2A (*p.Ile629Val) and *PDE3B* (Arg217Gln and p.Gly392Val) variants than in APAs without PDEs variants. APA, aldosterone-producing adenomas; BAH, bilateral adrenal hyperplasia; PA, primary aldosteronism; PDE, phosphodiesterase. All experiments were performed in triplicate.

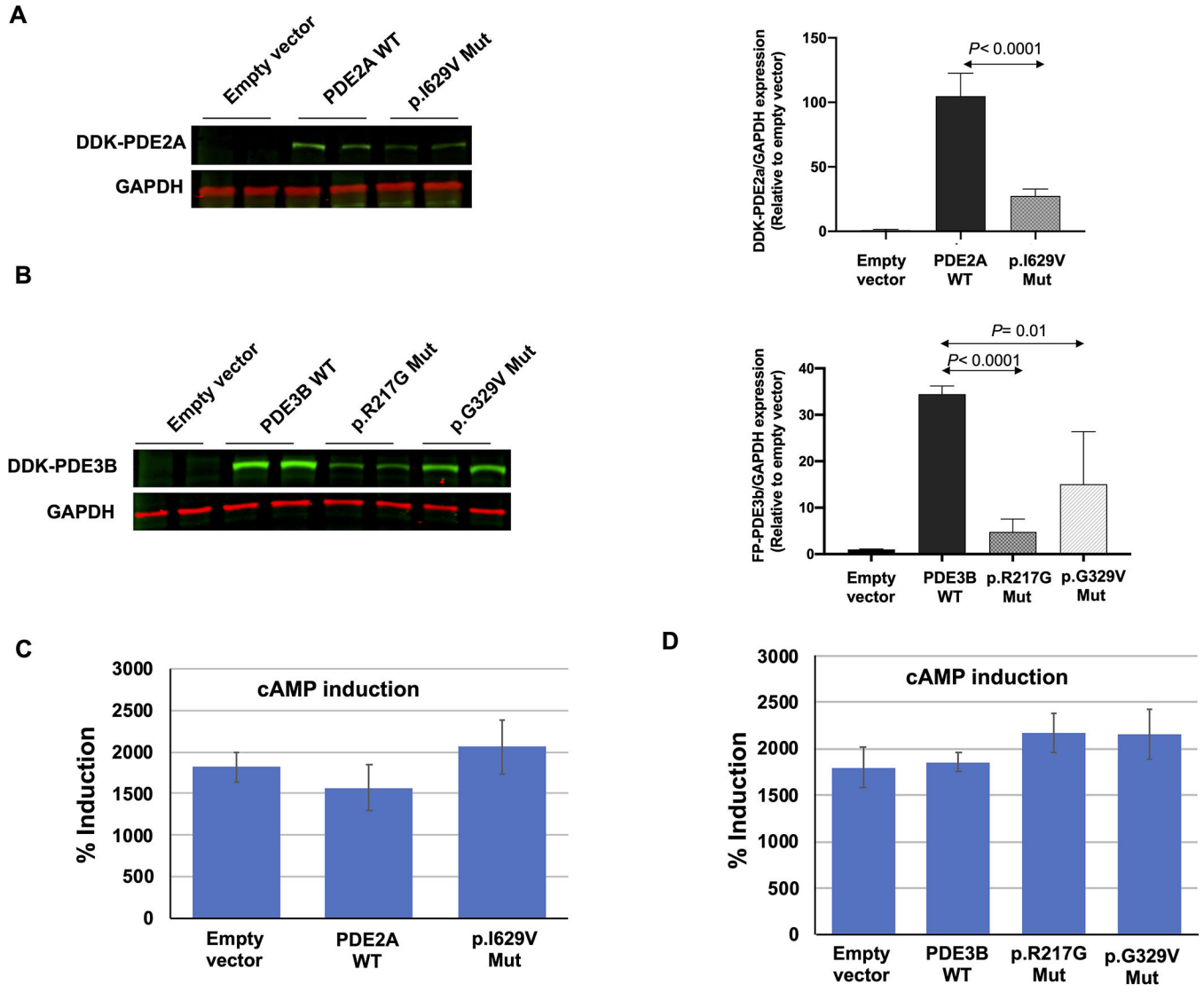

Figure 4.

A, PDE2A expression was markedly reduced in HEK 293T cells transfected with p.Ile629Val *PDE2A* mutant when compared to those transfected with WT *PDE2A*. **B,** The Arg217Gln and p.Gly392Val *PDE3B* variants significantly decreased PDE3B expression in HEK 293T cells in comparison with HEK 293T cells transfected with WT *PDE3B.* **C and D,** *PDE2A (*p.Ile629Val) and *PDE3B* (Arg217Gln and p.Gly392Val) variants did not increase *in vitro* PKA activity after cAMP induction in HEK 293T cells. All experiments were performed in triplicate.

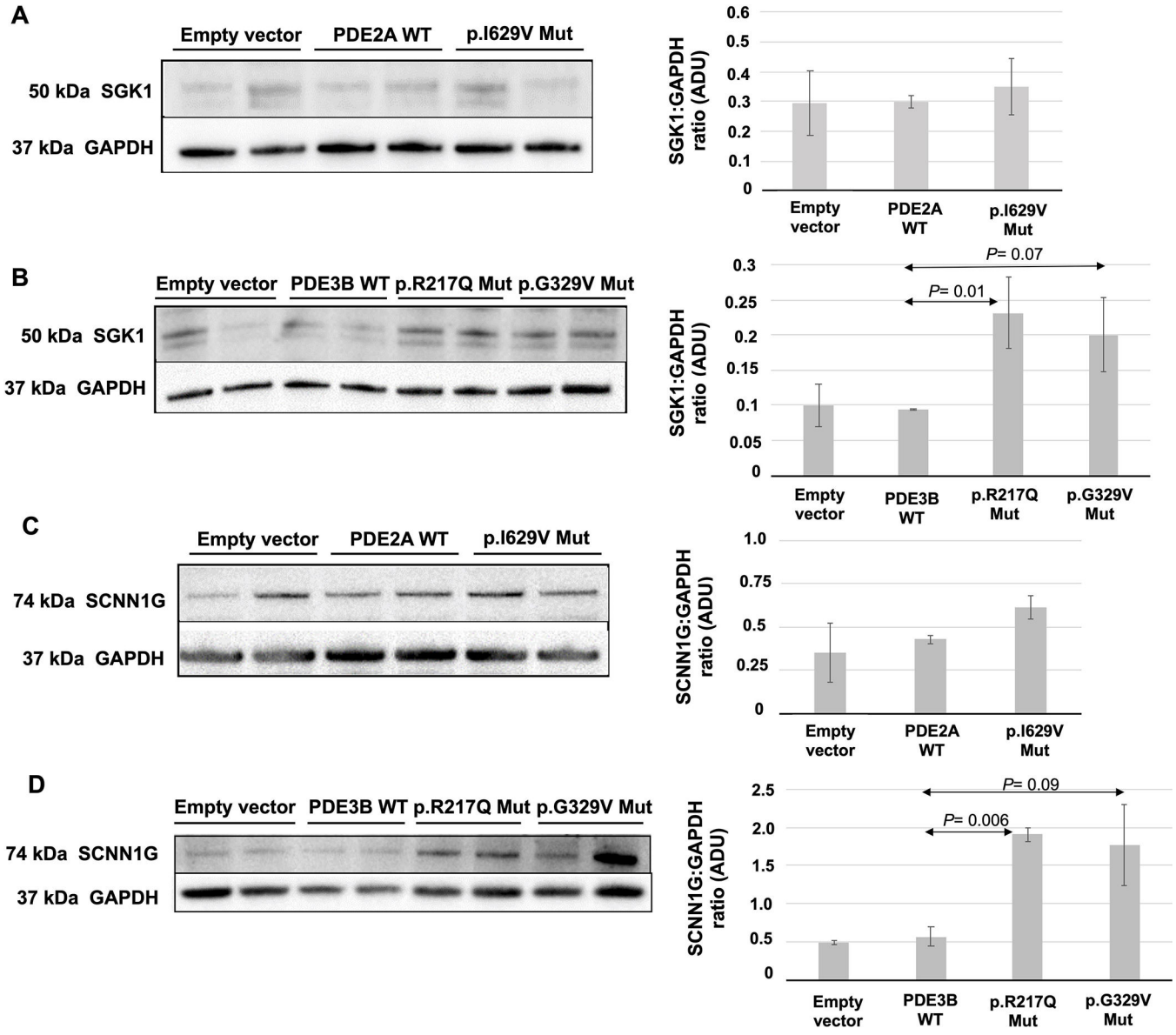

Figure 5.

A and B, The p.Ile629Val *PDE2A* variant did not modify SGK1 expression in HEK 293T cells (**A**), whereas both p.Arg217Gln and p.Gly392Val *PDE3B* variants increased SGK1 expression when compared to WT *PDE3B* (**B**). **C,** SCNN1G protein expression did not change after transfection with the p.Ile629Val *PDE2A* mutant. **D,** *PDE3B* (p.Arg217Gln and p.Gly392Val) variants increased SCNN1G expression in HEK 293T transfected cells. All experiments were performed in triplicate. In blots shown in 5B and 5D, the intensity of the bands for GAPDH differs somewhat across lanes despite the fact that equal amount was loaded; nevertheless, data were corrected for density on the same lane and not across lanes.

Table 1.

Clinical and biochemical data of patients with primary aldosteronism and bilateral adrenal hyperplasia.

Patient	**1**	**2**	**3**	**4**	**5**	**6**
Sex	F	M	F	F	M	F
Age at HT diagnosis (yr)	13	18	33	43	30	45
Age at PA diagnosis (yr)	40	58	56	50	65	68
Hypokalemia	Y	Y	Y	Y	Y	Y
A/PRA ratio	30.2	116.5	232.5	40	34.3	91
CT - Right adrenal	Normal	1.8 cm nodule	1.4 cm nodule	Normal	Normal	1.2 cm nodule
CT – Left adrenal	1.2 cm nodule	Thinckening	Thinckening	1cm nodule	1.1 cm nodule	2.0 cm nodule
AVS	-	LI 3.6 *	Unsuccessful AVS	-	-	-
Autonomous cortisol secretion	N	N	Y	N	-	-
Adrenalectomy	L	L	R	L	L	L
PA biochemical cure	N	N	N	N	N	N
HT remission	N	N	N	N	N	N
Histopathology (H&E)	Micro-nodular hyperplasia	Micro-nodular hyperplasia	Micronodular hyperplasia + dominant macronodule	Diffuse hyperplasia	Micro-nodular hyperplasia	Micronodular hyperplasia + dominant macronodule
CYP11B2 staining (positive areas)	Micro-nodules	Micro-nodules	Micronodules + Nodule	Micro-nodules	Micro-nodules	Micronodules

M, male; F, female; Y, yes; N, no; A/APR, aldosterone/plasma renin activity; CT, computed tomography; R, right; L, left adrenal; PA, primary aldosteronism; HT, hypertension; H&E, hematoxylin and eosine; AVS, adrenal venous sampling; LI, lateralization index.

* LI was inconclusive (between 3–4), but it indicated a higher aldosterone secretion in the left adrenal.

Table 2.

Germline variants selected in whole exome sequencing after filtration pipeline.

Gene	Variant	Type	MAF (%) gnomAD	MAF (%) ABraOM	Conservative AA	Deleterious *in silico* #	ACMG classification
***PDE2A* (Case 1)**	p.Ile629Val (ENST00000334456)	*missense*	0	0	Yes	Yes	VUS
***PDE3B* (Case 2)**	p.Arg217Gln (ENST00000282096)	*missense*	0.0006	0	Yes	No	VUS
***PDE3B** (Case 3)**	p.Gly392Val (ENST00000282096)	*missense*	0.001	0.08	Yes	Yes	VUS

≥3 *in silico* tools. Although *PDE3B* p.Arg217Gln variant was not deleterious in ≥3 *in silico* tools, we selected this variant to perform functional studies.

* Somatic *KCNJ5* mutation (p.Gly151Arg)

ABraOM, Brazilian population database; AA, aminoacid; ACMG, *The American College of Medical Genetics and Genomics* classification; MAF, minor allele frequency; VUS, variant of uncertain significance.

Table 3.

Immunoexpression and localization of PDE2A and PDE3B proteins in bilateral adrenal hyperplasia causing primary aldosteronism.

Case	PDE2A	PDE3B
1	+++ (ZG) ++ (micronodules)	+ (Diffuse)
2	++++ (ZG)	+ (Diffuse)
3	++++ (ZG and micronodules)	+++ (Diffuse)
4	+++ (Subcapsular hyperplasia and micronodules)	++++ (ZG)
5	++ (ZG and micronodules)	+ (Diffuse)
6	++ (ZG)	++ (Diffuse)

ZG, zone glomerulosa