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Insights in adrenal endocrinology: 2021

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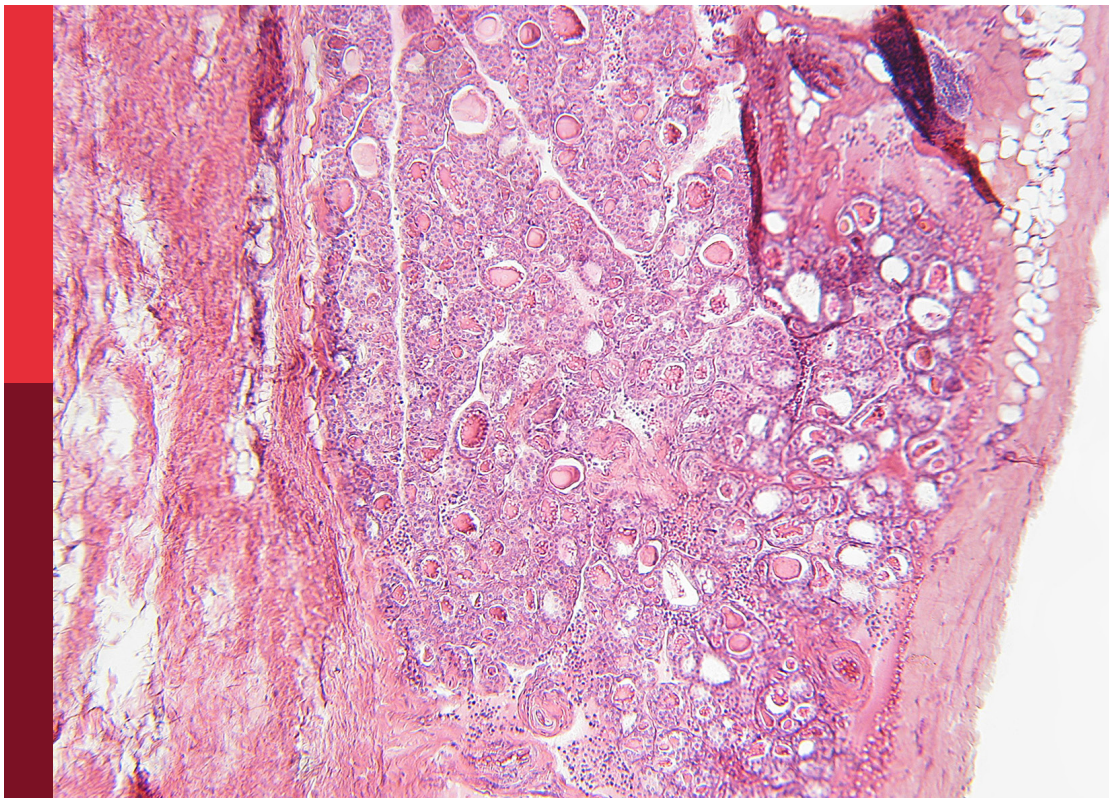

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Insights in adrenal endocrinology: 2021

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ORIGINAL RESEARCH
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Comparison of Bolus and Continuous Infusion of Adrenocorticotropic Hormone During Adrenal Vein Sampling

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Background: Adrenocorticotropic hormone (ACTH) is widely used in adrenal vein sampling (AVS) and can be administered as a bolus injection or continuous infusion. The optimal administration method has not been determined. We aimed to compare the effects of ACTH bolus with infusion on cannulation success, lateralization assessment and adverse events (AEs).

Methods: Retrospectively collected data from patients with primary aldosteronism who underwent AVS with ACTH at a tertiary hospital in China. Rate of successful cannulation, lateralization index (LI), complete biochemical remission and AEs related to AVS were analyzed.

Results: The study included 80 patients receiving ACTH bolus and 94 receiving infusions. The rate of successful cannulation was comparable between bolus and infusion groups (75/80, 93.4% vs 88/94, 93.6%). In those with successful cannulation, the bolus group had a higher selectivity index than the infusion group, while LI [6.4(1.8-17.5) vs. 7.6(2.0-27.8), P=0.48] and rate of complete biochemical remission (43/44, 97.7% vs 53/53, 100%, P=0.45) did not significantly differ between the two groups. One in the bolus and one patient in the infusion group had adrenal vein rupture but they recovered with conservative treatment. The bolus group reported more transient AEs such as palpitation (52.9% vs 2.2%) and abdominal discomfort (40.0% vs 2.2%) than the infusion group.

Conclusions: Due to their similar effects on cannulation success and lateralization, but a lower rate of transient AEs in the infusion group, the continuous infusion method should be recommended for ACTH stimulation in AVS.

Keywords: adrenocorticotropic hormone, adrenal vein sampling, primary aldosteronism, adrenal, hypertension

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INTRODUCTION

Primary aldosteronism (PA) is one of the most common causes of secondary hypertension (1). Diagnosis of PA requires screening, confirmation, and subtype differentiation. Current guidelines recommend computed tomography (CT) scanning and adrenal vein sampling (AVS) for the subtyping of PA, primarily to distinguish between unilateral aldosterone-producing adenoma (APA) and bilateral adrenocortical hyperplasia (BAH) (2, 3). Because CT scanning alone has been demonstrated to be misleading in some cases, AVS is considered the gold standard for assessing the lateralization of aldosterone secretion and thereby identifying surgically curable forms of PA (4–6). However, AVS remains markedly under-used due to its technically challenging nature, risk of complications (e.g. adrenal vein rupture) and the lack of a uniformly accepted protocol for its performance. Currently, the methods of AVS vary in different centers, including the use of adrenocorticotropic hormone (ACTH). ACTH stimulated-AVS is practiced by around 50% of expert centers (7) but has been reported to decrease the degree of lateralization and potentially mask unilateral APAs (8, 9). However, the potential benefits of using ACTH include higher rates of successful adrenal vein cannulation (10–12) and reduced impact of the time of AVS on aldosterone and cortisol concentrations especially when AVS is not performed in the early morning. Furthermore, ACTH is necessary for patients with contrast allergy who receive steroid treatment before AVS (13). Recently, a study found that ACTH-stimulated AVS might be useful in PA complicated by cortisol co-secretion (14) which has the potential to affect non-ACTH stimulated AVS results (15–17).

To introduce an additional variation in the AVS protocol, the method of ACTH administration varies in different centers, including bolus injection, continuous infusion and bolus plus continuous infusion (18). Some researchers consider continuous ACTH infusion to be preferable because it does not cause the supraphysiological stimulation of the bolus injection that might increase aldosterone production from the contralateral adrenal gland (13). In addition, as the bolus is usually a large dose delivered in a short time, more adverse events (AEs) might be induced by this method. However, no studies have specifically compared the different methods of ACTH stimulation in terms of AEs.

This study therefore aims to compare the effect of bolus ACTH injection and continuous infusion on the rates of cannulation success, assessment of lateralization together with post-operative cure, as well as AEs associated with AVS.

METHODS

Participants

This study was retrospectively conducted at the First Affiliated Hospital of Chongqing Medical University in China using the database of the Chongqing Primary Aldosteronism Study (CONPASS) (ClinicalTrials.gov: NCT03224312). The inclusion criteria were: 1) hypertensive patients underwent the screening and confirmatory test of PA and confirmed with PA diagnosis by at least one positive confirmatory test (described as follows); 2) completed AVS with ACTH stimulation. PA patients had missing data of AVS or complicated with autonomous cortisol secretion based on abnormal dexamethasone suppression tests were excluded from the analysis. When analyze the effects of different methods of ACTH administration on lateralization and surgical outcomes, those with unsuccessful cannulation (right and/or left selectivity index<3) were excluded. When analyze the blood pressure, heart rate and AEs during ACTH administration, the patients had missing data were excluded.

The ethics committee of the First Affiliated Hospital of Chongqing Medical University approved the protocol. Informed written consent was obtained from each participant.

Screening and Confirmatory Tests

The methods of screening and confirmatory tests have been reported before (19–21). For PA screening, treatment with diuretics, including mineralocorticoid receptor antagonists, was withdrawn for at least 4 weeks, and angiotensin-converting enzyme inhibitors, angiotensin-II receptor blockers and β-blockers were stopped for at least 2 weeks. Non-dihydropyridine calcium channel blockers and/or α-adrenergic blockers were allowed for uncontrolled hypertension. Samples for plasma renin concentration (PRC) and plasma aldosterone concentration (PAC) were collected in the morning after participants were out of bed for at least 2 hours and after they have been seated for 15 minutes. The screening test was considered positive when the ARR was ≥2.0 ng·dL−1/μIU·mL−1 (54pmol·L−1/μIU·mL−1).

Patients who tested positive proceeded to the confirmatory tests. For patients who tested negative, if PA was strongly suspected based on young age, hypokalemia, or resistant hypertension, they also proceeded to the confirmatory test. The diagnosis of PA was confirmed if at least one confirmatory test was positive: plasma aldosterone concentration (PAC) is not suppressed to less than 8 ng/dl (220 pmol/l) in the saline infusion test or PAC is not suppressed to less than 11 ng/dl (300 pmol/L) in the captopril challenge test or PAC is not suppressed to less than 6 ng/dl (166 pmol/L) in the fludrocortisone suppression test (2, 19). Patients confirmed with PA underwent thin-slice (1 to 3 mm thick) adrenal computed tomography (CT) scan and for those had willing for surgery, AVS was recommended.

Adrenal Vein Sampling

AVS was performed in the morning between 08:00 and 12:00 with ACTH stimulation (13, 18). During the AVS procedure, a nurse collected the data on AEs, blood pressure, and heart rate before and after (2 and 30 minutes) administration of ACTH.

From November 2016 to September 2019, ACTH was administered as a bolus injection. With local anesthesia, a 5F sheath was inserted into the right femoral vein followed by catheter insertion in the right and left adrenal veins. At this time, 25IU ACTH (amount to 250μg, Shanghai First Bio-chemical Pharmaceutical Company, H31022101) was injected, and 15 minutes later, blood samples were collected simultaneously from bilateral adrenal veins. Three tubes of blood in each adrenal vein were collected consecutively, and one tube of blood in the inferior vena cava (IVC) was collected immediately after the collection of each side of the adrenal vein blood. The adrenal vein blood samples

with the highest level of cortisol were usually used for index calculation. As the AEs were often reported by patients following bolus injections of ACTH, from Oct 2019, ACTH was administered as continuous infusion which was started 30 minutes before sampling and continued throughout the procedure at 5 IU/hr (50μg/hr).

The selectivity index (SI), namely plasma cortisol concentration (PCC) in adrenal vein/PCC in IVC>3, was considered to be successful cannulation. The ratio of PAC : PCC on the side with the higher ratio over the contralateral PAC: PCC ratio is defined as the lateralization index (LI). The cutoff for diagnosing lateralization was defined as LI>4 or LI between 3-4 together with a contralateral suppression (PAC/PCC of nondominant side < PAC/PCC of IVC) (13, 18). The diagnosis of APA required complete biochemical success following adrenalectomy, in accordance with the Primary Aldosteronism Surgery Outcome (PASO) criteria (22).

Biochemical Measurements

PRC and PAC were measured with automated chemiluminescence immunoassays (LIAISON; DiaSorin, Italy). The intra-and inter-assay coefficients of variation for PRC were from 1.2% to 3.7% and from 2.9% to 12.8%, respectively. The analytical sensitivity was 0.53 mIU/l, and the functional sensitivity was 1.6 mIU/l. The lower limit of detection was 0.5 mIU/l. The PAC assay has a measuring range from 2.2 ng/dl (analytical sensitivity) to 100 ng/dl, with a functional sensitivity of 3 ng/dl. The intra-assay coefficient of variation for PAC was from 2.4% to 4.8% and the inter-assay coefficient of variation was from 4.4% to 6.7%. Quality control was performed every day in the laboratory.

Statistical Analysis

Data distributions were analyzed with the Kolmogorov-Smirnov test. Normally distributed variables were expressed as the mean ± standard deviation (SD); variables with skewed distributions were expressed as the median (interquartile range); categorical variables were described as a percentage. Variables with skewed distributions were analyzed after natural logarithm transformation. Categorical variables were analyzed by the $\chi 2$ test, and quantitative variables were analyzed by Student's t-test. Comparisons at different time points were made using repeated measures of analysis of variance. SPSS 21 was used for statistical analysis. *P*-values <0.05 (two-tailed) were considered statistically significant.

RESULTS

Clinical Characteristics of the Subjects

From Nov 2016 to Jan 2021, a total of 85 patients with PA received an ACTH bolus and 99 received an ACTH infusion. One patient with missing data of AVS and nine PA patients complicated with autonomous cortisol secretion (four in bolus group, five in infusion group) were excluded from the study. Details of the ten patients were provided in the **Supplement Table 1**. Finally, a total of 174 patients with PA, including 80 in the bolus group and 94 in the infusion group, were included in the study. There were no significant differences in age, sex, blood pressure, BMI, concomitant diseases, medications, hypertension duration, serum potassium, PAC and PRC between the two groups (**Table 1**).

Effects of Different Methods of ACTH Administration on Cannulation Success

Using SI>3 in both adrenal veins as the criteria for bilateral successful cannulation, five and six patients had unsuccessful cannulation in the bolus and infusion groups respectively, resulting in comparable successful cannulation rates (75/80, 93.4% vs 88/94, 93.6%, P=1.0). In those with successful cannulation, the bolus group had a higher SI in the right adrenal vein and tendency of higher SI in the left adrenal vein when

TABLE 1 | Clinical and biochemical characteristics of study participants.

Parameters		Bolus (n=80)	Infusion (n=94)	*P*
Age (y)		48 (40-55)	51 (45-57)	0.099
Sex (female/%)		44/55.0	39/41.5	0.075
SBP (mmHg)		154 ± 18	152 ± 16	0.545
DBP (mmHg)		93 ± 13	92 ± 11	0.464
BMI (Kg/m^2)		25.1 ± 3.5	25.1 ± 3.6	0.615
Hypertension duration (month)		84 (42-120)	96 (36-144)	0.555
Complicated with diabetes (n/%)		9/12.2	7/9.3	0.557
Complicated with CVD (n/%)		6/10.5	9/11.9	0.804
History of hypokalemia (n/%)		57/77.0	64/83.1	0.348
Use of antihypertensive agents before screening	0-1 agent (n/%)	26/40.0	19/42.2	0.886
	2-3 agents (n/%)	32/49.2	24/53.3	0.671
	>3 agents (n/%)	7/10.8	2/4.4	0.105
Serum potassium (mmol/l)		3.3 (2.9-3.8)	3.3 (3.1-3.7)	0.663
PAC (ng/dl)		21.9 (16.2-33.5)	23.2 (19.3-31.4)	0.253
PRC (μIU/ml)		2.0 (1.2-6.8)	2.4 (0.9-5.4)	0.861
ARR ($ng•dl^{-1}/μIU•ml^{-1}$)		10.3 (5.0-20.2)	10.7 (4.3-23.3)	0.653
APA (n/%)		43/53.8	53/56.4	0.715

SBP, systolic blood pressure; DBP, diastolic blood pressure; PAC, plasma aldosterone concentration; PRC, plasma renin concentration; ARR, aldosterone/renin ratio; APA, aldosterone-producing adenoma; CVD, cardiovascular disease.

compared with the infusion group (right adrenal vein SI: 56.0(35.5-80.5) vs. 37.8(27.9-53.1), P<0.001; left adrenal vein SI: 26.9(16.7-42.3) vs. 22.1(15.1-35.0), P=0.075; **Figure 1**), but the difference had no impact on the assessment of cannulation success.

Effects of Different Methods of ACTH Administration on Lateralization

In those with successful cannulation, PAC, PCC and PAC/PCC ratio in bilateral adrenal veins did not significantly differ between the infusion group and the bolus group (**Table 2**). Compared with the bolus group, the infusion group had higher levels of PAC and PCC in the IVC, but the PAC/PCC ratio in the IVC was not significantly different between the two groups (**Table 2**). Accordingly, the LI was not significantly different between the bolus and infusion groups [6.4(1.8-17.5) vs. 7.6(2.0-27.8), P=0.48]. The number of patients with LI <3, 3-4, >4 in the bolus and infusion group were comparable at 26(35%), 5(7%), 43 (57%) and 34(39%), 4(5%), 49(56%), respectively (**Figure 2**).

Effects of Different Methods of ACTH Administration on Surgical Outcomes

For those with successful cannulation, 44 and 53 patients in the bolus and infusion group respectively underwent laparoscopic adrenalectomy. Among them, 43 and 53 showed complete biochemical remission. The rate of complete biochemical remission was not significantly different between the bolus and infusion groups (97.7% vs 100%, P=0.454).

Blood Pressure, Heart Rate, and AEs During ACTH Administration

Twenty-two patients had missing data on blood pressure and/or AEs after ACTH administration (10 in bolus group, 12 in infusion group), therefore, they were excluded from the analysis concerning the vital sign and AEs.

The changes in blood pressure and heart rate during ACTH administration were shown in **Table 3**. There was no significant difference in systolic blood pressure, diastolic blood pressure, and heart rate between the bolus and infusion groups before ACTH administration. Both methods of ACTH administration caused a decrease in systolic blood pressure within two minutes, however the systolic blood pressure was significantly lower in the bolus group (135 ± 20 vs 150 ± 20mmHg, P<0.001). The lowered systolic blood pressure returned to baseline at 30 minutes after ACTH administration in the bolus group but not in the infusion group, resulting in a much higher systolic blood pressure in the bolus group at 30 minutes (157 ± 18 vs 148 ± 20mmHg, P=0.003). The diastolic blood pressure was not significantly different between the two groups before and after ACTH administration.

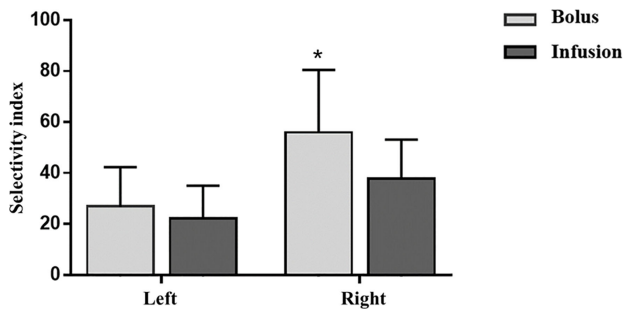

FIGURE 1 | Effects of ACTH infusion and bolus on Selectivity index. Selectivity index is defined as cortisol adrenal vein/cortisol peripheral vein. ACTH, adrenocorticotropic hormone. *P<0.05, bolus vs infusion.

ACTH bolus led to a significantly higher heart rate at 2 minutes when compared to ACTH infusion (85(77-93) vs 69(65-75) beats per min, P<0.001). The heart rate decreased to a level lower than the baseline at 30 min after ACTH bolus, resulting a significantly lower heart rate when compared to patients who had ACTH infusion (62(59-71) vs 68(63-74) per min, P=0.002) (**Table 3**).

One patient in the bolus and one in the infusion group had adrenal vein rupture but they recovered with conservative treatment. Palpitation (52.9%), abdominal discomfort (40.0%), chest tightness (24.3%) and hot flushes (22.9%) were common in the bolus group, while only 2% of subjects in the infusion group reported ACTH-related AEs during AVS (**Table 4**). All these AEs were transient and resolved spontaneously in 30min. None of the patients need special treatment and none of the AVS procedures were cancelled due to these AEs.

DISCUSSION

In patients who seek surgical cure of PA, current guidelines recommend the use of AVS for accurate subtyping (2, 3). ACTH stimulation has been used in AVS by many centers but the ACTH protocols vary between centers. Our study has demonstrated that bolus and continuous infusion of ACTH did not differentially affect the rates of successful cannulation or the assessment of lateralization. However, compared with infusion, bolus injections of ACTH led to more rapid and significant changes in blood pressure and heart rate as well as higher rates of transient AEs, including palpitation and abdominal discomfort, during AVS.

The history of ACTH use in AVS dates back to 1979, when Weinberger et al. introduced continuous infusion of ACTH (5 IU/h) in AVS to reduce errors induced by the episodic production of aldosterone as well as dilution by nonadrenal venous sources (23). Thereafter, ACTH became widely used. A multicenter study of AVS protocols in 24 eligible centers from Asia, Australia, North America, and Europe, found that half of the centers performed AVS with ACTH stimulation (7). The administration protocols included a high dose bolus, continuous infusion and a high dose bolus plus continuous infusion (18).Two previous studies investigated the performance of ACTH infusion compared to bolus injections in patients with PA (8, 10). Silvia et al. investigated the role of continuous ACTH infusion (50μg/h) and bolus (250μg) on the performance and interpretation of AVS in 76 patients with PA (10). The authors reported that LI was not significantly different between methods. In their published data, SI in both adrenal veins seemed to be lower after continuous ACTH infusion than after bolus, but the

TABLE 2 | Effects of ACTH bolus and infusion on aldosterone and cortisol concentration.

Parameters*	Bolus (n=75)	Infusion (n=88)	*P*
PAC left (ng/dl)	1774.0(598.5-3965.0)	1860.0(508.0-3950.0)	0.941
PAC right (ng/dl)	1610.0(553.0-3625.0)	2290.0(365.0-7100.0)	0.804
PCC left (nmol/l)	11973.0(7442.6-18400.3)	13968.0(8497.0-22305.0)	0.147
PCC right (nmol/l)	25274.0(17355.0-31530.8)	22907.0(17333.0-33997.0)	0.654
PAC/PCC left (ng/dl per nmol/l)	0.2(0.0-0.3)	0.1(0.0-0.2)	0.332
PAC/PCC right (ng/dl per nmol/l)	0.1(0.0-0.2)	0.1(0.0-0.2)	0.984
PAC in IVC (ng/dl)	32.1(22.6-49.6)	43.5(29.2-58.6)	0.002
PCC in IVC (nmol/l)	446.7(390.1-524.1)	630.2(552.9-705.5)	<0.0001
PAC/PCC in IVC (ng/dl per nmol/l)	0.1(0.0-0.1)	0.1(0.0-0.1)	0.897

**The parameters were analyzed only in those with successful canulation. PAC, plasma aldosterone concentration; PCC, plasma cortisol concentration; IVC, inferior vena cava; ACTH, adrenocorticotropic hormone.*

author did not directly compare the SI between the two protocols as one ACTH protocol was performed in a Japanese center and the other in an Italian center. The other study compared the effects of a high dose (250 μg IV as a bolus, n=47), an intermediate dose (50μg/h, n=14) and a very low dose (250pg IV, n=6) of ACTH on lateralization. Similarly, the authors did not demonstrate a significant difference in the LI between the high dose (bolus) and intermediate dose (infusion). In our study, the LI was not significantly different between the bolus and infusion groups, which confirmed the previous findings.

None of the prior studies evaluated the effect of different methods of ACTH administration on cannulation success, which is the main purpose of using ACTH. Our study reassuringly demonstrated comparable cannulation success between the bolus and infusion groups. While there was a significantly higher SI in the right adrenal vein in patients who received bolus ACTH, the SI in all groups were well above the threshold of 3 for determining successful cannulation and therefore no impact on cannulation success was observed.

Previous studies have reported the effects of ACTH on blood pressure and heart rate (24–28). In the study conducted by Connell et al, they found that muscle injection of ACTH 0.5mg every 12h for five days markedly raised blood pressure in normal man (24). Another study investigated the effect of a continuous 5-day ACTH infusion (40U/24 h) on blood pressure in normotensive and hypertensive subjects also observed increased blood pressure after ACTH infusion (25). Jackson et al. studied the effect of ACTH infusion on blood pressure and heart rate 1- 8h after administration (26). Significant increases in blood pressure and heart rate were observed by 2h but not 1h, and remained generally elevated for the duration of the infusion in their study. The results of the previous studies differ from ours. Notably, the time frame (1 hour-5 days) of the previous studies was much longer than our study (2-30min). Our study illustrated the acute effects of ACTH bolus injection which included an immediate decrease in blood pressure with normalization by 30 minutes, and an increase in heart rate that was accompanied by a drop below baseline after 30 minutes. The decrease in systolic blood pressure was also noted following ACTH infusion, but to a lesser extent, and no change was observed in the heart rate. The mechanism of these acute reactions is unclear. Previous studies reported that ACTH secretion was regulated by carotid body chemoreceptor (29, 30). There is a possibility that ACTH influences the chemoreceptor and related blood pressure regulating areas of the central nervous system. The changes in blood pressure might influence the circulating volume and dilution of adrenal hormones in peripheral blood, which might explain the difference in peripheral cortisol concentration and SI between patients who receive ACTH bolus and infusion.

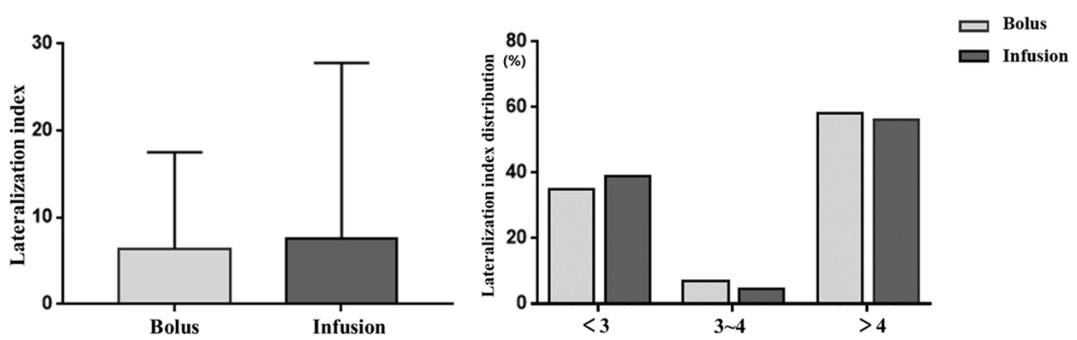

FIGURE 2 | Effects of ACTH infusion and bolus on lateralization index. Lateralization index is defined as aldosterone/cortisol adrenal vein/aldosterone/cortisol contralateral adrenal vein. Left picture: Lateralization index in the subjects; Right picture: Percentage of subjects with different lateralization index. ACTH: adrenocorticotropic hormone.

TABLE 3 | Adverse events reported during ACTH administration.

Parameters	Bolus (n = 70)	Infusion (n = 82)	*P*
Palpitation (n/%)	37/52.9	2/2.2	<0.0001
Chest tightness(n/%)	17/24.3	2/2.2	<0.0001
Dyspnea(n/%)	5/7.1	0/0	0.045
Nausea(n/%)	5/7.1	0/0	0.045
Abdominal discomfort(n/%)	28/40.0	2/2.2	<0.0001
Dizziness/headache(n/%)	17/24.3	0/0	<0.0001
Acroanesthesia(n/%)	10/14.3	0/0	0.001
Hot flush(n/%)	16/22.9	0/0	<0.0001

TABLE 4 | Influence of adrenocorticotropic hormone infusion and bolus on blood pressure and heart rate.

	0min			2min			30min		
	Bolus	Infusion	P	Bolus	Infusion	P	Bolus	Infusion	P
SBP(mmHg)	156 ± 15	156 ± 19	0.878	135 ± 20	150 ± 20	<0.001	157 ± 18	148 ± 20	0.003
DBP(mmHg)	93 ± 12	94 ± 14	0.607	91 ± 12	93 ± 14	0.534	95 ± 12	93 ± 14	0.282
HR(beat per min)	72(67-82)	71(66-79)	0.790	85(77-93)	69(65-75)	<0.001	62(59-71)	68(63-74)	0.002

SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate.

Up till now, no reported studies have specifically compared the two methods of ACTH stimulation in terms of AEs. As ACTH is mostly used in diagnostic procedures rather than treatment, and tend to be used only for a short period of time, the AEs related to ACTH are rarely investigated in published studies. However, the drug information documents for Cosyntropin (https://www.fda.gov/) as well as Synacthen (http://www.tga.gov.au) mention that tachycardia and bradycardia are the most common AEs of these drugs, in accordance with our findings. The faster changes in blood pressure and heart rate induced by ACTH bolus injection than infusion most likely contribute to the higher rate of AEs reported in the bolus group. In order of frequency, palpitations, abdominal discomfort, chest tightness, dizziness, hot flushes, acroanaesthesia, dyspnea and nausea were reported in up to half of the patients who received ACTH as a bolus injection compared to less than 2% in the group who received ACTH infusion. Although the serious AEs of AVS were not different between the two methods, the transient symptoms caused by bolus would contribute to patient discomfort and are almost entirely preventable using the infusion protocol.

A potential limitation of the present study is that the adrenal and peripheral venous blood before ACTH administration were not collected, therefore, the changes in PAC and PCC induced by different methods of ACTH administration could not be compared directly to the hormone levels before ACTH stimulation. Furthermore, the study is retrospective and the two protocols were applied to different patients over different periods of time. Cannulation success could have improved over time, but were comparable between the two groups mainly because AVS was performed by two dedicated interventional endocrinologists over the entire study period. The comparison of lateralization accuracy of one protocol over the other might be affected by potential differences in the underlying phenotypes of the two cohorts. However, clinical characteristics including age, sex, PAC, PRC and proportion of patients with APA were similar between the two cohorts.

PERSPECTIVES

Guidelines clearly state the importance of AVS in the subtyping of PA and ACTH-stimulated AVS is widely used. Unfortunately, despite being the gold-standard procedure, there are no standardized procedures for AVS. The present study demonstrated that bolus and continuous infusion of ACTH did not differ in their effect on cannulation success or PA subtyping, however, the bolus method caused a higher rate of transient AEs than the infusion method. Therefore, when using ACTH stimulation in AVS, continuous infusion should be recommended. These findings may contribute to the development of a standardized procedure for AVS. Furthermore, the acute decrease in blood pressure following ACTH administration has not been reported before. This phenomenon and its underlying mechanism need to be verified in future studies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The CONPASS study was approved by the ethical committee of Chongqing Medical University, and written informed consent was obtained from all patients participating in the study.

AUTHOR CONTRIBUTIONS

Conception and design: QL, SY, and JH. Analysis and interpretation of the data: YJ, JC, and QC. Drafting of the article: JH. Critical revision of the article for important intellectual content: JY, and SY. Obtaining of funding: QL, LM, and YS. Administrative, technical, or logistic support: ZD and YS. Collection and assembly of data: TL, YW, LM, and YY. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.784706/full#supplementary-material

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Attenuation Value in Adrenal Incidentalomas: A Longitudinal Study

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Context: A tendency to grow has been reported in adrenal incidentalomas. However, long-term data regarding attenuation value, a measure of lipid content, are not available.

Aim: This study aims to collect radiological data (diameter in mm and attenuation value in Hounsfield units, HU) with computed tomography (CT) in adrenal incidentalomas, in order to compare baseline characteristics with the last follow-up imaging.

Design: This is a longitudinal study which included patients with a new diagnosis of adrenal incidentaloma, evaluated from January 2002 to June 2020.

Setting: Referral University-Hospital center.

Patients: Two hundred seventy-seven patients with 355 different cortical adenomas (baseline group) were evaluated at the first outpatient visit; the follow-up cohort consists of 181 patients with 234 adenomas (12–175 months after baseline). Inclusion criteria were conservative management and radiological features able to minimize malignancy or risk of progression.

Main Outcome Measure: CT modification according to endocrine function: autonomous cortisol secretion (ACS) if cortisol >50 nmol/L after 1-mg dexamethasone test (DST).

Results: At baseline CT, mean diameter was 18.7 mm and attenuation value was 0.8 HU (higher in ACS, 66 cases >10 HU), without modification in early imaging (12–36 months). The size increased over time (r = 0.289), achieving the largest differences after at least 60 months of follow-up (mean diameter, +2 mm; attenuation value, –4 HU), combined with a reduction in the attenuation value (r = –0.195, especially in patients with ACS). Lipid-poor adenomas (>10 HU) presented a reduced cortisol suppression after 1-mg DST, an increase in size and the largest decrease in attenuation value during follow-up. Univariate analysis confirmed that larger adenomas presented reduced suppression after DST and increase in size during follow-up.

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Conclusions: Growth is clinically modest in adrenal incidentaloma: the first follow-up CT 5 years after baseline is a reasonable choice, especially in ACS. Mean density is increased in patients with ACS and overt hypercortisolism. Mean density reduces during follow-up in all adrenal adenomas, suggesting an increase in lipid content, especially in those with ACS.

Keywords: adrenal incidentaloma, autonomous cortisol secretion, attenuation value, computed tomography, Hounsfield Unit

INTRODUCTION

Adrenal incidentalomas are incidentally discovered masses, during a radiological study not performed for the suspicion of an adrenal-related disorder, as arterial hypertension, hypokalemia, early-onset diabetes, metabolic syndrome, or osteoporotic fractures (1). They represent an important challenge for differential diagnosis among several adrenal and extra-adrenal diseases (2, 3). The detection of adrenal incidentalomas is increasing over the last years, up to 10%–15% in subjects over 70 years old (2, 4), due to the large availability of imaging medical equipment in routine clinical practice, such as computed tomography (CT) and magnetic resonance (MR) (5). In most cases, adrenal incidentalomas are benign nonfunctioning cortical adenomas that do not require further studies or surgical/medical treatment (1).

Considering the endocrine function, most guidelines suggest to rule out at the baseline visit pheochromocytoma, primary aldosteronism, and Cushing's syndrome (CS) (1, 2). A consistent cohort of patients present a subclinical autonomous cortisol secretion (ACS), without the full-blown clinical picture of overt hypercortisolism (6, 7). Several studies reported a progression from a nonfunctioning adrenal incidentaloma (NFAI) to ACS in up to 11% of cases (8–10), especially in larger adenomas (11, 12).

The guidelines of the European Society of Endocrinology (ESE) in collaboration with the European Network for the Study of Adrenal Tumours (ENS@T) define benign adrenal incidentalomas as those with low attenuation value, homogeneous texture, and diameter <4 cm (1) [the diameter has been confirmed in a retrospective study with 233 patients (13)]. In clinical practice, a growth tendency has been observed in adrenal incidentalomas: an increase of >0.5 cm has been described in 20 out of 77 patients in a 5-year study (14). Larger cohorts with shorter follow-up (median 2 years) reported an increase >1 cm in 12/229 (9) and 25/139 patients (15). A positive correlation between adenoma diameters and serum cortisol levels after dexamethasone suppression test (DST) has been observed (16). A recent systematic review reported a minimal growth (2 mm in 4 years), an increase of >1 cm was observed in 2.5% of cases (17).

The calculation of tissue attenuation or density values, measured in Hounsfield units (HU), is the assessment of X-ray absorption during CT (18, 19). An inverse relationship between lipid content and the attenuation value obtained with unenhanced CT is described in adrenal adenomas (1, 2, 20): a density of <10 HU indicates a lipid-rich adenoma with 71% sensitivity and 98% specificity (18). At the best of our knowledge, scarce data have been reported regarding the modification in attenuation value during follow-up. Only one study by Hammarstedt et al. reported in 2012 a slight increase in the lipid content of adrenal incidentalomas after 2 years of follow-up; nonetheless, cortisol secretion was not considered.

In the present study, we investigated the radiological changes (diameter and attenuation value) in a cohort of patients with adrenal incidentalomas, according to their cortisol secretion, after a long-term follow-up.

MATERIALS AND METHODS

Patients

This longitudinal study included patients with a new diagnosis of adrenal incidentaloma, evaluated from January 2002 to June 2020. A dedicated query (incidental findings of an adrenal mass) was used in the web-based Padova University-Hospital database to extract the initial cohort of patients with a regular follow-up in the Endocrinology Unit (n = 460 patients). Then specific inclusion criteria for this study were:

- Rule out of malignancy and overt hormonal secretion. Patients with any active malignancies (not only adrenal) or clear signs/symptoms of endocrine excess were excluded. Normal serum aldosterone/renin ratio [after appropriate interfering drug wash-out (13)] and urinary fractionated metanephrine level (14) were used to rule out primary aldosteronism and pheochromocytoma. Overt adrenal CS was searched in all patients with clinical signs/symptoms consistent with endogenous hypercortisolism. In case of unsuppressed serum cortisol after 1-mg DST (>50 nmol/L, performed in all patients), urinary free cortisol (UFC) and late-night salivary cortisol (LNSC) were evaluated with a home-brew LC-MS/MS method previously described (21, 22).
- Radiological evidence at CT of a benign adrenal incidentaloma. All images of an unenhanced abdominal CT were available in the database of the University-Hospital of Padova. If attenuation value was not sufficient to indicate a lipid-rich adenoma in unenhanced CT (HU <10), a contrast-enhanced CT or a MR was performed. Adrenal adenoma was confirmed in case of absolute washout of >60% or a relative washout of >40% in the delayed images (15 min for the venous phase) during contrast-enhanced CT or a signal drop in the out-of-phase images of >20% during MR scan (23). Same criteria were used in the follow-up study.

- CT image consistent with a discrete cortical adrenal nodule. All cases of adrenal hyperplasia (one or both adrenals massively enlarged by the presence of multiple macronodules >1 cm) and adrenal myelolipoma (diagnosed by its peculiar imaging) were excluded (20).
- Conservative management of the adrenal incidentaloma during the follow-up. All surgical cases were excluded from baseline evaluation, in order to reduce selection bias. Surgical management was proposed in patients with suspected malignant features (24), adrenal secretion, or incidentalomas with a rapid increase in size (>5 mm in 6 months was considered clinically significant) after a multidisciplinary evaluation (3).

According to the aforementioned criteria, we selected 277 patients (157 females, 57%), median age 66 years old (range 29,–86). Two hundred eight one monolateral adrenal incidentaloma, 62 patients two adenomas, five patients three different adenomas, and two patients four adenomas (bilateral incidentaloma in 64 patients). Three hundred fifty-five different discrete cortical adenomas were considered for the baseline evaluation. A follow-up CT was available after at least 12 months (median interval 52 months, range 12–175) in 181 patients with 234 adenomas.

We evaluated also CT scan 8 in patients with a clinical and radiological diagnosis of adrenal CS (collection period 2014–2018). Evidence of monolateral adenoma, full-blown clinical picture at diagnosis, increased UFC and LNSC levels, unsuppressed serum cortisol after 1-mg DST, baseline ACTH <10 pg/m, histological confirmation of adenoma, and cortisol insufficiency after surgery were the criteria adopted to confirm adrenal hypercortisolism.

Endocrine data collected at baseline and during the last follow-up visits were serum cortisol after 1-mg DST, morning ACTH, and UFC levels. ACS was defined in case of cortisol secretion of >50 nmol/L after 1-mg DST; otherwise, incidentalomas were considered NFAI. Dexamethasone levels were measured with a home-made liquid-chromatography tandem-mass spectrometry method in all DST test (from 2014), previously described: dexamethasone levels <4.5 nmol/L were considered insufficient and 1-mg test was discarded (25). Clinical data collected included gender, age, body weight and height (to calculate BMI), blood pressure, glycated hemoglobin (HbA1c), presence of cortisol-related comorbidities as hypertension (systolic or diastolic blood pressure >130/90 mmHg or antihypertensive treatment), diabetes mellitus (increased fasting blood glucose or HbA1c, antidiabetic treatment), dyslipidemia, osteoporosis (lumbar or femoral t score <−2.5 or clinical evidence of frailty fractures).

Between 2002 and 2009, the CT scans were performed with a 16-slice scanner (Somatom Emotion™; Siemens Healthineers, Erlangen, Germany), craniocaudal image acquisition with 120 kV tube voltage, 225 mAs effective dose, 0.5 s rotation time, 0.75 mm detector collimation, and 0.8 pitch. Slice thickness was 1.5 mm for unenhanced acquisitions; all images analyzed had a soft-tissue reconstruction with a 30B kernel.

After 2009, all CT examinations were performed with a 128-slice scanner (Somatom Definition™; Siemens Healthineers, Erlangen, Germany), craniocaudal image acquisition with 120 kV tube voltage, 250 mAs effective dose, 0.5 s rotation time, 0.6 mm detector collimation, and 0.75 pitch. The slice thickness for unenhanced scans was 1.5 mm, and the reconstruction kernel was 30B. All CT scanners were calibrated every morning before the first patient, according to the University-Hospital of Padova standard procedures and were maintained according to the manufacturer's specifications.

CT images were reviewed on a dedicated workstation by one endocrinologist and one radiologist expert in abdominal imaging (GM and FCr), blinded to clinical and biochemical data of the patients. Discrepancies (maximal diameters, difference in results higher than standard error of mean, selected area) were resolved by discussion and, if in case of disagreement, a third senior radiologist (EQ) was involved. The diameter of the adenoma was considered the mean of the two perpendicular maximal diameters on an axial plane. Mean attenuation values of the adenoma (HU^m) was obtained by the average of three measurements with a circular or ovoid region of interest (ROI) cursors performed in the axial slice at unenhanced CT with the largest diameters of the lesion. Each ROI placed over the lesion included at least two-thirds of the area except edges to minimize partial volume effects; necrotic, cystic, hemorrhagic, and calcified areas within the ROI were avoided if possible. The same acquisition protocol was used to collect data in all CT scan (baseline and last follow-up available).

Our study complies with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement and guideline (26).

The Ethics Committee of Padova University Hospital (Comitato Etico per la Sperimentazione Scientifica) approved the study (protocol No. 53401-2021). The clinical data were obtained from the web-based database of Padova University Hospital, in the form of electronic case reports or records.

Statistical Analyses

Proportions and rates were calculated for categorical data. Continuous data were reported as means and standard deviation (SD), median and interquartile range (IQR), or difference (follow-up *versus* baseline, termed Δ). Groups were compared with the chi-square test for categorical variables (the raw *p*-values were adjusted with the Bonferroni method to take multiple comparisons into account), and with paired Student's *t*-test for quantitative variables. From a clinical perspective, data from the last visit available was compared with the baseline consultation, and then the follow-up was divided in short (12–36 months), intermediate (37–60 months), and long term (>60 months).

Analysis of variance (ANOVA) was computed to observe variance data; *post-hoc* Dunnett's test for multiple comparison procedure was used after a significant ANOVA, using the first quartile of mean diameter as a control group.

The SPSS 24 software package for Windows (SPSS, Inc., Chicago, IL, USA) was used to manage the database and

perform the statistical analysis. The significance level was set at $p < 0.05$ for all tests. All data analyzed during this study are included in the data repositories of the University of Padova - Research Data UniPD (27).

RESULTS

According to our definition, 44% of patients presented ACS (at baseline 122 out of 277, at last visit 80 out of 181). Patients with ACS were older than those with NFAI, and most of the women were postmenopausal (only six still had periods). Hypertension was diagnosed in 77% (214 out of 277), dyslipidemia in 44% (123 out of 277), diabetes mellitus in 21% (58 out of 277), and osteoporosis in 11% (30 out of 277) at the baseline visit in all patients with adrenal incidentaloma. Clinical and radiological features in patients with NFAI or ACS are reported in **Table 1**. Attenuation value presented a symmetrical distribution and a positive correlation with cortisol after 1-mg DST ($r = 0.219$, $p < 0.0001$, depicted in **Figure 1**).

At baseline CT, mean diameter was 18.7 mm (SD ± 6.9 mm; range 8–42.1 mm; IQR 13.1–23.1 mm), and attenuation value was 0.8 HU^m (SD ± 12.2 HU^m, range −39.5 to 44.9; IQR −6.4 to 7.5) in the whole cohort of 355 incidentalomas. Mean diameter was >40 mm in two patients (respectively 41 and 42.1 mm), attenuation value was >10 HU in 66 cases.

Eleven patients with NFAI developed ACS in the follow-up (8%); 19 patients with ACS at baseline reported serum cortisol <50 nmol/L after 1-mg DST at the last available visit. We observed during follow-up an increase in diameter and a reduction in attenuation values in the group of 234 patients with a control CT available, as reported in **Table 2**. Mean diameter at follow-up was 19.4 mm (SD ± 8.5 mm, range 8–42.2 mm, Δ +1 mm *versus* baseline); an increase ≥10 mm was observed in six incidentalomas. Age or age-based groups were not able to differentiate the radiological data reported, at baseline and during follow-up.

Mean attenuation value at last visit was −0.7 HU^m (SD ± 10.7 HU^m, range −28.4 to 30.1, Δ −2 HU^m *versus* baseline). An increase in attenuation value was observed in 73 adenomas (out of 234, 31%, range 0 to +19.8 HU^m), 32 were patients with ACS. An increase ≥10 HU^m was observed in 16 adenomas (range +10 to +19.8 HU^m, baseline attenuation value −27.8 to 20 HU^m), five were ACS patients. Size and lipid content at the last visit were similar to baseline when CT was performed early (in 12–36 months), while differences increased over time, with the greatest change (Δ diameter +2 mm and Δ attenuation value −4 HU^m) in case of long-term follow-up (>60 months in 101 cases, as described in **Table 2**). The reduction of lipid content was observed in NFAI and in ACS (respectively HU^m in the follow-up CT was lower than baseline in 52% and in 69% of patients, respectively, as reported in **Table 1**).

A positive linear regression was found between the duration of follow-up and Δ diameter ($r = 0.289$, $p < 0.0001$) and a negative regression between the duration of follow-up and Δ attenuation value ($r = -0.195$, $p = 0.003$, reassumed in **Figure 2**).

Considering endocrine secretion, during follow-up, an increase in diameter was observed either in patients with NFAI and in those with ACS (as reported in **Table 2**); however, a reduction in attenuation value was evident only in ACS, especially in the long-term follow-up (as depicted in **Figure 2**). Likewise, 69% of patients with ACS presented a lower attenuation value at follow-up CT with respect to baseline ($X^2 = 6.371$, $p = 0.013$, reported in **Table 1**).

Patients with lipid-poor adenoma (>10 HU^m at baseline CT scan) were more often female (postmenopausal, according to the age of onset), they presented a reduced cortisol suppression after 1-mg DST (see **Table 3**), an increase in size (as those with NFAI) and the largest decrease in attenuation value during follow-up (Δ −5.4 HU^m).

In order to predict the growth of an incidentaloma, we have divided the follow-up cohort according to their quartile of diameter: the groups obtained were homogeneous for number of adenomas, as reported in **Table 4**. ANOVA analysis (considering attenuation value, mean diameter, Δ difference, age, cortisol after 1-mg DST, baseline ACTH and UFC) revealed that cortisol after 1-mg DST at baseline ($F = 11.09$; $p < 0.0001$) or at last available visit ($F = 11.639$, $p < 0.0001$) and Δ

TABLE 1 | Clinical and radiological data, presented as mean and standard deviation, or percentage if appropriate.

	NFAI	ACS	*p*-value
Age at diagnosis (years)	63.6 ± 4.9	66.6 ± 5.3	0.05
Gender female/male (% female)	81/74 (52%)	74/48 (61%)	0.081
BMI (kg/m^2)	30.57 ± 4.81	28.48 ± 5.11	0.006
Basal ACTH (ng/L)	19.04 ± 11.18	13.69 ± 10.08	<0.0001
UFC (ULN)	0.53 ± 0.37	0.68 ± 0.21	0.007
HbA1c (mmol/mol)	41.69 ± 8.68	42.77 ± 9.21	0.33
Mean diameter (mm)	16.55 ± 5.81	20.94 ± 9.08	<0.0001
Attenuation value (HU^m)	−0.84 ± 11.34	2.16 ± 12.97	0.027
HU^m follow-up > baseline (%)	48 (48%)	25 (31%)	0.013
HU^m follow-up < baseline (%)	53 (52%)	55 (69%)	
Hypertension (%)	118 (76%)	101 (83%)	0.216
Diabetes mellitus (%)	23 (15%)	28 (23%)	0.067
Dyslipidemia (%)	71 (46%)	52 (43%)	0.656
Osteoporosis (%)	11 (7%)	23 (19%)	0.001

NFAI, nonfunctioning adrenal incidentaloma; ACS, autonomous cortisol secretion; HU^m, mean of Hounsfield Unit in unenhanced CT; UFC, urinary free cortisol; ULN, upper limit of normality; BMI, body mass index; HbA1c, glycated hemoglobin.

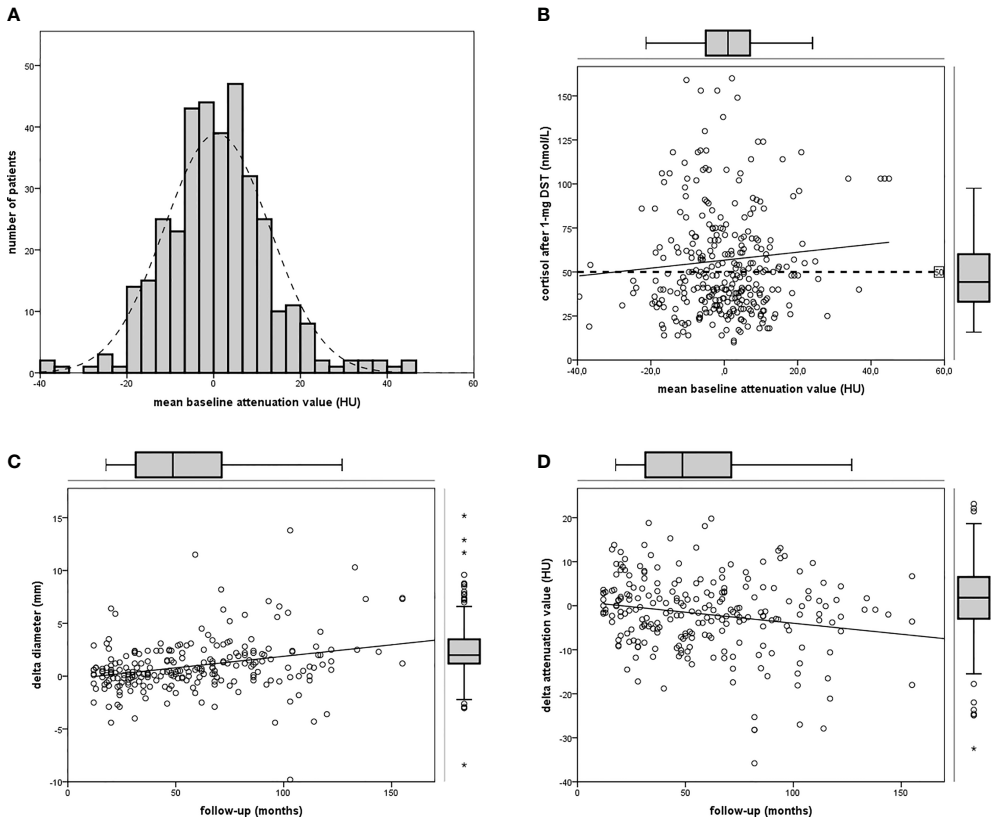

FIGURE 1 | Attenuation value in the cohort of patients. **(A)** Frequency histogram of mean attenuation value; **(B)** linear regression between attenuation value and cortisol after 1-mg DST (n = 355); **(C)** linear regression between follow-up and Δ diameter (n = 234); **(D)** linear regression between follow-up and Δ attenuation (pn = 234).

diameter (F = 5.251, p = 0.002) were significant for the variation. Larger adenomas presented reduced suppression after 1-mg DST and greater increase in size during the follow-up, according to the *post-hoc* Dunnet's test reported in **Table 5**.

Mean diameter in patients with adrenal CS was similar to those with ACS and larger than those with NFAI: 28.4 ± 6.63 mm, 24.48 ± 9 mm, and 18.98 ± 7.08 mm (respectively p = 0.231 and p = 0.005). Attenuation value was higher in adrenal CS than in ACS and NFAI: 22.28 ± 13.54 HU^m, 2.16 ± 12.97 HU^m, and −0.84 ± 11.34 HU^m (respectively p = 0.004 and p = 0.002), confirming the correlation between density of the adenoma and cortisol secretion. Univariate analysis confirmed these results (F = 21.162, p < 0.001 for diameter and F = 14.968, p < 0.001 for attenuation value), and *post-hoc* Dunnet's test revealed that the difference in attenuation value was mild between ACS and NFAI (Δ difference 3 HU^m, p = 0.027) and more evident between adrenal CS and NFAI groups (Δ difference 23.11 HU^m, p < 0.0001).

DISCUSSION

Adrenal masses, discovered incidentally during an imaging study not performed for the suspicion of adrenal-related diseases, are increasingly detected in the adult-elderly population. Their management is not a minor concern for patients and healthcare-related costs. After comprehensive radiological (to exclude adrenal or metastatic malignancies) and endocrine evaluation (to assess excessive cortical or medullary secretion), a conservative management is proposed in patients with NFAI (1, 2, 23).

It has been extensively reported that there is a tendency to grow (9, 14, 15) or to develop ACS (6, 7, 9–12, 14, 15, 28, 29) in a proportion of patients with adrenal incidentaloma during follow-up. The ESE-ENS@T guidelines suggest no further evaluation if basal radiological assessment is consistent with a benign cortical adenoma (as diameter <4 cm and attenuation value <10 HU) (1). According to these guidelines, one study reported a minimal growth (1 mm) in 54 patients with benign incidentalomas after follow-up, suggesting that a radiological and endocrine reassessment can be performed after 5 years (30). In 2017, a large Korean study proposed that a diameter of 3.4 cm and attenuation value of 20 HU are able to distinguish benign adenoma from adrenal malignancy, without change in size in patients with NFAI (31). Attenuation value at CT is a feature that indicates lipid content in adrenal adenoma: it has been evaluated mostly at the baseline CT, and no data are available in

TABLE 2 | Radiological data of patients with a follow-up CT scan.

Group	*n*, age at presentation (years)	Diameter baseline (mm)	Diameter follow-up (mm)	Δ Diameter (mm, 95% CI)	*p*-value *vs.* baseline	Attenuation value baseline (HU^m)	Attenuation value follow-up (HU^m)	Δ Attenuation value (HU^m, 95% CI)	*p*-value *vs.* baseline
12–36 months of follow-up	78, 67.9 ± 8.4	18.13	18.29	+0.16 (–0.56 to 0.24)	0.42	–0.32	–0.08	–2.24 (–1.87 to 1.38)	0.756
37–60 months of follow-up	55, 66.6 ± 9.8	18.14	18.98	+0.84 (0.3–1.38)	0.003	0.53	–1.31	–1.83 (–1.87 to 0.13)	0.066
>60 months of follow-up	101, 66.6 ± 8.4	18.74	20.38	+1.65 (1.04–2.25)	<0.0001	2.82	–0.73	-3.55 (–5.5 - -1.59)	<0.0001
Baseline CT *vs* last CT	234, 66.3 ± 8.9	18.39	19.35	+0.97 (0.64–1.3)	<0.0001	1.24	–0.67	–1.88 (–2.99 to –0.77)	<0.0001
NFAI	132, 64.4 ± 9.3	16.54	17.26	+0.72 (0.31–1.13)	0.001	–0.56	–1.52	–0.96 (–2.46 to 0.54)	0.209
ACS	102, 67.2 ± 8.3	20.31	21.6	+1.29 (0.82–1.76)	<0.0001	3	–0.04	–3.04 (–4.73 to –1.35)	0.001
Baseline $HU^m \leq 10$	185, 66.7 ± 8.8	18.60	19.39	0.79 (0.43–1.14)	<0.0001	–3.05	–4	–0.94 (–2.07 to 0.19)	0.105
Baseline $HU^m > 10$	49, 64.4 ± 9.7	17.59	19.2	1.61 (0.82–2.41)	<0.0001	17.40	11.97	–5.43 (–8.42 to –2.44)	0.001

NFAI, nonfunctioning adrenal incidentaloma; CT, computed tomography; ACS, autonomous cortisol secretion; Δ difference; 95% CI, 95% confidence interval; HU^m, mean of Hounsfield Unit in unenhanced CT.

longitudinal series. Therefore, the aim of our study was to collect radiological data (diameter and attenuation value) in a large cohort of patients with a long-term follow-up CT, according to cortisol secretion.

We describe a baseline cohort of 277 patients (with 355 different adenomas), and a follow-up group of 181 patients (234 adenomas) with available unenhanced CT after median 52 months (range 12–175 months, >60 months in 101 out of 181).

We confirmed that, if we consider radiological features consistent with a cortical adenoma [as diameter <10 cm and reduced attenuation value (32)], the overall growth of the adrenal mass was minimal, as recently reported (30, 31). We did not observe an increase in size if follow-up CT was performed early (<3 years after baseline imaging). Growth of the adenoma was significant (albeit modest) if the follow-up CT was performed after at least 5 years from diagnosis [close to 2 mm, as reported in a meta-analysis (17)]. Moreover, a positive linear regression was found between the duration of follow-up and increase in diameter: the longer the follow-up, the greater the growth, irrespective of endocrine secretion. Larger adenomas (>24 mm, the fourth quartile of diameter distribution) presented the greatest increase and the reduced cortisol suppression after 1-mg DST: the same threshold has been proposed by Morelli et al. for the risk of ACS development (12). As previously reported in several papers, we confirm a minimal growth and a tendency to develop ACS in case of long-term follow-up (6, 7, 9–12, 14, 15, 28, 29).

The change of the attenuation value during follow-up has been explored in our study. At baseline, mean density in patients with ACS was higher than in those with NFAI: reduced attenuation value indicate poor lipid content in patients with cortisol secretion (33). Overall, 66 incidentalomas presented at baseline an attenuation value >10 HU^m (reduced to 49 cases at the last CT evaluation), characterizing a lipid-poor adenoma: contrast-enhanced CT or MR were used to confirm the benign behavior of the mass, and a conservative management was indicated after multidisciplinary discussion (3). We confirmed a tendency to decrease the attenuation value, previously reported by Hammarstedt et al. in a smaller study (34), especially after a long-term follow-up: we observed a reduction of the attenuation value (–4 HU^m) in 101 cases at least after 60 months. A negative regression between the duration of follow-up and attenuation value was observed, especially in patients with ACS: up to 70% of them presented a mean density lower than baseline. The largest decrease in attenuation value (–5 HU) was observed in those patients with mean density >10 HU^m at baseline CT scan. Intracellular lipid content can be studied with MR chemical shift: the quantitative assessment of adrenal-to-spleen ratio and signal intensity index are able to distinguish patients with ACS (35). Further studies, ideally prospective, are needed to clarify the association between lipid content and cortisol secretion in patients with adrenal adenoma. Age was not able to predict size or lipid content; however, according to our data, we can speculate a kind of "adrenal aging". We described a growth in size and a reduction of the attenuation value during years of follow-up, irrespective of age at presentation, reflecting an

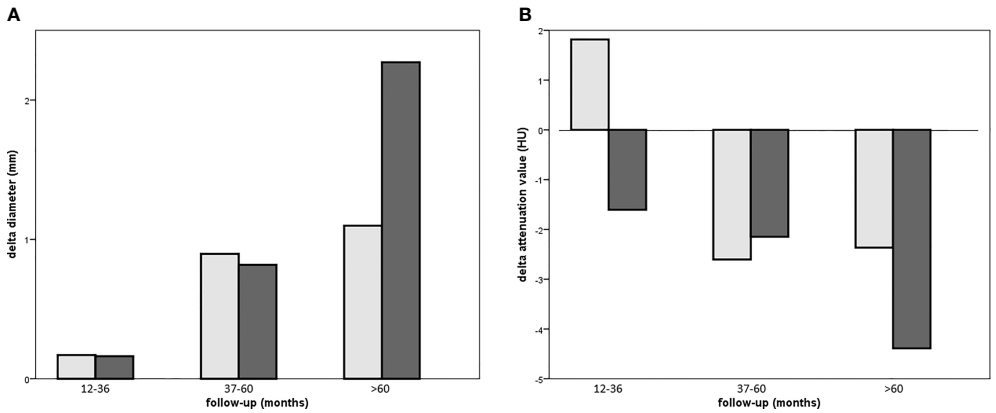

FIGURE 2 | Bar chart depicting Δ diameter **(A)** or Δ attenuation **(B)** in patients with NFAI (light gray bar) or ACS (dark gray bar) according to short, intermediate, or long-term follow-up.

TABLE 3 | Clinical and radiological data of patients, sorted by attenuation value.

	$HU^m \leq 10$	$HU^m > 10$	*p*-value
Age at diagnosis (years)	65.5 ± 2.9	62.9 ± 4.1	0.068
Gender female/male (% female)	119/105 (53%)	39/14 (74%)	0.001
Basal ACTH (ng/L)	17.15 ± 12.3	15.15 ± 9.59	0.236
Cortisol after 1-mg DST	59.6 ± 51.9	94.2 ± 80.1	<0.0001
UFC (nmol/24 h)	0.58 ± 0.22	0.59 ± 0.24	0.833
HbA1c (mmol/mol)	42.77 ± 9.23	41.7 ± 7.66	0.432
BMI (kg/m^2)	29.61 ± 5.01	28.36 ± 5.36	0.197
Mean diameter (mm)	18.73 ± 6.98	18.71 ± 6.83	0.982
Δ diameter (mm)	0.8 ± 2.44	1.62 ± 2.77	0.044
Δ attenuation value (HU)	−0.94 ± 7.84	−5.43 ± 10.41	0.001
HU^m follow-up > baseline (%)	96 (43%)	17 (33%)	0.203
HU^m follow-up < baseline (%)	128 (57%)	36 (67%)	
Hypertension (%)	181 (81%)	39 (73%)	0.159
Diabetes mellitus (%)	45 (20%)	12 (22%)	0.645
Dyslipidemia (%)	101 (45%)	23 (43%)	0.767
Osteoporosis (%)	25 (11%)	8 (15%)	0.347

Data are presented as mean and standard deviation. NFAI, nonfunctioning adrenal incidentaloma; ACS, autonomous cortisol secretion; HU^m, mean of Hounsfield unit in unenhanced CT; UFC, urinary free cortisol; BMI, body mass index; HbA1c, glycated hemoglobin; Δ, difference from baseline CT; DST, dexamethasone suppression test.

increased lipid content of the adenoma. "Adrenal aging" is only a food-for-thought consideration and need to be further validated with histological analysis and prospective radiological studies.

We also collected imaging data from eight patients with overt monolateral adrenal masses secreting cortisol. Their diameter was similar to those with ACS, both larger than NFAI group, therefore adrenal size could not help clinicians in indicating cortisol secretion in patients with unsuppressed serum cortisol after 1-mg DST. On the contrary, attenuation value was higher in adrenal CS than in ACS, confirming the correlation between density (or lipid content) and cortisol secretion (33). As a matter of fact, HU^m was higher in CS, but

TABLE 4 | Radiological data of patients with a follow-up available CT, grouped by quartile of mean diameter (reported in the first column).

Quartile (diameter in mm)	*n*	Diameter baseline (mm)	Diameter follow-up (mm)	Δ Diameter (mm, 95% CI)	*p*-value *vs.* baseline	Attenuation value baseline (HU^m)	Attenuation value follow-up (HU^m)	Δ Attenuation value (HU^m, 95% CI)	*p*-value *vs.* baseline
1st (8–14.1)	60	11.68	11.92	+0.25 (−0.26 to 0.52)	0.075	0.22	−0.59	−0.81 (−2.77–1.15)	0.413
2nd (14.1–18)	60	15.59	16.29	+0.7 (0.25–1.15)	0.003	0.61	−0.35	−0.96 (−3.42 to 1.49)	0.435
3rd (18.1–23.88)	56	19.85	20.8	+0.95 (0.18–1.72)	0.016	4.29	1.37	−2.92 (−5.36 to −0.49)	0.02
4th (23.9–42.2)	58	26.82	28.82	+1.98 (1.06–2.91)	<0.0001	1.01	−3.95	−2.94 (−5.09 to −0.78)	0.008

Δ, difference; 95% CI, 95% confidence interval; HU^m, mean of Hounsfield Unit in unenhanced CT.

TABLE 5 | Mean differences according to the quartile of diameter.

	Quartile	Mean difference *vs.* 1st quartile (95% CI)	*p*-value
Cortisol after 1-mg DST (nmol/L)	2nd (14.1–18 mm)	10.46 nmol/L (–14.84 to 35.77)	0.637
	3rd (18.1–23.88 mm)	19.23 nmol/L (–7.24 to 45.69)	0.207
	4th (23.9–42.2 mm)	60.08 nmol/L (34.03–6.1)	<0.0001
Diameter (mm)	2nd (14.1–18 mm)	0.46 mm (–0.6 to 1.53)	0.604
	3rd (18.1–23.88 mm)	0.72 mm (–0.363 to 1.81)	0.273
	4th (23.9–42.2 mm)	1.74 mm (0.67–2.82)	<0.0001

95% CI, 95% confidence interval.

significantly decreased in patients with ACS, and we can speculate that lipid content can be used to suggest an overt CS in patients with an adrenal adenoma and some peculiar features of hypercortisolism or cortisol-related comorbidities.

Our work presents some limitations. First, the observational longitudinal design of the study, without interventions: imaging follow-up in the absence of adrenalectomy is likely the general practice. Conservative management was one of the inclusion criteria: we included adrenal incidentaloma with a low-risk of progression based upon radiological features. According to our definition, also larger adenomas were excluded, because we selected those patients with benign adrenal incidentaloma, reflecting the suggestions of the ESE-ENS@T guidelines. Moreover, CT scans were performed as indicated during an outpatient visit, therefore follow-up was not homogenous. Our selection criteria reflect the clinical practice; however, a prospective and controlled study could help to further analyze the correlation between adrenal dimension, lipid content, and cortisol secretion.

To conclude, growth rate is significant but clinically modest in patients with adrenal incidentaloma selected with strict criteria indicating a benign behavior. According to our data, the first long-term imaging control with an unenhanced control CT can be proposed to patients with ACS or diameter >24 mm, at least 5 years after baseline. Lipid content, measured with attenuation value, is reduced in patients with ACS. In the follow-up, despite an increase in size, we observed a tendency to the reduction of mean density in adrenal incidentalomas.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: http://researchdata.cab.unipd.it/id/eprint/530.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Padova University Hospital (Comitato Etico per la Sperimentazione Scientifica). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FCe, IT, and FCr: writing—original draft, review, and editing. GV and GM: data acquisition and curation. IM, EQ, and CS: supervision and writing—review and editing. All authors contributed to the article and approved the submitted version.

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Primary Adrenal Lymphoma: Two Case Series From China

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Objective: Primary adrenal lymphoma (PAL) is a rare form of adrenal mass. We summarize our experience in its clinical presentation, biochemical indexes, radiological features, pathological information, therapy regimens, and outcomes.

Methods: This was an institutional review board-approved retrospective review of medical records and surgical pathology specimens of patients with a diagnosis of PAL at the Chinese People's Liberation Army General Hospital and the First Affiliate Hospital of Xiamen University between July 2007 and July 2017.

Results: Twenty-six patients were identified. The mean age at presentation was 60.84 ± 13.14 years with a male-to-female ratio of 2.25:1 (18:8). The most common presenting symptoms were loss of appetite (65%, 17/26), weight loss (62%, 16/26), abdominal pain (58%, 15/26), and fatigue (58%, 15/26). The levels of lactate dehydrogenase (75%, 15/20), β_2-microglobulin (100%, 10/10), C-reactive protein (82%, 14/17), and ferritin (88%, 7/8) and the erythrocyte sedimentation rate (83%, 10/12) were elevated. Bilateral involvement was seen in 21 of 26 patients (81%); 12 of 19 evaluated patients with bilateral lesions (63%) were confirmed to have adrenal insufficiency. On computed tomography (CT), the mean tumor diameter was 7.31 ± 3.35 cm and the median Hounsfield density was 37.0 HU (range: 31.0–45.0 HU); 67% (10/15) and 27% (4/15) of lesions presented with mild and moderate enhancement after injection of contrast medium. ^{18}F-fluorodeoxyglucose positron emission tomography (FDG PET)-CT revealed not only an adrenal tumor but also extra-adrenal lesions. Diffuse large B-cell lymphoma (DLBCL) was the most common phenotype (92%, 24/26). Ninety-two percent (24/26) of patients received chemotherapy while 8% (2/26) received unilateral adrenalectomy plus chemotherapy. The prognosis of PAL was poor, with a general survival time of 7.20 ± 5.18 months.

Conclusion: PAL is a rare disease. The clinical characteristics of PAL include loss of appetite and weight loss. Endocrine evaluation should be performed to determine whether

patients have adrenal insufficiency, especially patients with bilateral lesions. FDG-PET appears to be more accurate than other imaging modalities in revealing extra-adrenal sites. Better therapy is required to improve the poor prognosis of PAL.

Keywords: primary adrenal lymphoma, clinical features, adrenal insufficiency, imaging, histology

INTRODUCTION

Most lymphomas originate from lymph nodes, while up to a quarter develop from extra-lymph node lymphoid or non-lymphoid tissue. Primary extranodal lymphoma (PEL) has been reported to account for 25%–40% of all cases of lymphoma in Western countries (1) and 45.9% in Taiwan respectively (2). The most commonly involved sites in PEL are the gastrointestinal tract, central nervous system, and skin (3). Lymphoma originating from the endocrine system accounts for 3% of cases of PEL, and primary adrenal lymphoma (PAL) accounts for only 0.2% of those lymphomas (4). There are only 250 cases described in the English-language literature worldwide to date (5); the majority of published articles about PAL are case reports or case-series studies with only a limited number of patients. The common features of PAL are male sex, older age, bilateral lesions, adrenal insufficiency, and poor outcomes, based on data mostly from Western countries (6). Due to the extreme rarity of this disorder, difficulty in differential diagnosis such as adrenocortical carcinoma or pheochromocytoma, and the lack of guidelines, physicians may face the difficulty in diagnosing and treating PAL.

The objective of this study is to summarize the clinical features, biological and imaging characteristics, and outcomes of 26 patients with PAL in the northern and southern regions of China in order to characterize this entity, which may improve the differential diagnosis of adrenal masses and finally improve prognosis through early diagnosis and treatment.

MATERIALS AND METHODS

Nineteen and seven patients with PAL were diagnosed at the Chinese People's Liberation Army General Hospital in Beijing and the First Affiliated Hospital of Xiamen University in Xiamen, respectively, from July 2007 to July 2017. The diagnostic criteria for PAL were as follows (5): (a) histologically proven lymphoma that involves at least one adrenal gland; (b) no prior history of lymphoma; and (c) adrenal lesions are unequivocally dominant if lymph nodes or other associated organs involved. The demographics, clinical manifestations, biochemical examination, imaging features, pathological type, and prognosis of 26 patients with PAL were retrospectively reviewed. The diagnosis of adrenal insufficiency (AI) was based on an 8 am cortisol value <5 μg/dl in combination with a high adrenocorticotropic hormone level.

Statistical analyses were performed using the IBM Statistical Package for the Social Sciences version 16.0. Mean ± standard deviation (SD) and median (interquartile range) are used to describe variables with normal and non-normal distributions, respectively. Frequency and percentage are used to describe categorical data. Group variables with a normal distribution were compared using the t-test, while categorical data were compared using the χ^2 test.

RESULTS

Baseline Demographic

A total of 26 cases of PAL were identified (clinical characteristics are summarized in **Table 1**), which included 18 male (69%) and 8 female (31%) patients, with a sex ratio of 2.25:1. The mean age of the patients at diagnosis was 60.84 ± 13.14 years. The distribution of patients according to age is illustrated in **Figure 1**, and PAL was most commonly seen in patients aged 60–69 years. The mean body mass index (BMI) at diagnosis across all patients was 23.26 ± 3.92 kg/m^2. None of our patients had a history of autoimmune disease, other malignancy, or immune suppression therapy. In our cohort, the largest number of patients were diagnosed in the Endocrinology Department (12/26, 46%), followed by the Hematology Department (4/26, 15%), Urology Department (4/26, 15%), Gastroenterology Department (4/26, 15%), and Respiratory Department (2/26, 8%).

TABLE 1 | Clinical characteristics of the study population.

	Total
n	26
Age (years)	60.84 ± 13.14
Sex (male/female)	18/8
BMI (kg/m^2)	23.26 ± 3.92
Autoimmune disease (n)	0
Immune suppression (n)	0
Malignances (n)	0
Elevated LDH (n, %)	15, 75%
Elevated CRP (n, %)	14, 82%
Elevated ESR (n, %)	10, 83%
Elevated β2-MG (n, %)	10, 100%
Elevated ferritin (n, %)	7, 88%
Adrenal insufficiency (n, %)	12, 63%
Unilateral/bilateral involvement (n)	5/21
Tumor size (cm)	7.31 ± 3.35
CT value (HU)	37.0
Non-enhancement (n, %)	1, 6%
Mild-enhancement (n, %)	10, 67%
Moderate-enhancement (n, %)	4, 27%
Homogeneous enhancement (n, %)	10, 71%
Heterogeneous enhancement (n, %)	4, 29%
Mean SUV of adrenal lesion	17.72 ± 8.64
Mean SUV of extra-adrenal lesion	11.82 ± 6.08
Diffuse large B cell lymphoma/extranodal NK/T cell lymphoma (n)	24/2

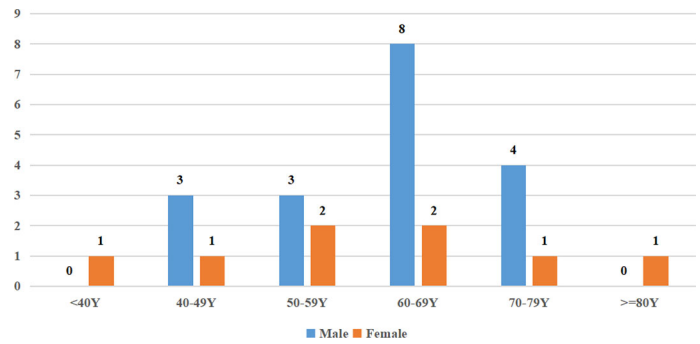

FIGURE 1 | Age distribution of PAL in this series.

Clinical Manifestations

The duration of disease ranged from 0.46 to 3.00 months, with a median duration of symptoms of 1.0 month. At the time of initial diagnosis, the most common presenting symptoms were loss of appetite (17/26, 65%), weight loss (16/26, 62%), abdominal pain (15/26, 58%), and fatigue (15/26, 58%). Less common presenting symptoms included fever (11/26, 42%), nausea and vomiting (6/23, 23%), back pain (5/26, 19%), hyperpigmentation (5/26, 19%), salt craving (2/26, 8%), and night sweats (2/26, 8%) (**Figure 2**). The initial presenting symptom in most cases (13/26, 50%) was abdominal pain. On physical examination, posture hypotension, lymphadenopathy, abdominal masses, and hepatosplenomegaly were present in 23% (6/26), 15% (4/26), 4% (1/26), and 4% (1/26) of our patients, respectively.

Biological Profile

The level of lactate dehydrogenase (LDH) was elevated in 15 (75%) out of the 20 patients in whom it was tested, with a mean value of 493.94 ± 382.63 U/l (40–250 U/l). Serum β_2-microglobulin (β_2-MG) was elevated in all 10 tested patients (100%), with a mean value of 0.40 ± 0.18 mg/dl (0.07–0.18 mg/dl). C-reactive protein (CRP) was elevated in 82% of patients (14/17) with a mean value of 3.66 ± 3.53 mg/dl (0–0.8 mg/dl). Twelve patients underwent erythrocyte sedimentation rate (ESR) tests, and 10 patients (83%) had elevated values, with a mean value of 42.17 ± 23.49 mm/h (0–20 mm/h). Plasma ferritin levels were elevated in seven of eight tested patients, with a mean value of 1,005.00 ± 756.45 ng/ml (30–400 ng/ml). Twelve of the 19 (63%) evaluated patients had AI, and all cases of AI were caused by bilateral lymphomatous involvement.

Imaging Features

Bilateral adrenal gland involvement was seen in 81% (n = 21) of cases. Among five patients with unilateral disease, the left–right ratio was 3:2. Six patients had only adrenal lesions with disease in no other locations. Four patients had involvement at one extra-adrenal site, five patients had involvement at two extra-adrenal sites, four patients had involvement at three extra-adrenal sites, and seven patients had involvement at four or more sites (**Figure 3**). The diameter of the tumors was 7.31 ± 3.35 cm.

Computed Tomography

On axial images, the proportion of PAL with a regular pattern was 68% (27/40) and 32% (13/40) with an irregular pattern. The frequency of well-defined and ill-defined tumors was 63% (25/40) and 37% (15/40), respectively. Non-contrast and contrast-enhanced computed tomography (CT) was performed in 26 and 8 cases, respectively. The median Hounsfield density was 37.0

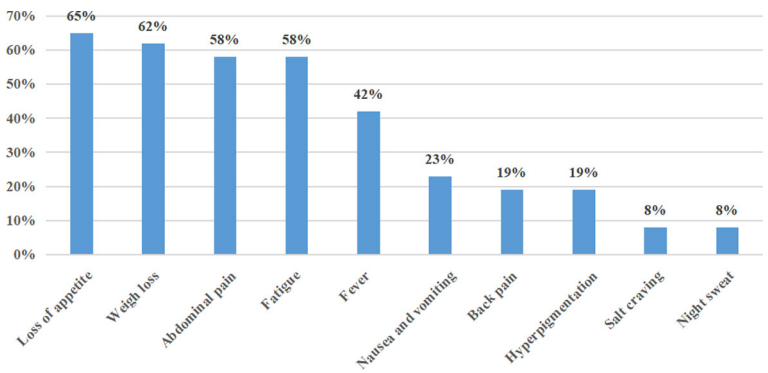

FIGURE 2 | Frequency of clinical presentations of PAL in this series.

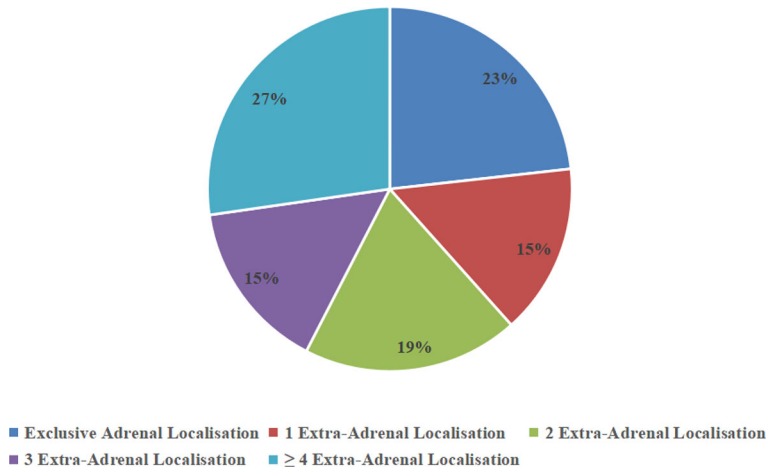

FIGURE 3 | Percentage of adrenal and extra-adrenal locations.

Hounsfield units (HU) (range: 31.00–45.00 HU). After intravenous injection of contrast medium, 7% (1/15) of lesions showed no enhancement, 67% (10/15) of tumors showed mild enhancement, and 27% (4/15) of lesions showed moderate enhancement. Homogeneous and heterogeneous enhancement on CT was seen in 71% (10/14) and 29% (4/14) of tumors, respectively (**Figure 4**).

^{18}F-Fluorodeoxyglucose Positron Emission Tomography-Computed Tomography

Twenty-one patients underwent 3.6 18F-fluorodeoxyglucose positron emission tomography-computed tomography (^{18}F-FDG PET-CT) examination in our study. The standard uptake value (SUV) of the adrenal gland was elevated in all patients, with a mean value of 17.72 ± 8.64, while mean SUV of ex-adrenal lesions was 11.82 ± 6.08. The uptake of ^{18}F-FDG in the adrenal gland was more intense than the uptake in extra-adrenal lesions. Fourteen patients had lymph node involvement, with abdominal lymph node involvement being the most common (93%, 13/14), followed by the mediastinal (50%, 7/14) and neck (29%, 4/14) involvement. There were 17 cases of extranodal. The most frequently affected locations were the bone (47%, 8/17), liver (29%, 5/17), and lung (24%, 4/17) (**Table 2**).

Histology

In 92% (24/26) of patients, the diagnosis was based on histopathological examination and immunohistochemistry of the adrenal tissue following adrenal biopsy. Only 8% (2/26) of patients underwent initial unilateral adrenalectomy. The most frequent pathology was diffuse large B-cell lymphoma (DLBCL) in 92% (24/26) of patients, while the histology in 8% (2/26) of patients was extranodal NK/T cell lymphoma.

Treatment and Prognosis

Twenty patients with DLBCL received treatment with prednisone (90 mg, days 1–5), vincristine (1.4 mg/m^2 day 1), cyclophosphamide (750 mg/m^2 day 1), or doxorubicin (45 mg/m^2, day 1) along with rituximab (375 mg/m^2, day 0) (R-CHOP regimen). Two patients with extranodal NK/T cell lymphoma received methotrexate (3 g, day 1), heterocyclic phosphamide (3 g, day 2; 2 g, day 3–4), dexamethasone (40 mg, days 2–4), etoposide (100 mg, days 2–4), and asparaginase (10,000 U; days 8, 10, and 13) (SIMLE regimen). To date, twenty-two patients have died till now, with a mean survival time of 7.20 ± 5.18 months; four patients were lost during the follow-up.

DISCUSSION

We discussed the demographic findings, clinical presentation, biological evaluations, imaging characteristics, pathology, treatment, and prognosis of PAL in two centers from China during the last 10 years. To our best knowledge, the current study is the only reported double center experience of PAL in China.

PAL usually develops in elderly male patients, with a median age at presentation ranging from 48 to 68 years and male/female ratio of 1.8:1–7:1 (5–9); our findings were consistent with the reported age and sex ratio ranges. Dobrinja et al. suggest that the highest incidence occurs in adults approximately 60 years old (10), which corresponds with the present series. The BMI was similar to the existing literatures noted by Yumi, Horiguchi and Huang (11–13), while it was lower than that reported by Laurent et al. (6). This may be attributed to the difference of ethnic, diet structure, and environment.

The median time from symptom onset to the initial diagnosis of PAL was 1 month, which is slightly shorter than that reported for Asian cases (median time, 1.5 months; range, 0.25–12 months) (14–24). Since the adrenal glands are concealed in the retroperitoneum and endocrine inactivity, the initial signs and symptoms are often unspecific. As established by findings from a meta-analysis (5), the most common symptoms were B-symptoms, which include fever, weight loss, night sweats

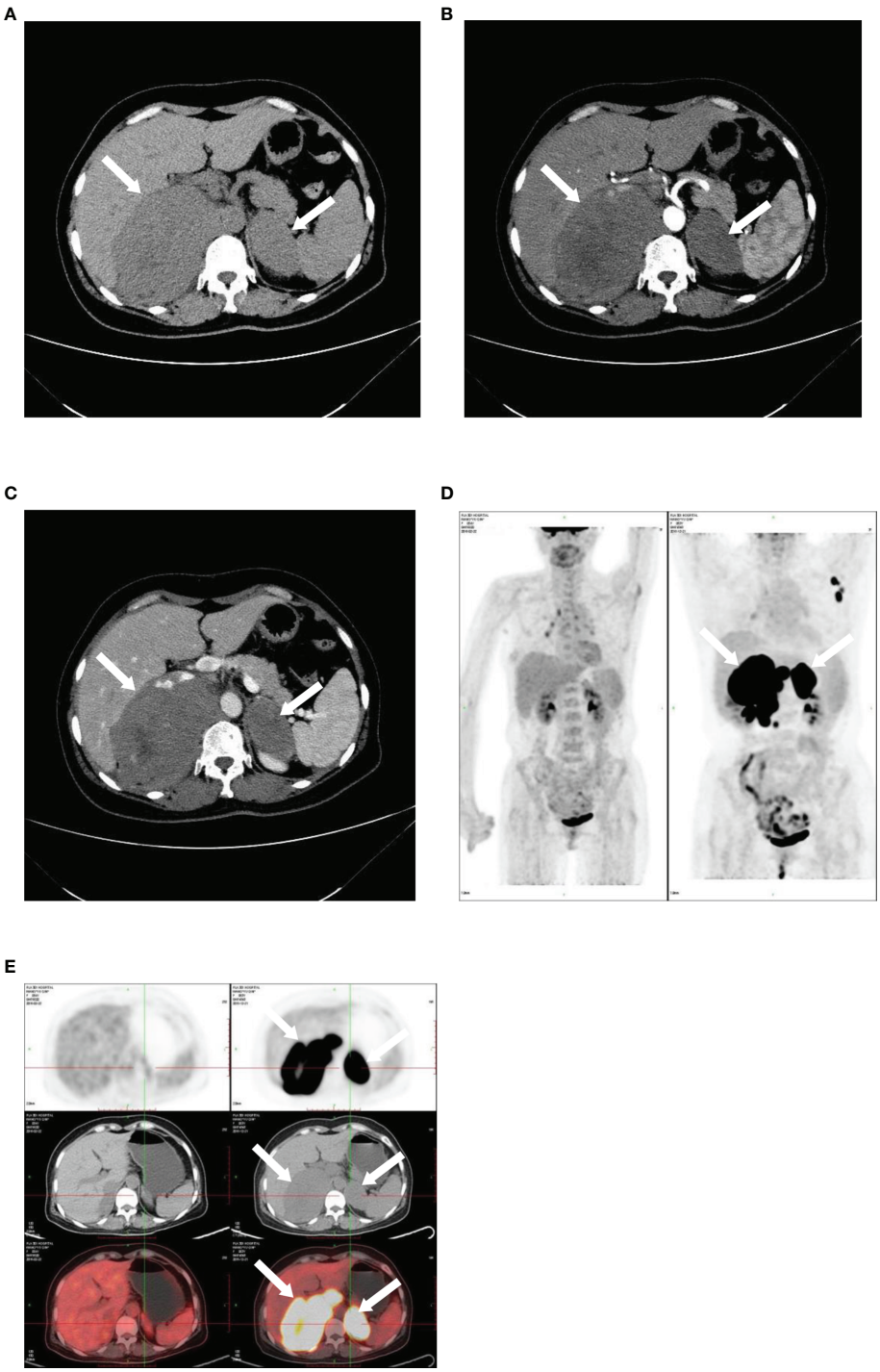

FIGURE 4 | Imaging of bilateral primary adrenal lymphoma. CT and PET images illustrating bilateral primary adrenal lymphoma. CT scans: **(A)**, non-contrast phase; **(B)**, arterial phase; **(C)**, venous phase. 18F-FDG PET-CT: **(D, E)**.

(68%), pain (42%), and fatigue (36%), which corresponds with our data. Regarding signs, adenopathy, splenomegaly, and hepatomegaly were present in 18%, 14%, and 11% in a cases series from Laurent et al. (6), which was slightly higher than the rates in our case series (15%, 4%, and 4%). More specific symptoms and signs, such as hyperpigmentation (41%–74%), salt craving (38%–74%), and postural hypotension (55%–58%), were related to AI in a previous review (25); these signs were

TABLE 2 | Summary of extra-adrenal infiltrations.

Affected lymph nodes	Cervical	4
	Axillary	1
	Mediastinal	7
	Abdominal	13
Affected organ/tissues	Bone	8
	Liver	5
	Lung	4
	Spleen	3
	Kidney	3
	Testicle	2
	Pancreas	1
	Colon	1
	Meningeal	1

present in 42%, 25%, and 50%, respectively, in our population, suggesting that there were few endocrinology consultations or not all patients were managed by the Endocrinology Department. In addition, AI often presented in patients with bilateral adrenal involvement. In the 12 confirmed cases of AI in 19 assessed patients, no patient with unilateral involvement had AI, which is in accordance with the principle that >90% adrenal parenchymal destruction will lead to AI given the huge reserve in adrenal function (26). Consequently, endocrinology evaluation should be performed in patients with bilateral adrenal involvement and/or clinical symptoms or signs of AI. Immune system dysfunction has been proposed as an etiology of PAL (27). Wang et al. reviewed 55 patients with PAL and found that 13% had autoimmune disease (28). However, none of our patients had any prior history of carcinoma or autoimmune disease, which is in accordance with the literatures noted by Kasaliwal and Rashidi (5, 9).

In our series, 75% (15/20) of patients had increased LDH levels, which is in accordance with data shown by Laurent et al. (74%, 20/27) (6), indicating that tumor burden increases with cell turnover but lower than that reported by Rashidi et al. (88%, 70/80) (5), which may be due to the small sample size in the current series. Elevated serum β_2-MG levels may indicate hematological disease, which was present in 100% (10/10) of our cases, higher than the incidence reported by Laurent el al. (71%, 15/21) (6). In addition, serum β_2-MG and CRP levels were the most important predictive factors for overall survival in DLBCL (29–31), which explains the lower median survival in our study compared to that noted by Laurent et al. (6). The ESR and ferritin levels may indicate an inflammatory syndrome, but neither of them lead to a diagnosis of DLBCL or correlate with prognosis.

Diagnostic imaging includes CT, magnetic resonance imaging (MRI), and PET-CT. CT is considered to be the most important imaging modality in evaluating adrenal masses as it can be used for their localization, visualization, and characterization. On CT, 81% of patients (21/26) had bilateral involvement, which was a slightly higher rate than that reported in previous related studies (32); the delayed diagnosis in our series may reflect that unilateral involvement is the process of bilateral lesions. The mean tumor diameter at the time of diagnosis in our case series was 7.31 ± 3.35 cm, which is similar to that in previous related studies (33). The Cleveland Clinic performed a large retrospective study including 299 cases and determined a threshold of 10 HU on non-enhanced CT to distinguish benign and malignant adrenal masses, with a sensitivity and specificity of 79% and 96%, respectively (34). In our series, the median attenuation of PAL was 37 HU, which is in accordance with the density reported by Zhou et al. (35). In addition, the dominant pattern of enhancement after intravenous injection of the medium showed that 88% of our patients had a "slight to moderate enhancement pattern", which was reported to occur in 78%–100% of cases in previous reports (5, 35), given that PAL is not a hypervascular tumor. This pattern is different from adrenocortical carcinoma and pheochromocytoma, which are hypervascular lesions and show significant enhancement. Furthermore, 71% of patients displayed "homogeneous enhancement." Conversely, previous studies showed that PAL tends to exhibit a heterogeneous enhancement pattern (36), whereas a study that included 28 cases demonstrated that most had homogeneous or slightly inhomogeneous enhancement (32). ^{18}F-FDG PET-CT provides anatomical and functional information, both in the adrenal gland and in other involved sites. Twenty-one patients underwent ^{18}F-FDG PET-CT, which makes our study the largest collection of patients with PAL examined using PET-CT. Due to the metabolic hyperactivity in PAL, SUV levels were elevated with a mean value of 17.72 ± 8.64 and 11.82± 6.08 in adrenal and extra-adrenal lesions, which is slightly lower than the values in a previous report (6). In addition, compared with CT and MRI, PET-CT provides more precise evaluation of PAL extension. Eighty-one percent (17/21) of our cases exhibited ex-adrenal extension on PET-CT, which is higher than the rate reported in the study by Laurent, consistent with the aggressive biological characteristics of PAL.

While clinical manifestations, biological, and imaging can be helpful, a definitive diagnosis can only be made histologically using tissue obtained *via* needle core biopsy, incisional or excisional biopsy, or autopsy. The DLBCL phenotype was predominant (92%) in our series and ranges 78%–94% in previous reports (12, 33, 37). PAL has a poor prognosis. Two decades ago, the median survival was 4 months (38), with the longest reported survival of 15 months (39). Recently, with the use of the R-CHOP regimen instead of the CHOP regimen, the clinical outcome has improved (40). Unfortunately, the mean survival in our case series was 7.20 ± 5.18 months, which was worse than that in earlier studies (40), suggesting hidden manifestation and that patients may have been diagnosed at a late stage of disease.

CONCLUSION

PAL is a rare entity that usually occurs in elderly men and more commonly presents with bilateral lesions. ^{18}F-FDG PET-CT is a major tool in the diagnosis of adrenal lesions and extra-adrenal extensions. The dominant histological phenotype is DLBCL, which can lead to adrenal insufficiency, and has a poor prognosis. Better therapy is required to improve outcomes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of West China Hospital of Sichuan University (No. 2019-229). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JZ and FY: collected the data and wrote the manuscript draft. YC, LZ, KC, ZL, JD, YM, and ML: contributed to the discussion and revision. GY: designed the study and revised the submission. All authors contributed to the article and approved the submitted version.

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Whole Transcriptome Profiling of Adrenocortical Tumors Using Formalin-Fixed Paraffin-Embedded Samples

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Whole transcriptome profiling is a promising technique in adrenal studies; however, whole transcriptome profiling of adrenal disease using formalin-fixed paraffin-embedded (FFPE) samples has to be further explored. The aim of this study was to evaluate the utility of transcriptome data from FFPE samples of adrenocortical tumors. We performed whole transcriptome profiling of FFPE and fresh frozen samples of adrenocortical carcinoma (ACC, n = 3), aldosterone-producing adenoma (APA, n = 3), and cortisol-producing adenoma (CPA, n = 3), and examined the similarity between the transcriptome data. We further examined whether the transcriptome data of FFPE samples could be used to distinguish tumor types and detect marker genes. The number of read counts was smaller in FFPE samples than in fresh frozen samples ($P < 0.01$), while the number of genes detected was similar ($P = 0.39$). The gene expression profiles of FFPE and fresh frozen samples were highly correlated ($r = 0.93$, $P < 0.01$). Tumor types could be distinguished by consensus clustering and principal component analysis using transcriptome data from FFPE samples. In the differential expression analysis between ACC and APA-CPA, known marker genes of ACC (e.g., *CCNB2*, *TOP2A*, and *MAD2L1*) were detected in FFPE samples of ACC. In the differential expression analysis between APA and CPA, known marker genes of APA (e.g., *CYP11B2*, *VSNL1*, and *KCNJ5*) were detected in the APA of FFPE samples. The results suggest that FFPE samples may be a reliable alternative to fresh frozen samples for whole transcriptome profiling of adrenocortical tumors.

Keywords: whole transcriptome profiling, RNA sequencing (RNAseq), formalin-fixed paraffin-embedded samples (FFPE samples), adrenal diseases, adrenocortical tumors

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INTRODUCTION

The adrenal cortex produces a variety of steroid hormones and maintains metabolic homeostasis (1). Aldosterone, produced in the zona glomerulosa, is involved in the regulation of both the Na/K balance and blood pressure, while cortisol, produced in the zona fasciculata, plays an important role in the regulation of glucose metabolism and the immune response. Some adrenocortical adenomas produce these steroid hormones inappropriately and cause a variety of clinical syndromes. Aldosterone-producing adenoma (APA) causes primary aldosteronism, and cortisol-producing adenoma (CPA) causes Cushing's syndrome (2, 3). Adrenocortical carcinoma (ACC) is a rare malignant tumor that arises in the adrenal cortex and has a very poor prognosis (4). In the clinical management of adrenocortical tumors, it is important to distinguish between these tumors, especially ACC and adenoma, although they can have overlapping clinical and pathologic findings that can be difficult to distinguish. Previous transcriptome studies have shown that APA, CPA, and ACC have distinct gene expression profiles, suggesting that transcriptome profiling can contribute to the accurate diagnosis of adrenocortical tumors (5, 6). Furthermore, transcriptome profiling is also a promising technique for elucidating the pathogenesis of adrenal diseases in terms of endocrine function and adrenal differentiation (7, 8).

Molecular profiling, including transcriptomics, usually requires fresh frozen tissue samples. However, these are difficult to obtain for most adrenocortical tumors because fresh frozen tissue is rarely preserved in routine clinical practice. This is a major obstacle to transcriptome studies of adrenocortical tumors. However, formalin-fixed paraffin-embedded (FFPE) samples are relatively easy to obtain, but the nucleic acids are degraded and less high-quality DNA and RNA can be extracted compared with fresh frozen samples (9, 10). In recent years, advances in sequencing technology have made it possible to perform molecular profiling using nucleic acids extracted from FFPE samples, and techniques for the genomic profiling of tumors using DNA extracted from FFPE samples have been established and are being used clinically (11). Whole transcriptome profiling using FFPE samples has been reported to be useful in malignant tumors such as hepatocellular carcinoma and breast cancer (12, 13). However, it remains unknown whether whole transcriptome profiling of adrenal disease using FFPE samples can be successfully performed.

In this study, we performed whole transcriptome profiling using FFPE samples of adrenocortical tumors (ACC, APA, and CPA) and compared the results with those of fresh frozen samples. We demonstrated the utility of FFPE samples in the whole transcriptome profiling of adrenocortical tumors.

MATERIALS AND METHODS

Sample Preparation

We used ACC (n = 3), APA (n = 3), and CPA (n = 3) tumor tissues obtained from adrenocortical tumor patients operated at Kyushu University Hospital from 2017 to 2020. Diagnosis was made based on clinical symptoms, imaging features, and postoperative histopathological data. Each tumor was stored by both FFPE and fresh frozen after surgery. A portion of each tumor was collected in microtubes immediately after surgical removal and stored in a deep freezer (-80°C) (fresh frozen sample). The rest of the tumor was formalin-fixed and paraffin-embedded (FFPE sample). FFPE samples were stored at room temperature and fresh frozen samples were stored at -80°C for 1-4 years until RNA extraction. The detailed characteristics of each tumor sample were summarized in **Supplementary Table 1**.

RNA Isolation

FFPE samples were sectioned into 10 μm slices and only the tumor area was collected under visual observation. Total RNA was extracted from the collected tumor using the Maxwell RSC RNA FFPE Kit (Promega) according to the manufacturer's instructions. Total RNA from fresh frozen samples was extracted using TRIzol reagent (Thermo Fisher) according to the manufacturer's instructions. The RNA yield and quality were determined by UV absorption using a NanoDrop 2000 spectrophotometer. The RNA fragment size of FFPE samples was analyzed using the RNA 6000 Pico Kit (Agilent Technologies) running on the 2100 Bioanalyzer. Similarly, the RNA fragment size of fresh frozen samples was analyzed using the RNA 6000 Nano Kit. RNA integrity number (RIN) values and DV200 (the percentage of RNA fragments above 200 nucleotides in length) were obtained by running Bioanalyzer according to the manufacturer's instructions. RNA quality was assessed using RIN and DV200 values.

Library Preparation, Sequencing, and Alignment

Ribosomal RNA was removed from 100 ng of total RNA of each sample using the NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (NEB, E6310). Libraries were then prepared using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB, E7760). Libraries were quantified using the Bioanalyzer DNA-sensitivity kit (Agilent, 5067-4626) and sequenced on the NextSeq 500 (Illumina) using a 36 cycle, paired-end protocol providing approximately 33 million reads per sample. Base call files were converted to the FASTQ format using Bcl2Fastq (Illumina). Using the CLC genomics workbench (v10.1.1), all reads were aligned to the reference genome (Human, hg19), and gene expression was quantified.

Data Analysis

We used R (version 4.1.1) to perform the data analysis. Gene expression data were used as inputs for the R package edgeR (14). Genes with low expression were excluded using the "filterByExpr" function of edgeR. The gene counts were normalized by applying the trimmed mean of M-values (TMM) normalization method using the "calcNormFactors" and "cpm" functions of edgeR. The resulting log2 counts per million (logCPM) were used as inputs for downstream analyses (e.g., sample-to-sample correlation and consensus clustering). Consensus clustering (with the number of clusters "k" evaluated

for k = 2–6) was performed using the R package ConsensusClusterPlus (15). Fifty iterations were performed with a sample inclusion probability of 0.8 and an item inclusion probability of 1. The number of clusters was selected based on the inspection of the delta area plot. Principal component analysis was performed using the "prcomp" function of R package stats (16–18). Differentially expressed genes (DEGs) between adrenocortical tumors (ACC and the other types, or APA and CPA) were detected using the "lmfit" and "eBayes" functions of the R package limma (19, 20). The Benjamini-Hochberg method was used to correct for multiple comparisons. DEGs were defined as genes with an absolute value of log2 fold-change (logFC) greater than 2 and an adjusted P value (adj. P) of less than 0.05. KEGG pathway analysis was performed using the R package pathfindR (21). The "run_pathfindR" function was used with default parameters to identify the enriched KEGG pathways. The "score_terms" function was then used to calculate the agglomerated z score of each enriched pathway per sample.

RESULTS

Differences in Transcriptome Data Obtained From FFPE and Fresh Frozen Samples

We extracted RNA from a total of nine FFPE and nine fresh frozen samples from three ACC, three APA, and three CPA cases. Total RNA extracted from FFPE samples had lower RNA yield and quality parameters compared to those from fresh frozen samples (RNA concentration [mean 36.0 vs 464.5 μL, Wilcoxon rank sum test: P value < 0.01], RIN [mean 2.2 vs 7.9, P value < 0.01], DV200 [mean 47.6 vs 71.0, P value < 0.01]). To examine the effect of storage period on RNA yield and quality for each storage method, we divided the samples into two groups by the median storage period from surgery to RNA extraction and compared the two groups. There was no significant difference in the RNA yield and quality (**Supplementary Figure 1**).

Next, we performed RNA sequencing (RNA-seq) using the extracted RNA. The number of raw reads obtained from FFPE samples was similar to those from frozen samples (mean $3.30E^7$ vs $3.29E^7$, P value = 0.86), but the percentage of mapped reads of FFPE samples was less than those of fresh frozen samples (mean 82.9% vs 87.7%, P value = 0.02) (**Supplementary Table 1**). Consequently, the read counts obtained from FFPE samples were less than those from the fresh frozen samples (mean $3.58E^6$ vs $5.13E^6$, P value < 0.01) (**Figure 1A**). In contrast, the number of unique genes detected in FFPE samples was similar to those in fresh frozen samples (mean 18160 vs 18001, P value = 0.39).

Using normalized gene expression data, we examined the correlation between samples. In each case, the FFPE and fresh frozen samples were highly correlated (mean Pearson's correlation coefficient [r] = 0.93) (**Figure 1B**). Therefore, we found that similar gene expression profiles could be obtained using FFPE samples, although the amount of detectable transcriptome was lower than that of fresh frozen samples.

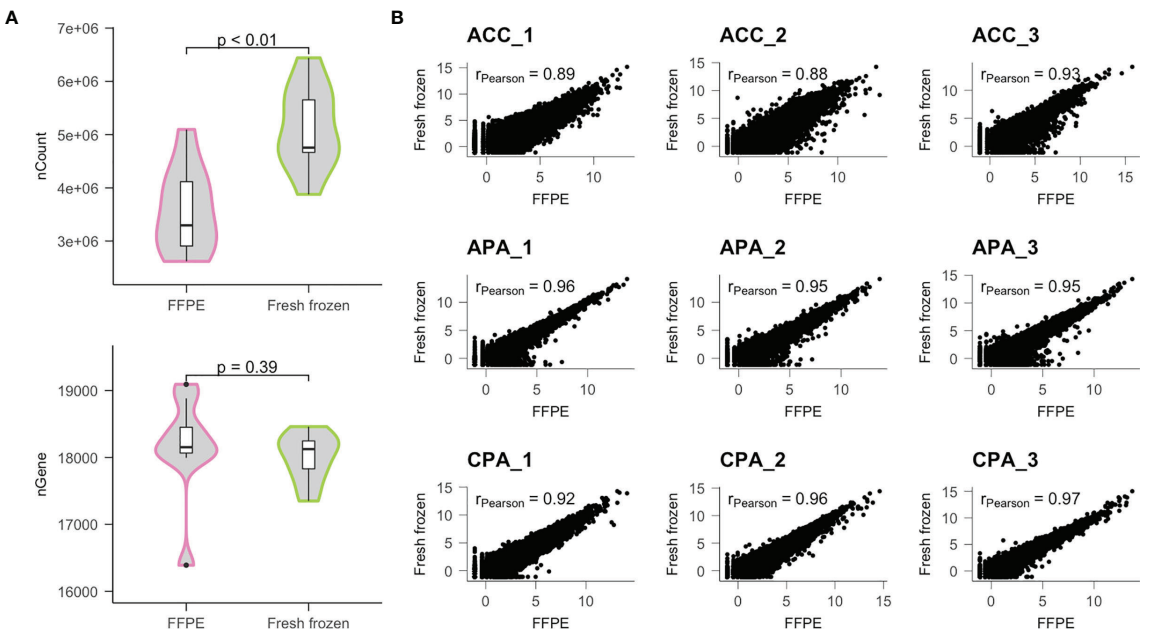

FIGURE 1 | Comparison of transcriptome data obtained from FFPE and fresh frozen samples. **(A)** Distributions of the number of read counts (nCount) and the number of genes detected (nGene) were compared between FFPE and fresh frozen samples. **(B)** Results of correlation analysis between FFPE and fresh frozen samples for each patient. r; Pearson's correlation coefficient.

Distinction Between ACC, APA, and CPA Using Transcriptome Data From FFPE Samples

We examined whether transcriptome data from FFPE samples could be used to distinguish between ACC, APA, and CPA. First, we validated whether the adrenocortical tumor types (ACC, APA, and CPA) could be separated at the transcriptome level using fresh frozen samples. Using consensus clustering, fresh frozen samples were classified into five clusters. APA and CPA were each classified into one cluster, and ACC was classified into one cluster per case (**Figure 2A**). Principal component analysis of fresh frozen samples was performed and visualized on two dimensions, with principal components 1 and 2 as the axes. APA and CPA each formed one cluster, whereas ACC was scattered in each case (**Figure 2B**). These results confirmed that the three types of tumors used in this study (ACC, APA, and CPA) had distinct gene expression profiles. In addition, ACC was considered to have a large difference in gene expression profiles among the cases.

Further, we performed consensus clustering and principal component analysis of the transcriptome data of FFPE samples. The FFPE samples were classified into five clusters by consensus clustering. APA and CPA were each classified into one cluster, and ACC was classified into one cluster per case (**Figure 2C**). Principal component analysis of FFPE samples was performed and visualized on two dimensions with principal components 1 and 2 as the axes. APA and CPA each formed one cluster, whereas ACC was scattered in each case (**Figure 2D**). The results of consensus clustering and principal component analysis of FFPE samples were both similar to those of fresh frozen samples. Therefore, we found that it was possible to distinguish between ACC, APA, and CPA using transcriptome data from FFPE samples.

Detection of Adrenocortical Tumor Marker Genes Using Transcriptome Data From FFPE Samples

We examined whether transcriptome data from FFPE samples could be used to detect previously reported adrenocortical tumor

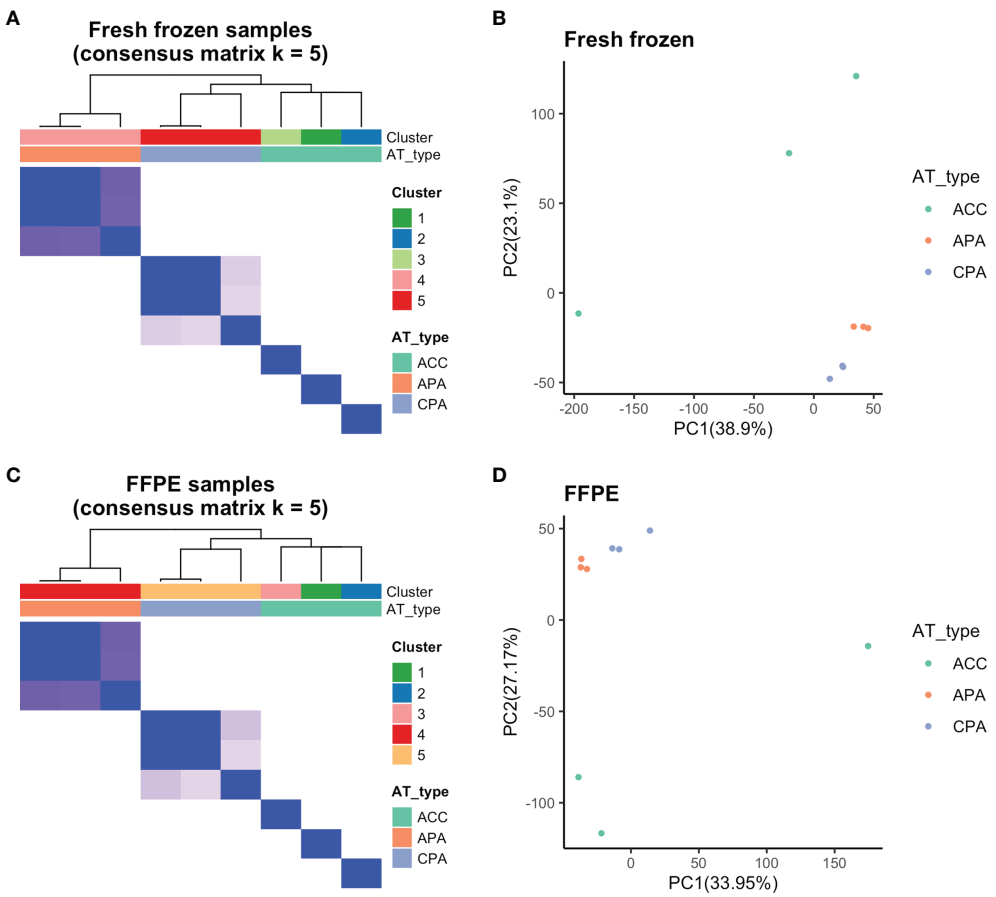

FIGURE 2 | Results of consensus clustering and principal component analysis. **(A)** Fresh frozen samples were divided into five clusters by consensus clustering (Cluster 1-3: ACC, Cluster 4: APA, Cluster 5: CPA samples). **(B)** Principal component analysis of fresh frozen samples confirmed separation based on tumor types. **(C)** By consensus clustering, FFPE samples were also divided into five clusters (Cluster 1-3: ACC, Cluster 4: APA, Cluster 5: CPA samples) similar to fresh frozen samples. **(D)** Principal component analysis of FFPE samples confirmed separation based on tumor types.

marker genes. First, we performed differential expression analysis between ACC and APA-CPA for each storage method (FFPE and fresh frozen). In FFPE samples of ACC, 402 DEGs were detected (161 upregulated and 241 downregulated). In fresh frozen samples of ACC, 356 DEGs were detected (148 upregulated and 208 downregulated). 193 DEGs were common between FFPE and fresh frozen samples (76 upregulated and 117 downregulated). The upregulated DEGs in FFPE samples of ACC included genes previously reported to be upregulated in ACC, such as *CCNB2* (encoding cyclin B2), *TOP2A* (encoding DNA topoisomerase II alpha), and *MAD2L1* (encoding mitotic arrest deficient 2 like 1) (**Table 1** and **Supplementary Table 2**) (22–24). These genes were also included in the upregulated DEGs in fresh frozen samples of ACC. *IGF2* (encoding insulin like growth factor 2), commonly used as a marker for ACC (25, 26), was not identified as an upregulated DEG in either FFPE or fresh frozen samples. This may be due to the low expression of *IGF2* in both FFPE and fresh frozen samples of case ACC_2 (**Supplementary Figure 2**). KEGG pathway analysis showed that upregulated DEGs in FFPE samples of ACC were enriched in tumor-related pathways including "cell cycle" and "p53 signaling pathway" (**Figure 3**). The upregulated DEGs in fresh frozen samples of ACC were also enriched in similar tumor-related pathways. Therefore, it was confirmed that the transcriptome data from FFPE samples could be used to detect the genes characteristic of ACC.

Next, we performed differential expression analysis between APA and CPA for each storage method (FFPE and fresh frozen). In FFPE samples of APA, 135 upregulated DEGs were detected. In fresh frozen samples of APA, 106 upregulated DEGs were detected. Forty-seven upregulated DEGs were common between FFPE and fresh frozen samples. The upregulated DEGs in FFPE samples of APA included genes reported to be associated with APA, such as *CYP11B2* (encoding cytochrome P450 family 11 subfamily B member 2, also known as aldosterone synthase), *KCNJ5* (encoding potassium inwardly rectifying channel subfamily J member 5), *VSNL1* (encoding visinin like 1), *CALN1* (encoding calneuron 1), and *HTR4* (encoding 5-hydroxytryptamine receptor 4) (**Table 2** and **Supplementary Table 3**) (27–31). These genes were also included in the upregulated DEGs in fresh frozen samples of APA. While in FFPE samples of CPA, 115 upregulated DEGs were detected. In fresh frozen samples of CPA, 97 upregulated DEGs were detected. Forty-eight upregulated DEGs were common between FFPE and fresh frozen samples. The upregulated DEGs in FFPE samples of CPA included genes reported to be upregulated in CPA, such as *FATE1* (fetal and adult testis expressed 1), *PITX1* (encoding paired like homeodomain 1) and *CXCL2* (encoding C-X-C motif chemokine ligand 2) (7, 32). KEGG pathway analysis showed that upregulated DEGs in FFPE samples of APA were enriched in pathways such as "serotonergic synapse" and "circadian entrainment", while DEGs in fresh frozen samples of APA were enriched in pathways such as "calcium signaling pathway" and "hippo signaling pathway" (**Figure 4**). The common upregulated DEGs between FFPE samples and fresh frozen samples were enriched in the pathway of "aldosterone synthesis and secretion" (**Supplementary Figure 3**). The upregulated DEGs in FFPE samples of CPA were enriched in metabolism-related pathways such as "steroid biosynthesis" and "cholesterol metabolism" (**Figure 4**). The upregulated DEGs in fresh frozen samples of CPA were also enriched in similar metabolism-related pathways. Thus, it was confirmed that transcriptome data from FFPE samples could be used to detect the characteristic genes of APA and CPA.

DISCUSSION

To evaluate the utility of transcriptome data obtained from FFPE samples in adrenocortical tumors, we compared transcriptome data from FFPE samples of ACC, APA, and CPA with those from fresh frozen samples of the same tumors. Transcriptome data from FFPE and fresh frozen samples showed a high degree of similarity. Using transcriptome data from FFPE samples, we were able to distinguish between ACC, APA, and CPA and detect the marker genes. Our study is the first to show that FFPE samples may be an alternative to fresh frozen samples for whole transcriptome profiling of adrenocortical tumors.

Recently, transcriptome profiling using FFPE samples has been performed in a variety of tissues (12, 13), but few studies

TABLE 1 | DEGs between ACC and APA-CPA.

Gene	FFPE sample		Fresh frozen sample		Gene description
	logFC	adj.P	logFC	adj.P	
ANLN	4.28	1.28E-02	4.44	3.99E-02	anillin actin-binding protein
ASPM	4.28	5.65E-03	4.65	1.47E-02	assembly factor for spindle microtubules
FOXM1	4.26	6.93E-03	3.99	1.14E-02	forkhead box M1
RRM2	4.02	1.37E-02	4.44	4.29E-02	ribonucleotide reductase regulatory subunit M2
DTL	3.77	1.87E-02	3.91	2.77E-02	Denticle-less E3 ubiquitin-protein ligase homolog
CCNB2	3.72	1.26E-02	4.49	5.09E-03	cyclin B2
TOP2A	3.71	6.92E-03	4.57	2.31E-02	DNA topoisomerase II alpha
TPX2	3.67	2.66E-02	3.85	2.16E-02	TPX2 microtubule nucleation factor
KIAA0101	3.41	1.19E-02	4.01	9.28E-03	PCNA clamp-associated factor

Showing genes related to ACC. Upregulated genes in ACC in the study by Giordano et al. were used as reference.
The higher the logFC, the higher the expression in ACC than APA-CPA.

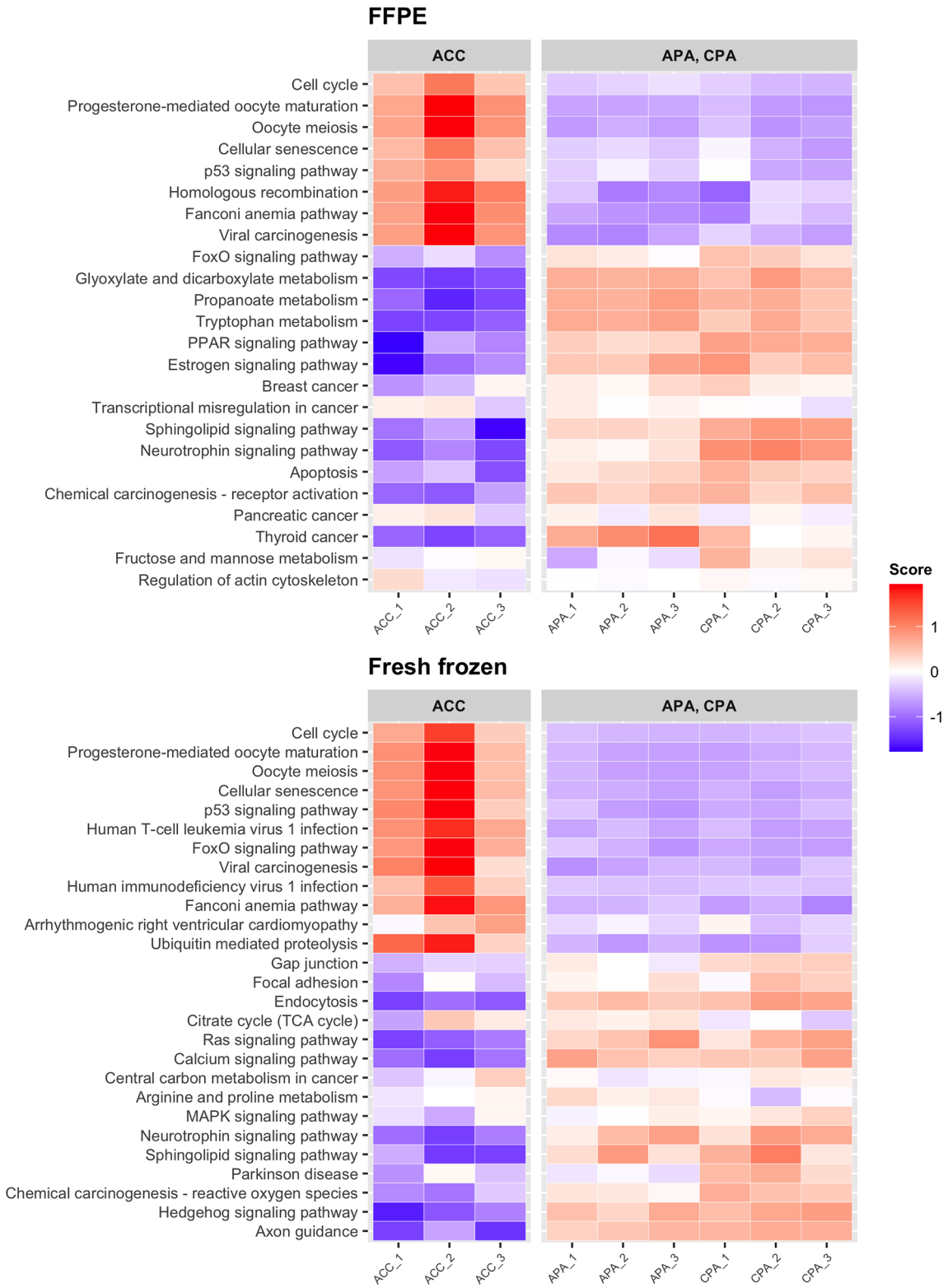

FIGURE 3 | Heatmap showing the results of KEGG pathway analysis of DEGs detected between ACC and APA-CPA by each storage type. Score; the agglomerated z score of each enriched KEGG pathway per sample.

TABLE 2 | DEGs between APA and CPA.

Gene	FFPE sample		Fresh frozen sample		Genn	Reference
	logFC	adj.P	logFC	adj.P		
CYP11B2	5.00	1.14E-02	7.00	1.41E-02	cytochrome P450 family 11 subfamily B member 2	Bassett MH et al. J Clin Endocrinol Metab. (27);90 (9):5446-5455.
VSNL1	3.57	8.92E-03	4.31	1.80E-02	visinin like 1	Williams TA et al. Hypertension. (28);59 (4):833-839.
CALN1	3.56	8.19E-03	5.81	3.87E-03	calneuron 1	Kobuke K et al. Hypertension. (29);71 (1):125-133.
HTR4	3.23	2.74E-03	4.54	4.92E-03	5-hydroxytryptamine receptor 4	Ye P et al. J Endocrinol. (30);195 (1):39-48.
KCNJ5	2.98	4.55E-03	4.27	2.21E-02	potassium inwardly-rectifying channel subfamily J member 5	Choi M et al. Science. (31);331 (6018):768-772.

Showing genes related to APA.
The higher the logFC, the higher the expression in APA than CPA.

have validated the accuracy of gene expression profiles obtained from FFPE samples using fresh frozen samples of the same tissue. Hedegaard et al. reported a comparative study of RNA-seq using FFPE and fresh frozen samples from the same tissue of six different human tissue types (bladder, prostate, and colon carcinoma; liver and colon normal tissue; reactive tonsil) (33). Shohdy et al. reported a comparative study of RNA-seq using FFPE and fresh frozen samples from the same tumors of seven different tumors (urothelial cancer, gastroesophageal junction adenocarcinoma, oligodendroglioma, cancer of unknown primary, leiomyosarcoma, papillary thyroid cancer, and colorectal cancer) in 11 patients (34). In this study, we showed that gene expression profiles from FFPE samples were highly similar to those from fresh frozen samples of the same adrenocortical tumors (ACC, APA, and CPA).

In transcriptome profiling using FFPE samples, targeted RNA-seq is often used because of the low yield and quality of RNA extracted from such samples (35, 36). Plaska et al. reported that targeted RNA-seq (194 target genes) using FFPE samples of adrenocortical tumors (ACC, APA, and CPA) could distinguish between benign and malignant tumors (36). However, targeted RNA-seq is not suitable for comprehensive genetic analysis (e.g., detection of novel pathogenic genes) because it restricts the genes that can be analyzed. In this study, we used whole transcriptome RNA-seq rather than targeted RNA-seq to obtain expression profiles of a large number of genes in each tumor (average number of genes detected in FFPE samples: 18001). We thus demonstrated that it is possible to distinguish ACC, APA, and CPA and detect their marker genes using transcriptome data from FFPE samples. Our results support the possible utility of whole transcriptome profiling using FFPE samples of adrenocortical tumors.

In the consensus clustering and principal component analysis of this study, APA and CPA each formed one cluster, and ACC differed greatly among cases. This result was similar for both FFPE and fresh frozen samples. The variation in ACC may be due to the different tumor traits in each case. Alternatively, it may be due to technical issues such as the storage conditions of each sample and the storage period until RNA extraction. A larger number of cases would be needed to examine the differences in tumor traits in ACC.

In the differential expression analysis of this study, it was possible to detect the marker genes of each adrenocortical tumor using FFPE samples. There were also differences in the DEGs that could be detected using FFPE samples and fresh frozen samples. In FFPE samples of ACC, genes such as *SPP1*, *PBK*, and *UBE2C* could not be detected. In FFPE samples of APA, genes such as *LGR5*, *HOPX*, and *ATP2B3* could not be detected. The read counts obtained from FFPE samples were lower than those of fresh frozen samples, which may result in the lower detection sensitivity of relatively low expression genes. When using FFPE samples for whole transcriptome profiling, a larger number of samples may be required compared to fresh frozen samples.

The use of FFPE samples for whole transcriptome profiling has advantages other than the ease of sample collection. FFPE samples are more suitable for morphological observation than fresh frozen samples, making it easier to collect transcriptomes from small regions of interest following microdissection. This may be applied, for example, to examine each layer of the adrenal cortex (which is composed of three layers) or small lesions such as aldosterone-producing cell clusters (presumed to be precursor lesions of APA) (37).

The limitation of this study is that the storage period of FFPE samples was relatively short (4 years at the longest). The longer the storage period, the lower the yield and quality of RNA, which may make it difficult to perform gene expression profiling equivalent to that using fresh frozen samples (38). Studies using FFPE samples with longer storage periods are required to validate our results. Another limitation is the lack of comparison between adrenocortical tumors and normal adrenocortical tissue adjacent to the tumors. In examining the intrinsic properties of each adrenocortical tumor, the normal adrenocortical tissue may be the ideal comparison target.

In conclusion, in this study, we demonstrated the utility of gene expression profiling of adrenocortical tumors using FFPE samples. FFPE samples are relatively easier to obtain, thus allowing large-scale adrenocortical tumor transcriptome studies.

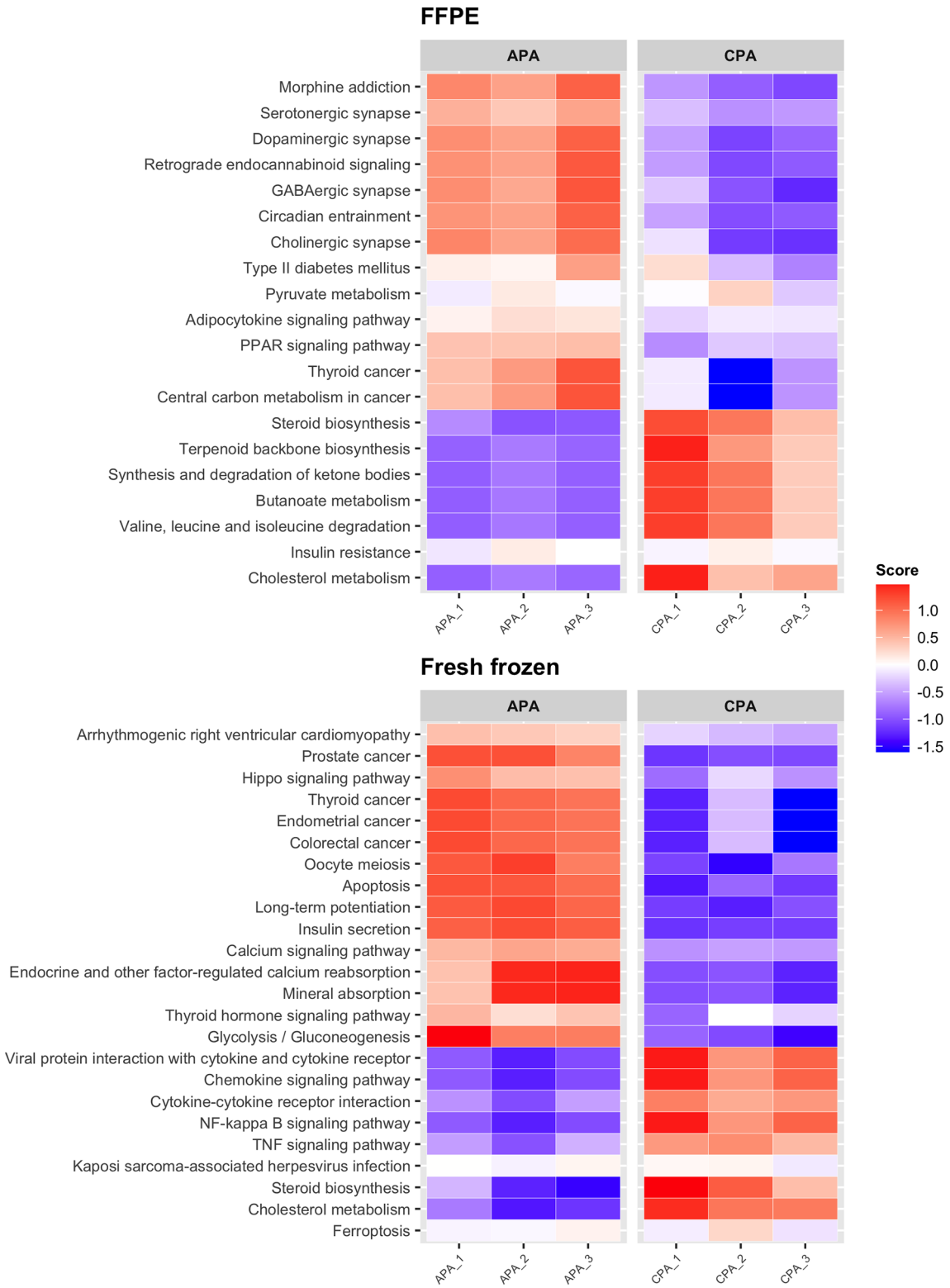

FIGURE 4 | Heatmap showing the results of KEGG pathway analysis of DEGs between APA and CPA by each storage type. Score; the agglomerated z score of each enriched KEGG pathway per sample.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/sra/PRJNA787399, PRJNA787399.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the medical ethics committee of the Kyushu University Hospital (approval #21025-00). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NI wrote the initial draft. HU and YO edited the manuscript. NI analyzed the results. HU and YO managed this study. HU contributed to data interpretation. MO, TF, HK, ET, SK, NU, KS, MY-U, YM, and RS curated the data and provided critical feedback and helped shape the research, analysis, and manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.808331/full#supplementary-material

Supplementary Figure 1 | Comparison of RNA yield and quality parameters between two storage period groups. FFPE samples were divided into two groups by 340 days, the median storage period. Similarly, fresh-frozen samples were divided by 427 days.

Supplementary Figure 2 | Distribution of the logCPM values of *IGF2*. Dots; each sample.

Supplementary Figure 3 | Heatmap showing the results of KEGG pathway analysis of DEGs between APA and CPA (common to FFPE and fresh frozen samples). Score; the agglomerated z score of each enriched KEGG pathway per sample.

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Limited Role of Hair Cortisol and Cortisone Measurement for Detecting Cortisol Autonomy in Patients With Adrenal Incidentalomas

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Several studies demonstrated the diagnostic accuracy of hair glucocorticoid measurement in patients with overt Cushing syndrome, but few data are available for patients with adrenal incidentaloma (AI) and cortisol autonomy. The aim of our study was to assess whether measurement of 5 corticosteroid hormones with the ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method in the keratin matrix is useful to stratify patients with AI by the presence of autonomous cortisol secretion [ACS] (defined as serum cortisol after 1 mg dexamethasone suppression test (DST) > 138 nmol/l) or possible ACS [PACS] (defined as serum cortisol after 1 mg DST > 50 nmol/l but ≤138 nmol/l). We analysed data of 67 AI patients (32 with cortisol autonomy) and 81 healthy subjects. We did not find any significant statistical difference comparing hair cortisol, cortisone, and 20β-dihydrocortisol concentrations between healthy controls and AI patients, while 6β-hydroxycortisol and 11-deoxycortisol were undetectable. Moreover, no significant difference was found in hair cortisol, cortisone, and 20β-dihydrocortisol levels of AI patients with or without cortisol autonomy. Finally, we did not find any correlation in patients with AI between hormonal concentrations in the keratin matrix and serum, salivary, and urinary cortisol levels, or with body mass index. In conclusion, our findings suggest that hair glucocorticoid measurement is not suitable as a diagnostic test for cortisol autonomy (ACS and PACS).

Keywords: subclinical Cushing, hair glucocorticoids, screening, diagnosis, adrenal adenoma, adrenal incidentaloma, Cushing, UPLC-MS/MS

INTRODUCTION

Adrenal incidentalomas (AIs) are masses incidentally and unexpectedly found in patients who undergo radiological exams due to diagnostic process or follow-up of extra-adrenal diseases (1). Since in the last decades the use of high-resolution cross-sectional imaging has become increasingly widespread, the serendipitous detection of adrenal tumours is common in clinical practice, accounting for 4.2%–7.3% in recent computed tomography (CT) series, up to 10% in elder patients (2–5).

In most cases, AI are benign adenomas, which secrete cortisol autonomously in up to 50% of patients (6, 7). In many cases, cortisol excess is mild and patients present without a typical phenotype, a condition previously called subclinical Cushing (8, 9) and now defined as autonomous cortisol secretion (ACS) or possible ACS (PACS) (1). Recent studies have demonstrated that this chronic, low-grade hypercortisolism can be associated with several cardio-metabolic comorbidities (5, 10–15) and increased mortality (16–18).

However, the diagnosis of ACS and PACS is challenging in practice and it can remain unrecognized for a long time, due to the subtle and heterogeneous clinical presentation and some methodological issues in laboratory screening tests (6).

According to the European Society of Endocrinology (ESE) and the European Network for the Study of Adrenal Tumors (ENSAT) guidelines, the main diagnostic tool is the 1-mg overnight dexamethasone suppression test (DST): cortisol levels ≤ 50 nmol/l exclude ACS, cortisol levels > 138 nmol/l define ACS, and levels between these two thresholds are considered expression of PACS (1). However, false positive or false negative results can occur, due to interferences in the absorption and/or the metabolism of dexamethasone (i.e., drugs, liver, or renal failure). The 24-h urinary free cortisol (UFC), midnight salivary cortisol (MSC), and plasma ACTH measurement at 8.00 a.m. support the diagnosis, but these tests are prone to several analytical errors or interference (6). Moreover, all these tools present the remarkable limit of measuring cortisol concentration at a single time point. Therefore, they cannot give an adequate representation of a chronic exposure to cortisol excess.

To overcome this issue, the measurement of hair cortisol has been recently proposed, given that cortisol progressively accumulates in hair shaft by passive diffusion from blood capillaries, according to the hair grow rate of approximately 1 cm per month. Cortisol remains sequestered in the keratin matrix with relatively little degradation over time, providing a window of detection which is much wider (weeks to several months) than that of serum or urine, in which cortisol levels decrease rapidly over a relatively short period of time (hours to days).

Although several studies confirmed the diagnostic accuracy of hair cortisol in patients with overt Cushing syndrome (19–23), only one study analysed the measurement of hair cortisol and cortisone in patients with AI (24).

In our study, we employed the method of the ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) for the simultaneous measurement of 5 corticosteroid hormones in the keratin matrix in AI patients with or without cortisol autonomy (ACS and PACS) and in healthy controls.

MATERIALS AND METHODS

Participants

We assessed consecutive patients affected by AI, which referred to the Unit of Internal Medicine at San Luigi Gonzaga Hospital, Orbassano (Italy), between March 2019 and February 2020. We compared this cohort with a group of healthy subjects, matched by sex and age with the AI group. Data of AI patients and healthy control were obtained from their medical records, interviews, and physical examinations and were reported on a detailed computerized database. Both patients and controls voluntarily participated in this study and gave written informed consent to the collection of data according to the local ethics committee indications (Registry and Repository of biological samples of ENSAT).

The inclusion criteria for AI patients were diagnosis of cortical adrenal adenoma, with specific CT characteristics (size less than 4 cm with well-defined margins, homogeneous and hypodense content). The exclusion criteria were suspected pheochromocytoma (i.e., patients with high levels of free plasma metanephrines or high urinary fractionated metanephrines), suspected primary hyperaldosteronism (i.e., patients with severe or resistant hypertension, hypokalemia, increased aldosterone/renin ratio), or suspected overt Cushing (i.e., patients with clinical signs of hypercortisolism or hyperandrogenism); patients who used topical or systemic corticosteroids in the last 3 months; and history of malignancy (breast, lung, and kidney cancer or melanoma) which can frequently metastasize in the adrenal gland.

The exclusion criteria for the healthy subjects were history of adrenal masses, Cushing syndrome, history of malignancy, suspected pseudo-Cushing states (i.e., chronic alcoholism, psychiatric disorders, poorly controlled diabetes mellitus, etc.), shift workers, and topical or systemic use of corticosteroids in the last 3 months.

Medical charts were reviewed to obtain clinical information: age, sex, body mass index (BMI), waist, and adrenal tumour size. Overweight was defined by BMI ≥ 25 and ≤ 30 kg/m^2, and obesity was defined by BMI > 30 kg/m^2. In all AI patients, we reported cortisol after 1 mg DST, 24-h UFC, and plasma ACTH at 8.00 a.m. and, when available, MSC.

Hair Cortisol Assessment

The determination and quantification of the analytes (cortisol, its metabolites cortisone, 20β-dihydrocortisol, and 6β-hydroxycortisol, and its precursor 11-deoxycortisol) in hair were carried out by the Regional Anti-Doping Center "Alessandro Bertinaria" (CAD), Orbassano (Turin). Methods of sample collection and UPLC-MS/MS analysis were similar to those commonly employed in toxicology and were adapted to the evaluation of the five targeted steroids (25).

Chemicals, Reagents, and Standard Solutions

Cortisol, cortisone, 20β-dihydrocortisol, 6β-hydroxycortisol and 11β-deoxycortisol, acetonitrile (ACN), methanol (MeOH), and dichloromethane were provided by Sigma-Aldrich (Milan, Italy). Cortisol-d2 was purchased by C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). Acetic acid was purchased by Carlo Erba (Cornaredo, MI, Italy). Ultrapure water was obtained by a Milli-Q Millipore system (Bedford, MA, USA). Stock standard

solutions of analytes and internal standard (IS), cortisol d2, were prepared in MeOH at a concentration of 1 mg/ml and stored at −20°C in the dark. Working MeOH solutions containing all the analytes at different concentrations were prepared by mixing the stock solutions at the proper dilution. The working solutions were used to spike negative hair samples at various levels.

Hair Sampling

Two small strands of hair were collected from the posterior vertex as close as possible to the scalp, according to the *Society of Hair Testing* and literature recommendations (26). For the analysis, we used the most proximal 3 cm of hair, corresponding to the most recent 3 months.

Analytical Method

All analyses were performed on a Shimadzu Nexera 30 UPLC-system (Shimadzu, Duisburg, Germany) interfaced to an AB Sciex API 5500 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) with an electrospray Turbo Ion source operating in the negative (ESI–) ion mode. An Acquity UPLC BEH C18 column 100 mm × 2.1 mm, 1.7 µm (Waters Corporation, Milan, Italy), protected by a C18 VanGuard pre-column, was used for the separation of the target analytes. The column oven was maintained at +40°C, and the elution solvents used were aqueous 0.1% aqueous acetic acid (solvent A) and ACN acidified with 0.1% of acetic acid (solvent B). The mobile phase was eluted under the following conditions (a/b; v/v): initial 90:10 ratio for 1 min, linear gradient to 55:45 in 6 min, linear gradient to 10:90 in 0.5 min, and final isocratic condition at 90% B for 0.5 min. The flow rate was 0.4 ml/min, and total run time was 8 min, plus 4 min of re-equilibration time. The MS system was operated in the selected reaction monitoring mode (SRM). In order to establish appropriate SRM conditions, each analyte was individually infused into the ESI capillary, while the declustering potential (DP) and the entrance potential (EP) were adjusted to maximize the intensity of the parent ions, the adducts of the corticosteroid molecules with acetic acid $[M+CH3COO]^-$ species. The collision offset voltage (CE) was adjusted to preserve approximately 10% of the precursor ion, and the cell exit potentials (CXP) were also optimized. The best results were obtained using a source block temperature of +500°C and an ion-spray voltage of -4.500 V. Both Q1 and Q3 were operated at unit mass resolution. Nitrogen was employed as the collision gas. The gas settings were as follows: curtain gas 35.0 psi, collision gas 8.0 psi, ion source gas GS1 40.0 psi, and ion source gas GS2 40.0 psi. Analyst 1.5.2 (AB Sciex) software was used for data processing. The chemical structures and biochemical interactions of the glucocorticoids investigated in this study are reported in **Figure 1**. All analytes and IS and their corresponding retention time, SRM transitions, and potentials are presented in **Table 1**.

Sample Preparation

About 200 mg of hair was twice-washed, once with dichloromethane and once with methanol (1.5 ml, vortex mixed for 3 min). After complete removal of solvent wash, the hair was dried at room temperature by a gentle nitrogen flow and subsequently cut with scissors into 1–2-mm segments. An aliquot of about 50 mg was weighted and then fortified with 25 µl of an IS working solution at 10 ng/ml, yielding a final concentration of 5 pg/mg. Sample extraction was carried out by addition of 1 ml of methanol, vortex shaking for 5 min, and centrifuging at 4,000 rpm for 3 min, to ensure the complete immersion of the matrix into the solvent, and final incubation at

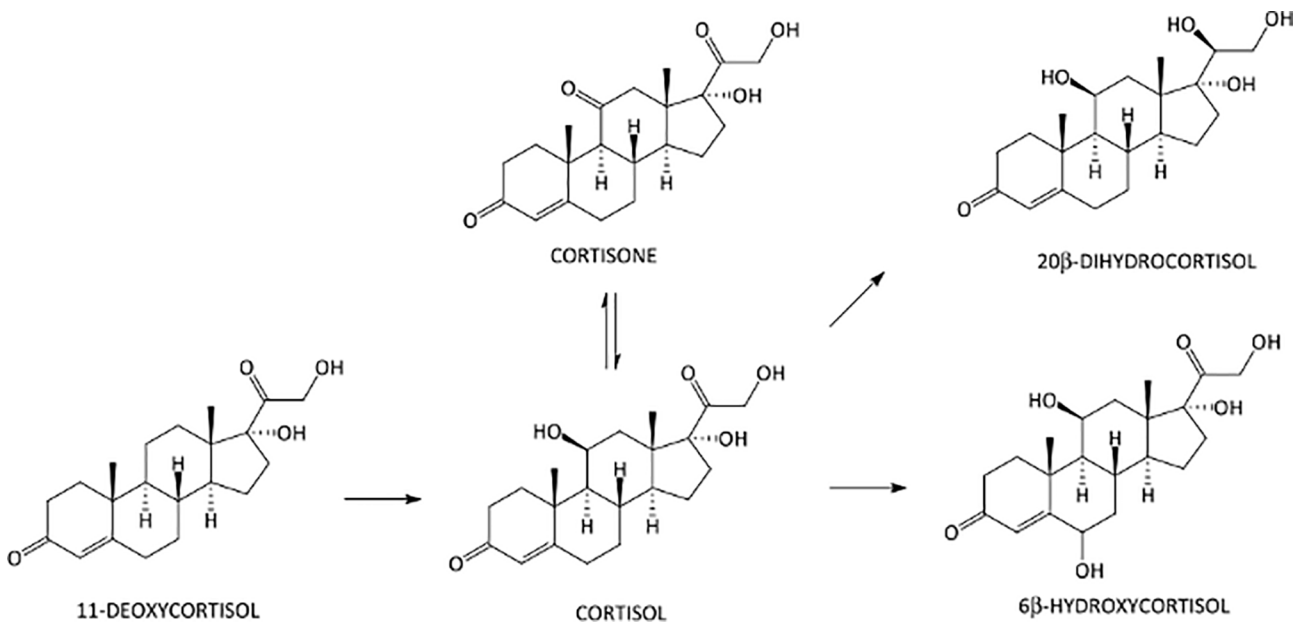

FIGURE 1 | Chemical structures of the steroids that were measured in the hair.

TABLE 1 | Mass spectrometry settings for the target compounds and the internal standard Cortisol-d2.

Analyte	t_r (min)	Precursor ion	DP (V)	EP (V)	Target fragment	CE (V)	CXP (V)	Qualifier fragment	CE (V)	CXP (V)
6β-Hydroxycortisol	2.96	437.2	-45	-12	347.3	-23	-13	313.4	-45	-10
20β-Dihydrocortisol	5.32	423.2	-56	-13	333.3	-35	-12	363.5	-35	-12
Cortisol	5.79	421.1	-57	-3	330.9	-24	-10	296.6	-43	-10
Cortisone	5.83	419.2	-58	-7	328.9	-25	-10	300.8	-30	-12
11-Deoxycortisol	6.84	405.3	-46	-12	315.3	-24	-13	345.4	-12	-13
Cortisol-d2	5.76	423.1	-69	-13	332.9	-38	-13	–	–	–

The values of the declustering potential (DP), exit potential (EP), collision offset voltage (CE), and cell exit potential (CXP) are reported.

55°C for 15 h. Lastly, 100 µl of the organic phase was collected and evaporated to dryness under a gentle stream of nitrogen and mild heating (25°C) using a Techne Sample Concentrator (Barloworld Scientific, Stone, UK). The residue was dissolved in 100 µl of mixture of the initial mobile phase of the following chromatographic run, transferred into a vial, and centrifuged at 4,000 rpm for 10 min. 10 µl of solution was injected into the UPLC–MS/MS system.

Validation

The analytical method was validated in accordance with the criteria and recommendations of international guidelines (27). The following parameters were investigated: specificity, selectivity, calibration range and model, detection and quantification limits (LOD and LOQ), and intra-assay and inter-assay precision and accuracy. Carryover and matrix effect were also investigated. Details about the validation procedure can be found elsewhere (28, 29). All the validation parameters resulted within the ranges of acceptability. In particular, lack-of-fit test and Mandel's test confirmed (95% confidence) the linearity of the calibration models for all steroids over the calibration range. Accuracy was verified at low, intermediate, and high concentrations, with bias% values largely below 15%. Intra-assay and inter-assay precision tests yielded coefficients of variation (CV%) below 10%. Calibration range, LOD and LOQ values, inter-day trueness, and inter-day precision are reported in **Table 2**.

Statistical Analysis

The occurrence of bias due to age or sex was initially investigated in the samples from healthy individuals. The Spearman correlation coefficient was computed on the levels of corticosteroids from individuals of the same sex along the sampled age range (20–83 years). The sex bias was investigated using the analysis of variance (ANOVA), and a significance threshold of 0.05 was chosen. The analysis demonstrated the absence of bias for these parameters.

The concentrations of the compounds in healthy individuals and patients with adrenal incidentaloma were compared using the Mann–Whitney or Kruskal–Wallis test as appropriate.

The group sizes (81 healthy subjects and 67 AI patients) achieved about 83% power to detect a difference in hair cortisol of at least 2.5 pg/mg (2.5 in healthy patients vs. 5 in AI patients) using a two-sided Mann–Whitney U or Wilcoxon rank-sum test when the significance level (alpha) of the test was 0.050 and the standard deviation was 5.0 in both groups.

The statistical calculations used in the analytical method validation were made by an *ad-hoc* Excel® sheet built in-house to adapt a published R-routine. The details are reported elsewhere (29).

RESULTS

We collected data from 81 healthy subjects and 67 patients with AI. The baseline characteristics are reported in **Table 3**.

No significant statistical difference was found in the hair concentrations of cortisol, 20β-dihydrocortisol, and cortisone between controls and patients (median 3.40, IQR 1.80–6.88, vs. 2.72, 1.24–6.78, pg/mg; median 1.55, 0.51–2.63, vs. 1.63, 0.98–3.36, pg/mg; median 13.21, 9.94–22.00, vs. 10.53, 6.43–22.91, pg/mg, respectively) (**Figure 2**). 6β-Hydroxycortisol and 11-deoxycortisol were undetectable in all samples.

TABLE 2 | Method's validation parameters, including calibration range, adjusted R^2, limit of detection (LOD), limit of quantitation (LOQ), inter-day trueness (bias %), and inter-day precision (CV%).

Analyte	Calibration range (ng/g)	Determination coefficient (adj R^2)	LOD (ng/g)	LOQ (ng/g)	Bias % 0.5 ng/g[a]	Bias % 5 ng/g[a]	Bias % 25 ng/g[a]	CV % 0.5 ng/g	CV % 5 ng/g	CV % 25 ng/g
6β-Hydroxycortisol	0.5–50	0.9990	0.2	0.5	93.3%	100.8%	95.3%	9.1%	6.4%	6.0%
20β-Dihydrocortisol	0.5–50	0.9963	0.2	0.7	103.0%	102.2%	97.0%	7.0%	8.6%	5.1%
Cortisol	0.5–50	1.0000	0.1	0.4	100.2%	104.6%	97.6%	9.0%	6.5%	5.2%
Cortisone	0.5–50	0.9996	0.1	0.3	98.4%	103.9%	98,3%	9.6%	6.8%	6.4%
					5 ng/g	25 ng/g	50 ng/g	5 ng/g	25 ng/g	50 ng/g
11-Deoxycortisol	2–50	0.9931	0.9	2.7	102.9%	104.5%	97.0%	7.2%	7.4%	5.6%

Inter-day trueness and precision were calculated from n. 30 repeated analyses for each concentration level executed during a period of 27 days.
[a]*Coincidence with the expected value corresponds to 100%.*

TABLE 3 | Baseline characteristics of healthy subjects and patients with AI.

Characteristics	Healthy controls N° 81	AI patients N° 67	*p* value
Gender			0.10
Male, *N* (%)	48 *(59.3)*	30 *(44.8)*	
Female, *N* (%)	33 *(40.7)*	37 *(55.2)*	
Age, year			0.22
Median (IQR)	64 (58–71)	68 (61–74)	
BMI, kg/m^2			/
Median (IQR)	/	27.1 (25.6–31.0)	
Waist, cm			/
Median (IQR)	/	102 (90.5–109.5)	
Cortisol after 1 mg-DST, nmol/L			/
Median (IQR)	/	49 (35–64)	
24-h UFC, nmol/24 h			/
Median (IQR)	/	185 (148–257)	
ULN median (IQR)	/	0.8 (0.7–1.2)	
MSC, nmol/L			/
Median (IQR)	/	11 (7–13)	
ULN median (IQR)	/	1.3 (0.8–1.6)	
ACTH, pmol/L			/
Median (IQR)	/	3.5 (2.4–5.4)	
Adrenal tumor size, mm			/
Median (IQR)	/	24 (18.5–30)	
Hair cortisol levels, pg/mg			0.26
Median (IQR)	3.40 (1.80–6.88)	2.72 (1.24–6.78)	
Hair cortisone levels, pg/mg			0.25
Median (IQR)	13.21 (9.94–22.00)	10.53 (6.43–22.91)	
Hair 20β-dihydrocortisol levels, pg/mg			0.39
Median (IQR)	1.55 (0.51–2.63)	1.63 (0.98–3.36)	
Ratio hair cortisol/cortisone levels			0.18
Median (IQR)	0.26 (0.18–0.36)	0.22 (0.14–0.34)	

AI, adrenal incidentalomas; N, number of patients; IQR, interquartile range; BMI, body mass index; DST, dexamethasone suppression test; UFC, urinary free cortisol; MSC, midnight salivary cortisol; ACTH, adrenocorticotropic hormone.

Among the AI patients, 35 had cortisol values ≤ 50 nmol/l (excluding cortisol autonomy), while the remainders had biochemical evidence of cortisol autonomy, either ACS (n = 3) or PACS (n = 29). The baseline characteristics of the two groups are reported in **Table 4**.

Hair cortisol, 20β-dihydrocortisol, and cortisone concentrations were similar in AI patients with serum cortisol after 1 mg-DST ≤ 50 nmol/l and AI patients with values > 50 nmol/l, as shown in **Table 4**. None of the three compounds in the keratin matrix showed a correlation with serum cortisol after 1 mg DST, 24-h UFC, plasma ACTH, or MSC (**Figure 3**).

Moreover, there was no correlation between cortisol and cortisone concentrations in the keratin matrix and BMI and no difference among normal weight, overweight, or obese patients (**Figure 4**).

DISCUSSION

This is the first study that has assessed specifically the value of hair glucocorticoid measurement as a possible diagnostic tool in patients with AI. This clinical setting is of particular interest due to the challenge in the identification of a mild cortisol excess, in a large number of patients who are indistinguishable from the general population, lacking a typical phenotype. Worthy of note is that all tests currently used for the diagnosis of cortisol excess (1 mg DST, 24-h UFC, MSC) reflect only instant or short-term secretion and cannot measure the chronic exposure to cortisol excess. For these reasons, the use of hair glucocorticoid measurement is appealing as a diagnostic test in these patients, arguing that a measure of chronic accumulation of cortisol in a tissue may improve the recognition of a minimal excess rather than using a point-like determination.

In our study, however, we did not find any difference in cortisol, cortisone, and 20β-dihydrocortisol hair concentrations between healthy controls and AI patients. Moreover, no significant difference was found in cortisol, cortisone, and 20β-dihydrocortisol levels of AI patients with or without cortisol autonomy (either ACS or PACS).

To date, several studies demonstrated a good performance of hair cortisol measurements to identify overt Cushing syndrome. Some studies conducted on a small number of patients found that hair cortisol levels were higher in patients with overt Cushing syndrome compared with healthy controls (19–21). In 2017, Wester et al. confirmed the diagnostic utility of hair cortisol in 43 patients with overt Cushing syndrome (26 with Cushing disease, 10 with adrenal Cushing, and 7 with ectopic ACTH secretion), reporting a sensitivity and a specificity of hair cortisol measurements for CS of 93% and 90%, respectively (22). In 2019, Savas et al. for the first time applied the LC-MS/MS technique for the measurements of hair cortisol and hair cortisone in a large cohort of 89 patients with endogenous

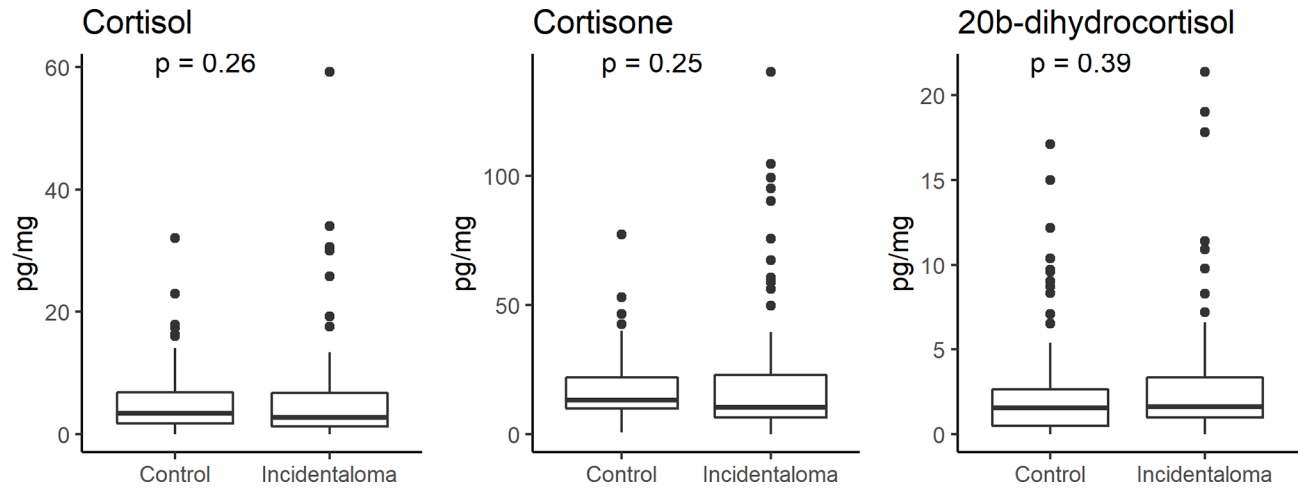

FIGURE 2 | Comparison of hair cortisol, cortisone, and 20β-dihydrocortisol levels between healthy controls and patients with adrenal incidentaloma showing the lack of any significant difference.

TABLE 4 | Comparison of baseline characteristics of AI patients, according to the presence of cortisol autonomy.

Characteristics	AI patients without cortisol autonomy N° 35	AI patients with cortisol autonomy N° 32	*p* value
Sex			0.74
Male, *N* (%)	15 *(42.9)*	15 *(46.9)*	
Female, *N* (%)	20 *(57.1)*	17 *(53.1)*	
Age, year			**<0.001**
Median (IQR)	64 (58–70)	70.5 (68–75)	
BMI, kg/m^2			
Median (IQR)	27.1 (25.9–30.3)	27.5 (25.4–31.4)	0.81
Normal	5 *(14.3)*	7 *(21.9)*	
Overweight	20 *(57.1)*	12 *(37.5)*	
Obese	10 *(28.6)*	13 *(40.6)*	
Waist, cm			0.14
Median (IQR)	100 (89.5–105)	104.5 (92–115)	
Cortisol after 1 mg-DST, nmol/L			**<0.01**
Median (IQR)	35 (28–41)	69 (59–91)	
24-h UFC, nmol/24 h			0.66
Median (IQR)	188 (145–248)	185 (158–268)	
ULN median (IQR)	0.9 (0.7–1.1)	0.8 (0.7–1.2)	
MSC, nmol/L			0.62
Median (IQR)	10 (7–14)	11 (7–13)	
ULN median (IQR)	1.3 (0.8–1.6)	1.3 (0.9–1.6)	
ACTH, pmol/L			**0.03**
Median (IQR)	3.9 (3.2–5.7)	3.0 (1.8–4.3)	
Adrenal tumor size, mm			**<0.01**
Median (IQR)	20 (16–24)	30 (24–36)	
Hair cortisol levels, pg/mg			0.51
Median (IQR)	2.33 (1.16–6.54)	2.97 (1.38–7.40)	
Hair cortisone levels, pg/mg			0.86
Median (IQR)	11.47 (6.43–24.73)	9.97 (6.55–22.53)	
Hair 20β-dihydrocortisol levels, pg/mg			0.15
Median (IQR)	1.35 (0.69–2.90)	1.97 (1.20–3.81)	
Ratio hair cortisol/cortisone levels			0.21
Median (IQR)	0.22 (0.10–0.32)	0.22 (0.17–0.40)	

AI, adrenal incidentalomas; N, number of patients; IQR, interquartile range; BMI, body mass index; DST, dexamethasone suppression test; UFC, urinary free cortisol; MSC, midnight salivary cortisol; ACTH, adrenocorticotropic hormone.
Bold characters indicate statistically significant difference.

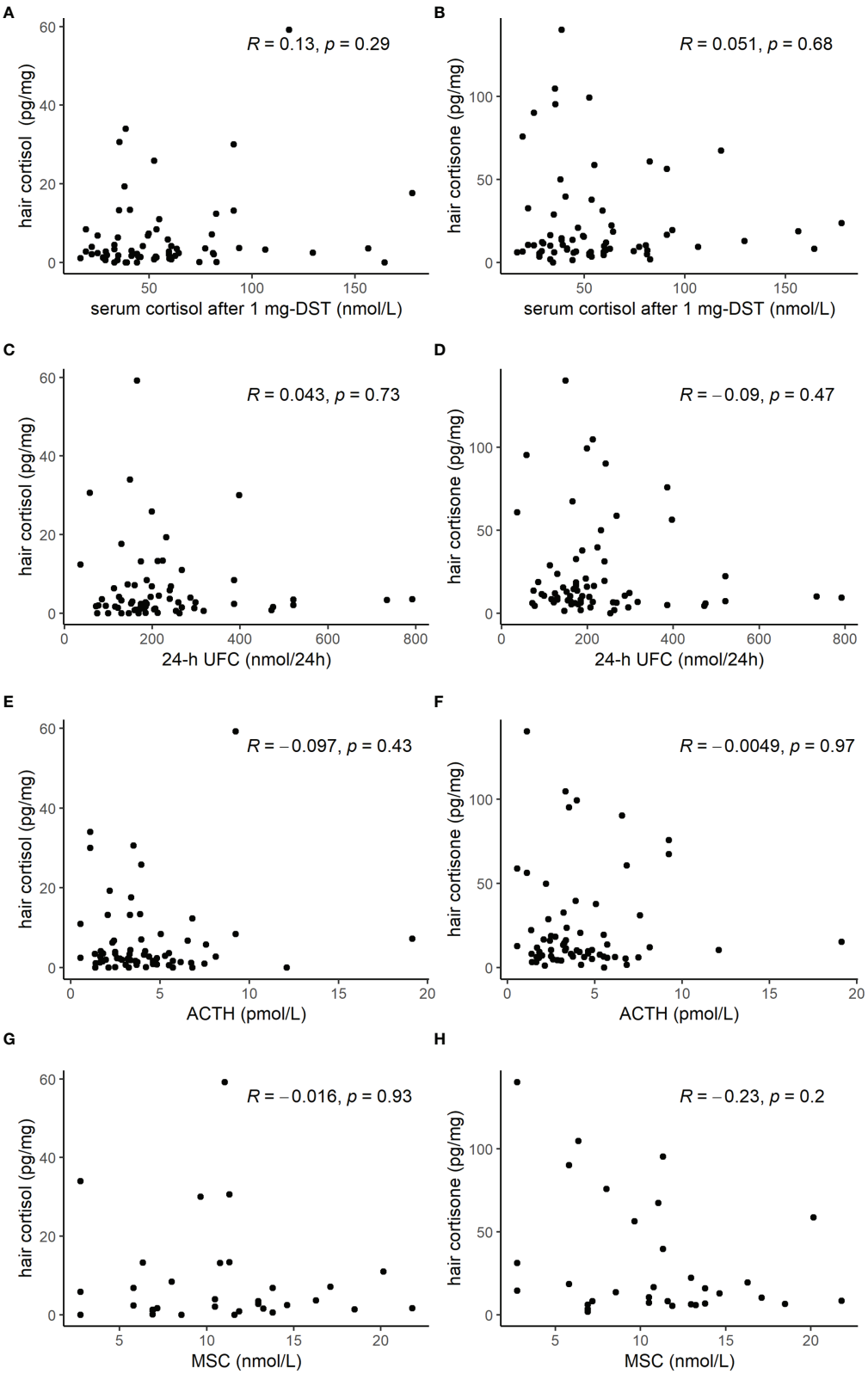

FIGURE 3 | Scatterplot of hormone tests [cortisol after 1 mg dexamethasone suppression test (DST), 24-h urinary free cortisol (UFC), adrenocorticotropic hormone (ACTH), and midnight salivary cortisol (MSC)] and hair cortisol **(A, C, E, G)** or hair cortisone **(B, D, F, H)** levels, showing the lack of any significant correlation.

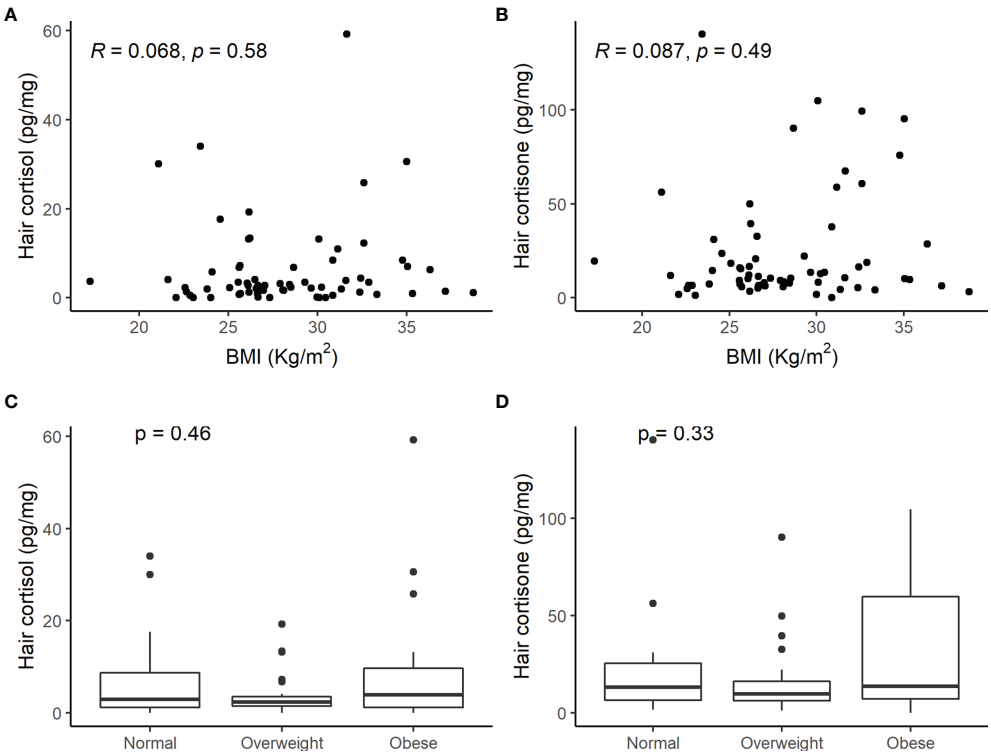

FIGURE 4 | Scatterplot of hair cortisol **(A)** and hair cortisone **(B)** levels and body mass index (BMI) and comparison of hair cortisol **(C)** and cortisone **(D)** concentrations in normal weight, overweight, or obese patients with adrenal incidentaloma. No significant correlation between hair steroids and weight was observed.

Cushing syndrome, compared to 295 controls (23). Although both glucocorticoids were significantly higher in Cushing patients than in controls, hair cortisone was even most accurate in differentiating Cushing patients from healthy subjects (87% sensitivity, 90% specificity, 96% negative predictive value) than hair cortisol (81% sensitivity, 88% specificity, 94% negative predictive value) (23).

Conversely, literature on hair glucocorticoid measurement in patients with AI and cortisol autonomy is very limited. Only a study reported data on 23 patients affected by mild Cushing with 24 overt Cushing and 84 healthy subjects (24). Brossaud and colleagues reported that in patients with mild Cushing, the hair cortisol and cortisone levels were lower than in overt Cushing, but higher than in healthy controls. However, only 8 out of 23 patients with mild Cushing had a unilateral adrenocortical incidentaloma and 6 bilateral macronodular adrenal hyperplasia (BMAH), while the remainders had ACTH-dependent Cushing syndrome (24).

A possible explanation for the lack of any significant difference in hair glucocorticoids levels in our study may rely on our inadvertent inclusion of patients with a low level of cortisol secretion, since most patients had normal levels of 24-h UFC and plasma ACTH was suppressed only in few patients. Brossaud and colleagues included patients in which mild Cushing was defined as serum cortisol > 50 nmol/l after 1 mg DST and at least one of the following endocrine alterations: mildly increased UFC (≤1.5 ULN), suppressed plasma ACTH levels (< 10 pg/ml) in patients with adrenal tumours, late-night salivary cortisol or midnight serum cortisol. Another hallmark which shows that in our study the degree of cortisol autonomy in the group of AI was rather limited is that the median cortisol after 1 mg dexamethasone suppression test was of 2.5 μg/dl. However, our cohort is representative of the current series of patients with AI, with only a minimal degree of hypercortisolism (5). It may be speculated that a higher amount of cortisol excess is needed to have a significant accumulation in hair.

Interestingly, in our cohort hair cortisone levels were higher than those of cortisol, similarly to what was found in saliva (30). This finding is in line with previous studies and may be explained with the presence in the scalp of the 11β-hydroxysteroid dehydrogenase type 2 enzyme (23, 24).

The strengths of this study are the detailed characterization of the patients and the use of the UPLC-MS/MS method, which offers high specificity and accuracy together with the possibility to simultaneously analyze several target analytes. These analytical features overcome the limitations typical of immunoassay, including cross-reactivity and single-component determination (26, 31). Moreover, the high sensitivity provided by the UPLC-MS/MS method allows to conduct the analysis on small amounts of hair, which are easily collected and stored at room temperature (31).

However, we have to underline that the measurement of hair glucocorticoid levels entails pre-analytical issues. It has been

demonstrated that shampoo use and hair dying can interfere with the measurement, while differences in race and age can influence cortisol incorporation in the scalp (32, 33), potentially increasing the interindividual variability and affecting the diagnostic performance of this tool.

Moreover, we should acknowledge the limits of the retrospective nature of our study and of a rather limited sample size. Although the monocentric nature of the study avoided bias due to inter-laboratory variability, it may limit the validity of our results to a specific population. Therefore, we are aware that only multicentric and prospective studies on a larger AI cohort can provide definitive results.

Despite these limits, our study provides useful evidence for the practical management of adrenal incidentalomas and assessment of cortisol autonomy.

CONCLUSION

This is the first study that focused on the diagnostic use of hair cortisol and cortisone measurement in patients with incidentally discovered adrenal masses given that this is an appealing, easy-to-perform and non-invasive tool.

Our findings do not support the use of hair glucocorticoid measurement as a diagnostic test for cortisol autonomy in patients with AI.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of San Luigi Gonzaga Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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Recent Advances in the Clinical Application of Adrenal Vein Sampling

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We reviewed clinical research investigating the applications of adrenal vein sampling (AVS). AVS could be applied not only to primary aldosteronism (PA) but also to other endocrine diseases, such as adrenocorticotropic hormone (ACTH) independent Cushing syndrome (AICS) and hyperandrogenemia (HA). However, the AVS protocol requires improvements to increase its success rate. Using the computed tomography image fusion, coaxial guidewire technique, and fast intraprocedural cortisol testing (CCF) technique could improve the success rate of catheterization in AVS for PA. ACTH loading could be considered in medical centers with a low selectivity of AVS for PA but is not essential in those with mature AVS technology. The continuous infusion method should be recommended for ACTH stimulation in AVS for PA to reduce adverse events. AVS has not been routinely recommended before management decisions in AICS, but several studies verified that AVS was useful in finding out the source of excess cortisol, especially for distinguishing unilateral from bilateral disease. However, it is necessary to reassess the results of AVS in AICS with the use of reference hormones to fully normalize cortisol levels. In addition, it is essential to determine the optimal model that combines AVS results and mass size to guide the selection of surgical plans, including identifying the dominant gland and presenting the option of staged adrenalectomy, to minimize the impact of bilateral resection. For HA, AVS combined with ovarian intravenous sampling to locate excess androgens could be considered when imaging results are equivocal.

Keywords: adrenal vein sampling (AVS), primary aldosteronism (PA), Cushing syndrome (CS), hyperandrogenism (HA), adrenal

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INTRODUCTION

Adrenal vein sampling (AVS) was first developed in the 1960s (1). The most important and extensive use of AVS was its use as the gold standard method for localizing the overproduction of aldosterone in primary aldosteronism (PA) (2). PA is the most common form of secondary hypertension. The sensitivity and specificity of AVS for detecting unilateral PA (UPA) were 95% and 100%, respectively (2, 3). Moreover, AVS could also be used for other endocrine disorders that are caused by excess secretion of cortisol or androgens from the adrenal gland. Adrenocorticotropic

hormone (ACTH) independent Cushing syndrome (AICS) is mostly caused by cortisol-producing adrenocortical tumors. However, in some cases, it is difficult to accurately localize the responsible focuses, especially in the presence of bilateral adrenal masses. AVS might be ideal for distinguishing between the responsible focuses that are causing excess secretion of cortisol. Although hyperandrogenism (HA) is a common endocrine disorder among women, at times, imaging fails to detect the source of overproduction of androgens. Consequently, AVS could be an attractive alternative option for the etiological diagnosis of HA. Theoretically, AVS could be used for pheochromocytoma, although to the best of our knowledge, there are currently no articles on this. Despite AVS being safe and effective, the success rate has varied, ranging from 8 to 95%, which has largely limited its wide application (4–6). Therefore, increasing the use of AVS requires improving the AVS protocol. Furthermore, the reference ranges are still controversial. In addition, the application of AVS in diseases other than PA is not yet known. Hence, in this review, we discussed in detail the latest improvements of AVS use in PA and briefly summarized the expanded application of AVS in AICS and HA, in order to help clinicians better understand the use of AVS in clinical practice. We searched all eligible original articles on PubMed with following keywords: (adrenal vein sampling [Title/Abstract]) AND (primary hyperaldosteronism [Title/Abstract]); (adrenal vein sampling [Title/Abstract]) AND Cushing syndrome [Title/Abstract]); (adrenal vein sampling [Title/Abstract]) AND hyperandrogenism [Title/Abstract]). There were no exclusion criteria in our review because the number of eligible studies was relatively small. The main results of representative studies are presented in **Table 1**.

PRIMARY ALDOSTERONISM

Primary aldosteronism (PA) is the most common cause of secondary hypertension. The prevalence of PA ranges from 3.2 to 12.7% in primary practice and from 1 to 30% in referral centers. This mainly depends on the degree of hypertension in the population being examined. Excess aldosterone is a strong risk factor for heart and kidney damage, independent of sex, age, and blood pressure. The prevalence of PA among patients with recently detected hypertension in China was at least 4%. It is critical to discriminate between the main subtypes to determine the correct therapeutic strategies, which is surgery for unilateral forms or medical therapy for bilateral forms (21–23). There has always been a degree of clinical difficulty in classifying the PA subtypes. Imaging often fails to visualize microadenomas and distinguish nonfunctioning incidentalomas from aldosterone-producing adenomas. The sensitivity and specificity of imaging were 78 and 75%, respectively (2). Clinicians needed to comprehensively analyze the biochemical indicators, imaging manifestations, and AVS results. Despite AVS being the gold standard method and having high sensitivity and specificity for the diagnosis of different primary aldosteronism subtypes, it has varied success rates. Therefore, it is paramount to improve the success rate of AVS.

In 2016, the Endocrine Society Clinical Practice Guideline recommended that for PA, adrenal computed tomography (CT) should be performed before AVS, by an immobilized experienced AVS angiographer, with sufficient time for the operation and with simultaneous bilateral adrenal vein cannulations, while limiting the use of contrast during the procedure to help minimize the failure risk and postoperative complications (2). Many studies focused on increasing the success rate of AVS, including successful right adrenal vein catheterization, the role of ACTH loading during AVS, and the evaluation index of the AVS results, which we have described below.

Correct Right Adrenal Vein Catheterization

It is well known that AVS failure is often owing to unsuccessful catheterization of the right adrenal vein (the right adrenal vein is usually shorter than the left and typically enters the inferior vena cava (IVC) at an acute angle). A recent study at our center found that the Computed tomography image fusion, Coaxial guidewire technique, Fast intraprocedural cortisol testing (CCF) technique significantly improved technical success rates and reduced procedure time, radiation exposure, and contrast medium volume (7). Previously, the only access for AVS was *via* the femoral vein, but according to the study of J Xu et al., the forearm vein may provide a complementary or alternative approach to catheterization. The success rate of AVS of the right and left adrenal vein was 93.8 and 100%, respectively (24). However, since only 48 patients were included in the study, the reliability of AVS access *via* the forearm vein remained unclear. Another viable method was using imaging to obtain an accurate visualization of the right adrenal vein, which was vital both before and during AVS. With the development of imaging technology, 3-dimensional reconstruction has frequently been applied in clinical settings. Using multi-detector CT (MDCT) with 3-dimensional reconstruction may reduce operation time and the quantity of contrast required and improve the success rate of catheterization. Previous studies have suggested that using Dyna CT, cone-beam CT during AVS could improve successful cannulation of the adrenal vein (25–27). Another study suggested that a 70-kilovoltage-peak (kVp) contrast-enhanced CT scan may provide better visualization and identification of the right adrenal vein (8). Thus, promoting the use of advanced CT techniques at medical centers may be necessary for visualizing the adrenal vein for accurate and successful cannulation.

The Role of ACTH Loading During AVS

At most medical centers, the medical staff did not possess the skills for simultaneous cannulation and as such, at about 40% of them the use of ACTH infusion during AVS is advocated for to overcome the limitations of non-synchronous catheterization (28, 29). A meta-analysis in 2018 showed that ACTH loading could significantly reduce the number of unsuccessful cannulations, without significantly increasing incorrect lateralization (9). However, a large retrospective study conducted by Tekada et al., in 2019 compared the two techniques (AVS with ACTH stimulation and AVS without

TABLE 1 | Summary of main studies involving advances about application of AVS.

Authors, year	Population	Results	Conclusions	Limitations
Primary Aldosteronism (PA)				
Correct right adrenal vein catheterization				
Liu (7)	105 Patients with PA (51 in the AVS–CCF group; 54 in the AVS group)	The technical success rate was higher for AVS–CCF than for AVS without CCF (98 vs. 83.3% for bilateral adrenal veins, P = 0.016).	The CCF technique during AVS not only contributed to improved technical success rates but also associated with decreased procedure time, radiation exposure, and contrast medium volume.	The AVS–CCF procedures were performed more recently than the AVS without CCF procedures.
Maruyama (8)	90 Patients with PA (43 in the 120-kVp group; 47 in the 70-kVp group)	In comparison with the 120-kVp group, the 70-kVp group had significantly superior conspicuity scores for the RAV (P < 0.001), higher RAV detection rates (P = 0.015–P = 0.033), and lower size-specific dose estimates (P < 0.001).	70-kVp contrast-enhanced CT has advantages over conventional 120-kVp contrast-enhanced CT.	The single-center, retrospective design, and use of 2 different CT scanners and different reconstruction techniques.
The role of ACTH loading during AVS				
Laurent (9)	14 Studies comparing the 2 techniques (AVS with ACTH stimulation and AVS without ACTH stimulation in patient with PA)	AVS with ACTH stimulation significantly reduced the number of unsuccessful cannulations of both adrenal veins more than AVS without ACTH stimulation in patients with PA (OR: 0.26, 95% CI: 0.17, 0.40; P<0.00001).	AVS with ACTH stimulation can significantly reduce the number of unsuccessful cannulations, without significantly reducing the number of incorrect lateralization.	Variability in institutional protocols and shortage of expert interventional radiologists.
Takeda (10)	2197 Japanese patients with PA from 28 centers	ACTH loading during AVS improved the success rate from 67 to 89%, while lateralization indices decreased from 62 to 28%.	The use of ACTH during AVS was helpful for improving the success rate, but did not contribute to better outcomes.	The limitation of the retrospective study.
Hu (11)	174 Patients with PA (80 receiving ACTH bolus; 94 receiving ACTH infusions)	The LI and rate of complete biochemical remission (43/44, 97.7% vs 53/53, 100%, P=0.45) did not significantly differ between the two groups. The bolus group reported more transient AEs such as palpitation (52.9% vs 2.2%) and abdominal discomfort (40.0% vs 2.2%) than the infusion group.	Due to the similar effects on cannulation success and lateralization, but a lower rate of transient AEs in the infusion group, the continuous infusion method should be recommended for ACTH stimulation in AVS.	The adrenal and peripheral venous blood before ACTH administration were not collected.
The evaluation index of AVS results				
Li (12)	37 Patients with PA	SI ≥ 3 for androstenedione or DHEA provided optimal sensitivity and specificity in AVS. Given the much larger AV/PV ratios and reduced variability compared to cortisol, the adrenal androgens are useful for assessing the selectivity of AVS without cosyntropin stimulation.	The adrenal androgens may be superior analytes in conditions with marked variability of cortisol levels or with adrenocortical tumors consecrating cortisol and aldosterone.	The sample size was small and the study did not compare the lateralization indices.
Dekkers (13)	86 Patients with PA (52 in the cosyntropin-stimulated group; 34 in the nonstimulated AVS group)	The adrenal to peripheral vein ratio of metanephrine was 6-fold higher than that of cortisol (94.0 versus 15.5; P<0.0001). ROC analysis indicated a plasma metanephrine SI cutoff of 12.	Metanephrine provides a superior analyte compared with cortisol in assessing the selectivity of adrenal vein sampling during procedures without cosyntropin stimulation.	The sample size was small and the study did not compare the lateralization indices.
Wolley (14)	80 Patients with PA	The degree of contralateral suppression was independently and significantly correlated with postoperative SBP.	Contralateral suppression should be a factor in deciding whether to offer surgery for treatment of PA.	Patients without contralateral suppression were a relatively selected group.
Adrenocorticotropic hormone independent Cushing's syndrome (AICS)				
Chen (15)	a Case of woman with ACTH-independent ectopic CS	Adrenal CT scan indicated no abnormality. A mass was discovered by pelvic ultrasonography. Combined ovarian and adrenal venous sampling together with a cortisol assay were conducted. Results revealed a right-side ovarian origin.	Combined ovarian and adrenal venous sampling is valuable in the localization of ACTH-independent ectopic CS.	The sample size.
Maghrabi (16)	a Patient with subclinical CS and AIMAH	AVS results were consistent with bilateral autonomous cortisol hypersecretion without lateralization. A left adrenalectomy was performed. The patient improved clinically after the surgery.	AVS is a useful diagnostic tool that helps localize the source of autonomous cortisol hypersecretion in ACTH-independent subclinical CS with bilateral adrenal masses.	The sample size.
Gu (17)	a Patient with CS and BAAs	AVS results were consistent with bilateral autonomous cortisol hypersecretion without lateralization. A left adrenalectomy was performed, followed by resection of the right-side adrenal mass.	AVS is of great significance for obtaining information on the functional state of BAAs before surgery.	The sample size.

(Continued)

TABLE 1 | Continued

Authors, year	Population	Results	Conclusions	Limitations
Raje (18)	6 Patients with CS (3 with bilateral adrenal enlargement or nodules; 3 with unilateral nodules)	AVS results aided management planning in five patients, definitively changing treatment from surgery to medical management in one patient.	AVS offered useful information for determining appropriate management of adrenal CS.	The sample size.
Hyperandrogenism (HA)				
Tng (19)	3 Studies including women with HA who underwent catherization	The summary sensitivity of the dexamethasone suppression test is 100% and that for selective venous sampling is 100%. The summary specificity of the dexamethasone suppression test is 89.2% and that for selective venous sampling is 100%.	There is limited evidence for the use of selective venous sampling in identifying virilizing tumors in postmenopausal hyperandrogenism.	Poor methodological quality.
Kaltsas (20)	38 Patients who underwent ovarian and adrenal venous catheterization and sampling for investigation of HA	The overall catheterization success rate was: all four veins in 27%, three veins in 65%, two veins in 87%. The success rate for each individual vein was: right adrenal vein 50%, right ovarian vein 42%, left adrenal vein 87% and left ovarian vein 73%.	Venous catheterization and sampling should be considered only for patients in whom uncertainty remains.	The low successful catherization rate.

AVS, adrenal vein sampling; PA, primary aldosteronism; CCF, computed tomography image fusion, coaxial guidewire technique, fast intraprocedural cortisol testing technique; RAV, right adrenal vein; CT, computerized tomography; ACTH, adrenocorticotropic hormone; AE, adverse event; LI, lateralization index; CI, confidence interval; SI, selective index; ROC, receiver operator characteristic; DHEA, dehydroepiandrosterone; AV, adrenal vein; PV, peripheral vein; SBP, systolic blood pressure; CS, Cushing syndrome; AIMAH, ACTH-independent macronodular adrenal hyperplasia; BAAs, bilateral adrenocortical adenomas; HA, hyperandrogenism.

ACTH stimulation among 2197 patients with PA from 28 centers in Japan and found that the use of ACTH loading during AVS increased the success rate from 67 to 89%, while decreasing the lateralization rate from 62 to 28% (10). Consequently, ACTH loading could be considered in medical centers with a low selectivity of AVS, but it is not essential in those with mature AVS technology. In addition, a recent study found that the continuous infusion method should be recommended for ACTH stimulation in AVS, due to the similar effects on cannulation success and lateralization, but a lower rate of transient adverse events among patients in the infusion group (11).

The Evaluation Index of AVS Results

The evaluation of AVS results comprised three indicators, including the selectivity index (SI), lateralization index (LI), and contralateral suppression index (CSI). In 2020, the European Society of Hypertension recommended that the SI of>2 for unstimulated and>5 for stimulated procedures be used to demonstrate correct cannulation of the adrenal veins. The LI of>4 for both unstimulated and stimulated procedures was considered unilateral PA. The CSI of<1 may indicate unilateral PA on the opposite side (22). However, criteria for AVS interpretation may vary between centers, owing to the large heterogeneity in AVS procedures and hormone measurements. Notably, although cortisol-based SI is currently a widely used indicator to evaluate the success of AVS, the secretion of cortisol varies (28, 30, 31). Hence cortisol-based SI may not be the best indicator. With the higher adrenal vein (AV)/peripheral vein (PV) ratio of adrenal androgens, and more importantly, with the lower variability of adrenal androgens than cortisol, adrenal androgen-based SI may be more useful than cortisol-based SI for assessing the selectivity of non-ACTH stimulated AVS. It has also been demonstrated that adrenal androgen-based SI may be superior to cortisol-based SI in adrenal masses producing both aldosterone and cortisol (12). The study also found that the androgen-based SI of≥3 was an optimal cut-off point for assessing the selectivity of AVS. In comparison to catecholamines, there is relatively little increase in metanephrines in response to stress. Furthermore, compared with cortisol, metanephrines provided a superior analyte in assessing the selectivity of AVS, with a SI cut-off point of 12 (13). Although the LI was widely used to determine whether there was dominant secretion of aldosterone, CSI may be a good substitution for some cases where catheterization on the dominant side has been unsuccessful. A retrospective study conducted by Wolley et al., in 2015 showed that the CSI of<1 correlated with positive blood pressure and biochemical outcomes following surgery (14).

ADRENOCORTICOTROPIC HORMONE INDEPENDENT CUSHING SYNDROME

Endogenous Cushing syndrome (CS) is a rare and severe disease with an annual incidence of 0.2 to 5.0 per million and a prevalence of 39 to 79 per million (32). CS has a high mortality rate, with a standard mortality rate (SMR) of approximately 2.0 to 4.0; cardiovascular disease is the most common cause of CS-related death (32). Adrenocorticotropic hormone independent CS (AICS) accounted for about 20% of CS cases, including unilateral adrenal adenoma or carcinoma, bilateral macronodular adrenal hyperplasia, bilateral micronodular adrenal hyperplasia, primary pigmented nodular adrenocortical disease, McCune-Albright syndrome, and bilateral adrenal adenomas or carcinomas. Among patients with AICS with bilateral adrenal masses, it is often difficult to determine the source of excess cortisol, which largely affected surgical planning. However, the source of excess cortisol in AICS could be localized using AVS. Multiple case

reports have suggested that AVS may be used to successfully localize excess cortisol in patients with AICS (16, 33–37). In the case of a 40-year-old woman from China with a large pelvic mass, AVS also aided localization of ectopic AICS (15).

The interpretation of AVS results in AICS was controversial. Young et al., stated that epinephrine concentrations exceeding that of PV concentrations by more than 100 pg/mL indicates successful catheterization, an AV/PV cortisol gradient of>6.5 indicates cortisol-secreting adrenal adenoma, and a high-side to low-side AV cortisol lateralization ratio of≥2.3 is consistent with autonomous cortisol secretion from predominantly one adrenal gland (38). A case report showed that a patient with subclinical CS and ACTH-independent macronodular adrenal hyperplasia (AIMAH) underwent AVS and the result indicated no lateralization using criteria stated by Young et al. A left adrenalectomy was performed, and the patient improved clinically after the surgery (the left mass was larger than the right mass) (16). However, in another case with bilateral adrenocortical adenomas (BAAs) and bilateral autonomous cortisol secretion without laterality, the patient required left adrenalectomy followed by the resection of the right-side adrenal mass to gain clinical recovery (17). In both cases with similar AVS results, distinct management was necessary to cure CS, which posed challenges in the interpretation of AVS results, particularly in the question of how to choose the reference hormone to calculate the cortisol to reference hormone ratios from the right and left adrenal veins and finally calculate the lateralization index in non-synchronous catheterization approaches, due to the rapid fluctuation in adrenal hormones during the AVS procedure. In 2018, Wei Jie el at., reported that aldosterone concentrations could be used as reference hormones to calculate the lateralization index (36). However, many factors can interfere with aldosterone concentrations. In addition, aldosterone has a shorter half-life (20 min) than cortisol (60–70 min) that may interfere with the interpretation of AVS findings. Therefore, aldosterone as a reference hormone still requires further validation (33). New criteria stated that a ratio of >12 for metanephrine was considered as correct cannulation. The LI of≥2 was interpreted as unilateral disease, using aldosterone, adrenaline, noradrenaline, and dehydroepiandrosterone sulfate as references (18, 39). Because universal criteria for the evaluation of laterality among patients with hypercortisolism in multi-centers have not yet been established, more multi-center studies are required. Moreover, in clinical practice in the presence of AIMAH, the excision of the larger mass is suggested in order to avoid bilateral adrenalectomy. Thus, it is essential to determine the optimal model that combines AVS results and mass size to guide the selection of surgical plans, to minimize the impact of bilateral resection.

HYPERANDROGENISM

Hyperandrogenism (HA) is a common endocrine disorder among women of a reproductive age, with a prevalence of approximately 5-10% (40). Causes of HA included endogenous neoplasms, non-neoplastic androgen overproduction, and exogenous pharmacologic agents. Endogenous neoplasms included adrenocortical adenomas or carcinomas, Sertoli-Leydig cell tumors, hilus cell tumors, teratomas, pituitary adenomas, and others. Adrenal and ovary vein sampling could be considered to locate the source of excess androgen. However, there were only limited reports from studies with small sample size (19, 41–51). A study involving 38 patients with HA showed that the successful catheterization rate of the four veins (bilateral AVs and ovary veins (OVs)) was 27%, and the failure rate of OV catheterization was higher than AV catheterization. OV/PV ratio of estradiol of >2 was considered as successful cannulation of the OV; AV/PV ratio of cortisol of>2 was considered as successful cannulation of the AV; A ratio of testosterone >2 was considered as androgen overproduction (20). In this study, no complications were observed. However, the low success rate of catheterization limited the application of venous sampling in HA, thus it may be only considered when imaging results are equivocal.

CONCLUSIONS

Conclusions from our review are presented in a summary figure (**Figure 1**). AVS could be applied not only to PA but also to other endocrine diseases, such as AICS and HA. However, the AVS protocol requires improvements to increase its success rate. Using the CCF technique could improve the success rate of catheterization

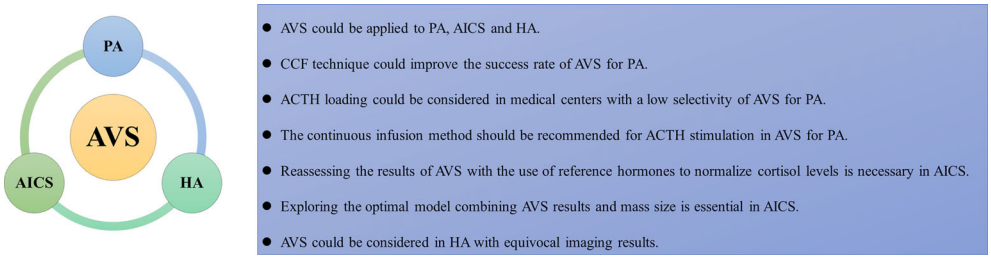

FIGURE 1 | The summarizing figure of the review. AVS, adrenal vein sampling; AICS, adrenocorticotropic hormone independent Cushing syndrome; HA, hyperandrogenism; CCF, computed tomography image fusion, coaxial guidewire technique, fast intraprocedural cortisol testing technique; ACTH, adrenocorticotropic hormone.

in AVS for PA. ACTH loading could be considered in medical centers with a low selectivity of AVS for PA but is not essential in those with mature AVS technology. The continuous infusion method should be recommended for ACTH stimulation in AVS for PA to reduce adverse events. AVS hasn't been routinely recommended before management decisions in AICS, but several studies verified that AVS was useful to find out the source of excess cortisol, especially for distinguishing unilateral from bilateral disease. However, it is necessary to reassess the results of AVS in AICS with the use of reference hormones to fully normalize cortisol levels. In addition, it is essential to determine the optimal model that combines AVS results and mass size to guide the selection of surgical plans, including identifying the dominant gland and presenting the option of staged adrenalectomy to minimize the impact of bilateral resection. For HA, AVS combined with ovarian intravenous sampling to locate excess androgens could be considered when imaging results are equivocal. This study had some limitations. First, since this review only explored the PubMed database and only included articles written in English, some articles may have been missed. Second, most studies included had limited sample sizes. Hence, more research should be conducted to improve the understanding of the clinical application of AVS in endocrine diseases.

AUTHOR CONTRIBUTIONS

XS and XX came up with the idea. SZ and TZ wrote the main text. MH, HY, ZheL, and ZhoL collected information. All authors were involved in drafting the manuscript, and have read and approved the final version.

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REVIEW
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Pathogenesis of Primary Aldosteronism: Impact on Clinical Outcome

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Primary aldosteronism (PA) is the most common form of secondary arterial hypertension, with a prevalence of approximately 20% in patients with resistant hypertension. In the last decade, somatic pathogenic variants in *KCNJ5*, *CACNA1D, ATP1A1* and *ATP2B3* genes, which are involved in maintaining intracellular ionic homeostasis and cell membrane potential, were described in aldosterone-producing adenomas (aldosteronomas). All variants in these genes lead to the activation of calcium signaling, the major trigger for aldosterone production. Genetic causes of familial hyperaldosteronism have been expanded through the report of germline pathogenic variants in *KCNJ5, CACNA1H* and *CLCN2* genes. Moreover, *PDE2A* and *PDE3B* variants were associated with bilateral PA and increased the spectrum of genetic etiologies of PA. Of great importance, the genetic investigation of adrenal lesions guided by the CYP11B2 staining strongly changed the landscape of somatic genetic findings of PA. Furthermore, CYP11B2 staining allowed the better characterization of the aldosterone-producing adrenal lesions in unilateral PA. Aldosterone production may occur from multiple sources, such as solitary aldosteronoma or aldosterone-producing nodule (classical histopathology) or clusters of autonomous aldosterone-producing cells without apparent neoplasia denominated aldosterone-producing micronodules (non-classical histopathology). Interestingly, *KCNJ5* mutational status and classical histopathology of unilateral PA (aldosteronoma) have emerged as relevant predictors of clinical and biochemical outcome, respectively. In this review, we summarize the most recent advances in the pathogenesis of PA and discuss their impact on clinical outcome.

Keywords: primary aldosteronism, aldosterone, aldosterone synthase, genetics, outcome

INTRODUCTION

Arterial hypertension (AH) represents one of the main risk factors for premature death, affecting about 10 to 40% of the world population (1, 2). Primary aldosteronism (PA) is the most frequent cause of endocrine AH, with a prevalence of around 4% and 10% in hypertensive patients treated in primary and tertiary care services, respectively, reaching around 20% of patients with resistant AH (3–6).

PA is characterized by autonomous production of aldosterone, independent of the renin-angiotensin system. As a consequence, sodium retention, plasma renin suppression, blood pressure (BP) elevation and K^+ excretion increase occur, with consequent cardiovascular damage (7). The latter is due to the fact that excess of aldosterone exerts its deleterious cardiovascular effects independent of blood pressure levels, resulting in higher cardiovascular morbidity and mortality in patients with PA when compared with patients with essential AH (8, 9).

Aldosterone is a mineralocorticoid hormone, which is synthesized by the zona glomerulosa (ZG) of the adrenal cortex. Its play a major role in electrolyte regulation through sodium and water renal reabsorption (10, 11). Aldosterone is synthetized from cholesterol and its biosynthesis is under the control of two principal factors: angiotensin II (Ang II) and extracellular potassium concentration (K^+) (10).

Stimulation of ZG cells by Ang II or an increase in plasma K^+ concentration leads to cell membrane depolarization and increase in intracellular Ca^{2+}, by opening of voltage-gated Ca^{2+} channels and inositol triphosphate-dependent Ca^{2+} release from the endoplasmic reticulum. The increase of intracellular Ca^{2+} leads to activation of a phosphorylation cascade that positively regulate aldosterone synthesis and cell proliferation, specifically by increasing the *CYP11B2* gene transcription (10, 12, 13).

Effects of aldosterone are mediated through the mineralocorticoid receptor (MR), a hormone dependent transcription factor that is expressed in non-epithelial tissues, such as the heart and vessels, and in epithelial tissues such as the salivary glands and kidney distal tubule, where aldosterone regulates sodium/water reabsorption and potassium excretion (10).

The main causes of PA are bilateral cortical adrenal hyperplasia (idiopathic hyperaldosteronism) and aldosteronomas (14). Idiopathic hyperaldosteronism is caused by bilateral nodular hyperplasia originating from the cortical zona glomerulosa, whereas aldosteronomas are aldosterone-producing adenomas usually measuring between 1-3 cm (but can even measure less than 1 cm). Each of these accounts for about 50-60% and 40-50% of PA cases, respectively (7, 14).

The two major causes of PA account for more than 95% of cases, with approximately 5% of bilateral hyperplasia occurring in a familial context. Thus, bilateral hyperplasia remains without a defined genetic etiology in most cases. Although somatic allelic variants are identified in about 90% of aldosteronomas, few advances have been made in the genetic elucidation of bilateral PA (10, 15).

Several genes that encode ion channels that modulate zona glomerulosa cell depolarization and aldosterone synthesis pathways have already been associated with the pathogenesis of PA (**Figure 1**), differing in prevalence among aldosteronomas and familial PA cohorts (12). The aim of this review is to discuss the most recent discoveries about the PA pathogenesis, as well as the clinical and prognostic impact of the genetic characterization of this very prevalent disorder, associated with a high cardiovascular morbidity.

DIAGNOSIS AND CLINICAL MANAGEMENT

According to the American Endocrine Society (The Endocrine Society), the following scenarios are indicated for PA screening in hypertensive patients: I) AH and hypokalemia (spontaneous or induced by diuretic therapy); II) AH and adrenal incidentaloma; III) Blood pressure >150x100 mmHg on three different occasions; IV) AH not controlled (≥140/90 mmHg) on three or more antihypertensive drugs (resistant AH); V) controlled AH (<140x90 mmHg) on four antihypertensive drugs (resistant AH); VI) AH associated with obstructive sleep apnea; VII) AH and family history of AH or cerebrovascular disease of the young (<40 years); VIII) AH in first-degree relatives of patients with PH (7).

PA screening should be performed with plasma aldosterone (A) and renin (R) measurements, with hypokalemia correction before the test. To avoid false negative results, diuretics and spironolactone should be withheld for at least 4 to 6 weeks before the test. Aldosterone concentration >10 ng/dL and an A/PRA ratio (plasma renin activity) ≥ 30 ng/dL/ng/mL/h or A/R ≥2.0 confer a sensitivity and specificity greater than 90% for PH diagnosis (7, 16, 17). It should be emphasized that A/PRA or A/R ratio should be calculated only for patients with suppressed or very low renin levels.

After laboratorial PA confirmation, patients should undergo adrenal computed tomography (CT) for etiologic characterization and exclusion of adrenal cortical carcinoma. Adrenal CT has limited accuracy (around 60-70%), especially for detection of small (<1 cm) aldosteronomas (7) or for patients with bilateral nodules (to differentiate non-functioning or cortisol producing-adenomas). Therefore, adrenal vein sampling (AVS) is indicated for the majority of patients with PA for the proper characterization of the lateralization of aldosterone production (7, 16). Although AVS is the gold standard approach to define aldosterone lateralization, it should be carried out only in centers with expertise for this procedure and by a very experienced interventional radiologist. In addition, AVS should be considered only if laparoscopic surgery is a treatment option. A detailed description of PA work-up investigation is beyond the scope of this review.

Treatment of unilateral PA consists of laparoscopic adrenalectomy. The bilateral hyperplasia is treated with a mineralocorticoid antagonist (spironolactone or eplerenone). Both approaches are associated with reduced cardiovascular

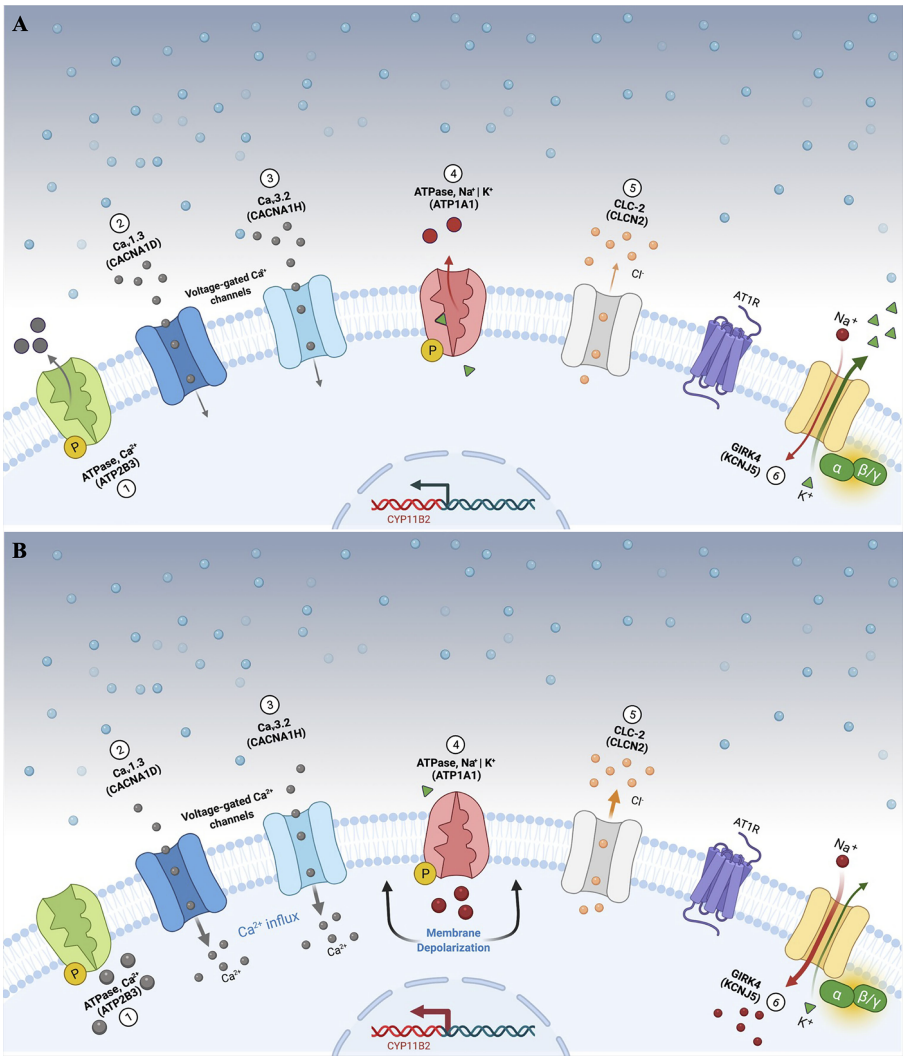

FIGURE 1 | Aldosterone secretion in adrenal zona glomerulosa cells under physiological **(A)** and pathological **(B)** conditions. **(A)** Adrenal glomerulosa cell resting. The normal resting potential of zona glomerulosa cells is hyperpolarized (highly negative membrane potential). Activation of the angiotensin receptor (AT1R) by angiotensin II or extracellular hyperkalemia results in membrane depolarization and calcium influx *via* activated voltage-gated calcium channels. Calcium influx activates signaling to increase expression of aldosterone synthase (*CYP11B2*) and ultimately aldosterone production. **(B)** Genetic alterations leading to cell membrane depolarization, intracellular ionic modification, and autonomous aldosterone secretion in PA. Pathogenic variants in the KCNJ5 gene (G-protein-activated inward rectifier potassium channel GIRK4) [6] promote loss of channel K^+ selectivity and increase permeability to Na^+, leading to membrane depolarization and calcium influx via voltage-gated calcium channels. Similarly, impaired ATPase, Ca^{2+} (ATP2B3) [1]; $Ca_v1.3$ (CACNA1D) [2]; $Ca_v3.2$ (CACNA1H) [3]; ATPase, Na^+ | K^+ (ATP1A1) [4], and CLC-2 (CLCN2) [5] function results in cell membrane depolarization, calcium influx and autonomous aldosterone secretion. PA, primary aldosteronism.

morbidity caused by excess of aldosterone (18, 19). The reduction of cardiovascular risk after medical treatment for PA is associated with normalization of renin levels (PRA >1 ng/mL/h) (20).

FAMILIAL HYPERALDOSTERONISM

Familial hyperaldosteronism (FH) is rare, but likely a highly underdiagnosed entity due to lack of routine screening (**Table 1**). Therefore, there is a lack of prevalence data for most of pathogenic variants listed in **Table 1**. The first report of FH occurred in 1966 (30), with subsequent characterization of its molecular etiology (21). This autosomal dominant form of PH was caused by a chimeric gene consisting of the 11β-hydroxylase promoter (*CYP11B1*) and aldosterone synthase (*CYP11B2*) coding region, resulting from a non-homologous pairing during crossing-over. Therefore, aldosterone synthesis becomes now regulated by adrenocorticotropic hormone (ACTH) instead of Ang II (21). This presentation of familial PH was then termed FH type 1 (OMIM #103900), or glucocorticoid-suppressible hyperaldosteronism.

TABLE 1 | Genetic causes of familial hyperaldosteronism.

Gene (OMIM)	First Report	Prevalence	Discovery Approach	Features
CYP11B1 (*610613)	1992 (21)	–	Kindred | Linkage	Chimeric *CYP11B1/CYP11B2* gene; PA remission by glucocorticoid treatment; variable age at onset (childhood to adult) (21, 22)
KCNJ5 (*600734)	2011 (23)	7% (FH) | 0.3% (PA) (24)	Cohort | Exome	Early onset (first decade of life); medication-resistant hypertension; hypokalemia; bilateral adrenal macronodular hyperplasia (24)
CACNA1D (*114206)	2013 (25)	–	Cohort | Exome	Early onset (at birth/first decade of life); seizures; neurologic abnormalities; cardiomyopathy (25)
CACNA1H (*607904)	2015 (26)	–	Cohort | Exome	Early onset (usually in the first decade of life); incomplete penetrance (26, 27)
CLCN2 (*600570)	2018 (28)	–	Cohort | Exome	Early onset (usually before 20 years of age); incomplete penetrance; variable expressivity; favorable response to spironolactone (29)

FH, familial hyperaldosteronism; PA, primary aldosteronism.

A diagnosis of FH 1 is highly suggestive if aldosterone suppression (<4 ng/dL) occurs after a dexamethasone suppression test (0.5 mg each 6h for 48h). However, the FH 1 diagnosis should be confirmed by the presence of the chimeric gene in a long range PCR (31). The treatment of FH 1 consists of low dose dexamethasone administration in adults (0.125–0.25mg/d) to suppress ACTH and block aldosterone synthesis (32, 33). If additional blood pressure control is required, a mineralocorticoid antagonist can be added.

The molecular pathogenesis of Type 2 FH (OMIM #605635) consists of gain-of-function heterozygous germline variants in the *CLCN2* gene (**Table 1**). Type 2 FH is characterized by autosomal dominant inheritance, incomplete penetrance and a family history of aldosteronoma or bilateral PA (34, 35). *CLCN2* was mapped as a FH gene in 2018 and encodes an inwardly rectifying chloride channel (ClC-2), a member of the CLC voltage-gated Cl^- channels family which is expressed in the cortical zona glomerulosa (28, 29).

So far, 6 missense pathogenic variants in *CLCN2* have been reported in the literature associated with FH 2 (**Table 2**) (48). The presence of these alleles causes an increase in Cl^- conductance through the channel, leading to a continuous

TABLE 2 | Germline allelic variants identified in probands with (familial) primary hyperaldosteronism/(early onset) hypertension.

Gene	Nucleotide change[1]	Aminoacid change[1]	Region	Families	ACMG[2,3]	Reference (first report)
(familial) Primary Hyperaldosteronism						
CLCN2	c.65T>A	p.(Met22Lys)	Exon 2	1	VUS-Cool	Scholl et al., 2018 (29)
	c.71G>A	p.(Gly24Asp)		1	VUS-Hot	Fernandes-Rosa et al., 2018 (28)
	c.76T>A	p.(Tyr26Asn)		1	VUS-Cool	Scholl et al., 2018 (29)
	c.515G>A	p.(Arg172Gln)	Exon 5	8	P	
	c.1084A>T	p.(Lys362*)	Exon 10	1	VUS-Tepid	
	c.2593A>C	p.(Ser865Arg)	Exon 24	1	VUS-Cool	
KCNJ5	c.155G>A	p.(Arg52His)	Exon 2	2	VUS-Tepid	Murthy et al., 2014 (36)
	c.433G>C	p.(Glu145Gln)		3	LP	Monticone et al., 2015 (37)
	c.452G>A	p.(Gly151Glu)		3	P	Mulatero et al., 2012 (38)
	c.451G>A	p.(Gly151Arg)		2	P	Scholl et al., 2012 (39)
	c.455A>G	p.(Tyr152Cys)		1	VUS-Hot	Monticone et al., 2013 (40)
	c.470T>G	p.(Ile157Ser)		1	VUS-Warm	Charmandari et al., 2012 (41)
	c.472A>G	p.(Thr158Ala)		3	LP	Choi et al., 2011 (23)
	c.736G>A	p.(Glu246Lys)		1	VUS-Warm	Murthy et al., 2014 (36)
	c.446_448del	p.(Thr149del)		1	VUS-Hot	Pons Fernández et al., 2019 (42)
CACNA1H	c.587C>T	p.(Ser196Leu)	Exon 5	1	VUS-Tepid	Daniil et al., 2016 (27)
	c.2669G>A	p.(Arg890His)	Exon 12	1	VUS-Warm	Wulczyn et al., 2019 (43)
	c.4645A>G	p.(Met1549Val)	Exon 25	5	LP	Scholl et al., 2015 (26)
	c.4647G>C	p.(Met1549Ile)		1	VUS-Hot	Daniil et al., 2016 (27)
	c.6248C>T	p.(Pro2083Leu)	Exon 35	1	VUS-Cold	
CACNA1D	c.1208G>A	p.(Gly403Asp)	Exon 8	1	LP	Scholl et al., 2013 (25)
	c.2310C>G	p.(Ile770Met)	Exon 17	1	LP	
	c.776T>A	p.(Val259Asp)	Exon 6	1	VUS-Warm	Semenova et al., 2018 (44)
	c.812T>A	p.(Leu271His)		1	VUS-Warm	De Mingo Alemany et al., 2020 (45)
(early onset) Hypertension						
KCNJ5	c.775G>A	p.(Val259Met)	Exon 2	1	VUS-Tepid	Markou et al., 2015 (46)
	c.834T>A	p.(His278Gln)		1	VUS-Tepid	Qin et al., 2019 (47)
	c.1042T>A	p.(Tyr348Asn)	Exon 3	1	VUS-Tepid	Markou et al., 2015 (46)
	c.1123C>T	p.(Arg375Trp)		1	LB	Qin et al., 2019 (47)

[1] RefSeq reference transcript: NM_004366.6 (CLCN2)/NM_000890.5 (KCNJ5)/NM_021098.3 (CACNA1H)/NM_000720.4 (CACNA1D); [2] ACMG/AMP five-tier system: B (Benign), LB (Likely benign), P (Pathogenic), LP (Likely pathogenic), VUS (Variant of uncertain significance); [3] ACGS (Association for Clinical Genomic Science) VUS temperature scale: Ice Cold, Cold, Cool, Tepid, Warm, Hot.

depolarization of the plasma membrane, resulting in an increase of *CYP11B2* expression and consequent stimulus for aldosterone synthesis (**Figure 1B**) (28, 29).

In 2008, individuals with childhood-onset PA, resistant AH, hypokalemia and bilateral macronodular adrenal hyperplasia were reported (31). In 2011, an inactivating germline variant in the *KCNJ5* gene was identified in a case with a similar clinical presentation. Named FH 3 (OMIM #613677), this autosomal dominant PA subtype is caused by an impaired function of K^+ GIRK4 (Kir3.4) potassium channel, which is encoded by *KCNJ5* gene (23).

The molecular defect in the K^+ GIRK4 potassium channel leads to the loss of its ionic selectivity, with a consequent increase in sodium conductance (**Figure 1B**). Naturally responsible for maintaining the zona glomerulosa membrane potential, it starts to act as a channel in favor of sodium influx, promoting continuous membrane depolarization and subsequent activation of voltage-dependent Ca^+ channels. These increased intracellular calcium concentrations trigger *CYP11B2* overexpression and aldosterone synthesis (39).

The genetic study of numerous PA cohorts and the consequent mapping of new *KCNJ5* pathogenic variants allowed, over the years, to expand the phenotypic heterogeneity of this PA subtype (39–41) (**Table 2**). Certain alleles between amino acids residues 151-158 of the K^+ GIRK4 potassium channel, more specifically p.(Gly151Arg), p.(Ile157Ser), and p.(Thr158Ala), are correlated with a more severe PA clinical presentation, with early-onset hypertension, more resistant to drug treatment and with a frequent need for bilateral adrenalectomy (24). On the other hand, some substitutions in this same region, namely p.(Gly151Glu) and p.(Tyr152Cys), result in a mild clinical presentation, with an adequate blood pressure control with aldosterone antagonists and without evidence of adrenal hyperplasia in CT evaluation (13, 39). Interestingly, *in vitro* experiments showed that mutant *KCNJ5* channels can be undermined with the use of macrolide antibiotics such as roxithromycin and clarithromycin, suppressing *CYP11B2* expression and aldosterone production (49).

Four *KCNJ5* germline variants were reported in cohorts of patients with AH without a typical familial and biochemical diagnosis of PA (**Table 2**) (46, 47). The p.(His278Gln) variant, for example, was reported in a patient with resistant AH with normal serum K^+ levels, plasma renin activity and aldosterone levels. The allele was inherited from his father who had essential AH without PA (47). None of the other reported cases had a phenotype similar to FH 3 patients, with early-onset medication-resistant hypertension, hypokalemia and bilateral adrenal macronodular hyperplasia (24).

Type 4 FH (OMIM #617027), the rarest subtype of PA, is caused by gain-of-function germline variants in *CACNA1H* gene (autosomal dominant inheritance), which encodes calcium voltage-gated channel subunit α1 H ($Ca_v3.2$) (26) (**Tables 1**, **2**). Scholl et al. identified a recurrent heterozygous variant in the *CACNA1H* gene in five patients with early-onset PA (26). *In silico* studies with the identified p.(Met1549Val) mutant demonstrated an increase in calcium influx into zona glomerulosa cells, resulting in continual stimulation of aldosterone synthesis (50). Later studies demonstrated a late and incomplete penetrance of this PA subtype (27).

In 2013, Scholl et al. sequenced the candidate *CACNA1D* gene in 100 unrelated individuals with early-onset PA and identified two *de novo* heterozygous alleles in two girls with an undescribed syndrome featuring PA, AH, seizures and neuromuscular abnormalities (OMIM #615474) (25) (**Table 2**). This gene

TABLE 3 | Genetic causes of unilateral primary aldosteronism.

Gene (OMIM)	First Report	Prevalence	Discovery Approach	Features
KCNJ5 (*600734)	2011 (23)	>40%	Cohort | Candidate Gene	Larger APAs with predominance of ZF-like clear cell composition; More frequent in younger, females, and East Asian patients; High aldosterone levels and severe hypokalemia (55–58)
ATP1A1 (*182310)	2013 (59)	5.3%	Cohort	More frequent in male patients; APA with predominance of compact ZG-like cells, smaller size* (56, 60)
ATP2B3 (*300014)	2013 (59)	1.7%	Cohort	APA with predominance of compact ZG-like cells; Severe hypokalemia (56, 60)
CACNA1D (*114206)	2013 (61)	9.3%	Cohort | Candidate Gene	More frequent in black and male patients; APA with predominance of compact ZG-like cells, smaller size* (56, 62)
CTNNB1 (*116806)	2015 (63)	5%	Cohort | Candidate Gene	More frequent in female and older patients; Associated with pregnancy and menopause; Higher *LHCGR* and *GNRHR* gene expression (63, 64)
CLCN2 (*600570)	2018 (28)	<1%	Cohort | Candidate Gene	Found in younger patients with high aldosterone levels; APA with smaller size** (65, 66)
CACNA1H (*607904)	2020 (67)	<1%	Cohort | Candidate Gene	Intra-tumoral *CYP11B2* expression heterogeneity; Composed of compact ZG-like cells** (67)

**Compared with KCNJ5 tumors; **Few (<3) cases reported in the literature, no statistical relevance; APA, aldosterone-producing adenomas (aldosteronomas); ZF, zona fasciculata; ZG, zona glomerulosa.*

TABLE 4 | Somatic variants identified in adrenal lesions associated with unilateral primary aldosteronism.

Gene	Nucleotide change[1]	Aminoacid change[1]	Region	Reference(first report)
KCNJ5	c.451G>A	p.(Gly151Arg)	Exon 2	Choi et al., 2011 (23)
	c.503T>G	p.(Leu168Arg)		
	c.433G>C	p.(Glu145Gln)		Akestrom et al., 2012 (69)
	c.472A>G	p.(Thr158Ala)		Mulatero et al., 2012 (38)
	c.451G>C	p.(Gly151Arg)		Taguchi et al., 2012 (70)
	c.467_469del	p.(Ile157del)		Azizan et al., 2012 (58)
	c.433G>A	p.(Glu145Lys)		Azizan et al., 2013 (61)
	c.446insAAC	p.(Thr149_Ile150insThr)		Kuppusami et al., 2014 (71)
	c.376T>C	p.(Trp126Arg)		Williams et al., 2014 (72)
	c.461T>G	p.(Phe154Cys)		Scholl et al., 2015 (73)
	c.470_471delinsAA	p.(Ile157Lys)		
	c.450_451insATG	p.(Ile150_Gly151insMet)		
	c.433_434insCCATTG	p.(Ile144_Glu145insAlaIle)		
	c.445_446insGAA	p.(Thr148_Thr149insArg)		Zheng et al., 2015 (74)
	c.439G>C and c.448_449insCAACAACCA	p.(Glu147Gln) and p.(Thr149_Ile150insThrThrThr)		Wang et al., 2015 (75)
	c.457_492dup	p.(Gly153_Gly164dup)		
	c.343C>T	p.(Arg115Trp)		Cheng et al., 2015 (76)
	c.737A>G	p.(Glu246Gly)		
	c.445A>T	p.(Thr149Ser)		Nanba et al., 2016 (77)
	c.443C>T	p.(Thr148Ile)		
	c.432_439delinsCA	p.(Glu145_Glu147delinsLys)		Zheng et al., 2017 (78)
	c.414_425dup	p.(Ala139_Phe142dup)		Hardege et al., 2015 (79)
	–	p.(Gly184Glu)*		Kitamoto et al., 2018 (80)
	–	p.(Ile157_Glu159del)*		
	–	p.(Gly151_Tyr152del)*		
	c.420C>G	p.(Phe140Leu)		Nanba et al., 2018 (81)
	c.447_448insATT	p.(Thr149delinsThrIle)		
	c.445_446insTGG	p.(Thr149delinsMetAla)		Nanba et al., 2019 (62)
CACNA1D	c.4007C>G	p.(Pro1336Arg)	Exon 32	Azizan et al., 2013 (61)
	c.4062G>A	p.(Met1354Ile)		
	c.2239T>C	p.(Phe747Leu)	Exon 16	
	c.2969G>A	p.(Arg990His)	Exon 23	
	c.776T>A	p.(Val259Asp)	Exon 6	
	c.2241C>G	p.(Phe747Leu)	Exon 16	
	c.2250C>G	p.(Ile750Met)		Scholl et al., 2013 (25)
	c.4012G>A	p.(Val1353Met)	Exon 33	
	c.2239T>G	p.(Phe747Val)	Exon 16	
	c.1207G>C	p.(Gly403Arg)	Exon 8A	
	c.1955C>T	p.(Ser652Leu)	Exon 14	Fernandes-Rosa et al., 2014 (56)
	c.2222A>G	p.(Tyr741Cys)	Exon 16	
	c.2993C>T	p.(Ala998Val)	Exon 23	
	c.3455T>A	p.(Ile1152Asn)	Exon 27	
	c.3451G>T	p.(Val1151Phe)		
	c.2936T>A	p.(Val979Asp)	Exon 23	
	c.1964T>C	p.(Leu655Pro)	Exon 14	
	c.2943G>C	p.(Val981Asn)	Exon 23	
	c.2248A>T	p.(Ile750Phe)	Exon 16	
	c.2992_2993delinsAT	p.(Ala998Ile)	Exon 23	
	c.2182G>A	p.(Val728Ile)	Exon 15	Wang et al., 2015 (75)
	c.2240T>G	p.(Phe747Cys)	Exon 16	Nanba et al., 2016 (82)
	c.3458T>G	p.(Val1153Gly)	Exon 27	Tan et al., 2017 (83)
	c.776T>G	p.(Val259Gly)	Exon 6	Nanba et al., 2018 (81)
	c.1201G>C	p.(Val401Leu)	Exon 8	Akerstrom et al., 2015 (84)
	c.1229C>T	p.(Ser410Leu)	Exon 9	Backman et al., 2019 (85)
	c.3019T>C	p.(Cys1007Arg)	Exon 24	Nanba et al., 2019 (62)
	c.3044T>G	p.(Ile1015Ser)		
	c.926T>C	p.(Val309Ala)	Exon 7	
	c.2978G>C	p.(Arg993Thr)	Exon 23	
	c.2968C>G	p.(Arg990Gly)		
	c.1856G>C	p.(Arg619Pro)	Exon 13	
	c.3452T>C	p.(Val1151Ala)	Exon 27	Guo et al., 2020 (86)
	c.2240T>C	p.(Phe747Ser)	Exon 16	
	c.2261A>G	p.(Asn754Ser)		

(Continued)

TABLE 4 | Continued

Gene	Nucleotide change[1]	Aminoacid change[1]	Region	Reference(first report)
	c.2978G>T	p.(Arg993Met)	Exon 23	
	c.2906C>T	p.(Ser969Leu)	Exon 22	Nanba et al., 2020 (87)
	c.3044T>C	p.(Ile1015Thr)	Exon 24	De Sousa et al., 2020 (88)
ATP1A1	c.311T>G	p.(Leu104Arg)	Exon 4	Beuschlein et al., 2013 (59)
	c.299_313del	p.(Phe100_Leu104del)		
	c.995T>G	p.(Val332Gly)	Exon 8	
	c.2878_2887delinsT	p.(Glu960_Ala963delinsSer)	Exon 21	Azizan et al., 2013 (61)
	c.295G>A	p.(Gly99Arg)	Exon 4	Williams et al., 2014 (72)
	c.306_317del	p.(Met102_Trp105del)		Akerstrom et al., 2015 (84)
	c.304_309del	p.(Met102_Leu103del)		
	c.308_313del	p.(Leu103_Leu104del)		
	c.2867_2882delinsG	p.(Phe956_Glu961delinsTrp)	Exon 21	
	c.2877_2882del	p.(Phe959_Glu961delinsLeu)		
	c.2879_2890del	p.(Glu960_Leu964delinsVal)		
	c.2864_2878del	p.(Ile955_Glu960delinsLys)		Nanba et al., 2019 (62)
	c.2878_2892delinsGCCGTG	p.(Glu960_Leu964delinsAlaVal)		Nanba et al., 2018 (81)
	c.2874_2882del	p.(Phe959_Glu961del)		Guo et al., 2020 (86)
	c.2877_2888del	p.(Glu960_Ala963del)		
	c.2878_2895delinsGCCCTGGTT	p.(Glu960_Ala965delinsAlaLeuVal)		Nanba et al., 2020 (87)
ATP2B3	c.1272_1277del	p.(Leu425_Val426del)	Exon 8	Beuschlein et al., 2013 (59)
	c.1277_1282del	p.(Val426_Val427del)		
	c.1273_1278del	p.(Leu425_Val426del)		
	c.1270_1275del	p.(Val424_Leu425del)		Fernandes-Rosa et al., 2014 (56)
	c.1277_1298delinsACA	p.(Val426Aspfs*10)		Scholl et al., 2015 (73)
	c.1264_1278delinsAGCACACTC	p.(Val422_Val426delinsSerThrLeu)		Zheng et al., 2015 (74)
	c.1276_1287del	p.(Val426_Val429del)		Akerstrom et al., 2015 (84)
	c.1228T>G	p.(Tyr410Asp)		Wu et al., 2015 (89)
	c.1269_1274del	p.(Val424_Leu425del)		Murakami et al., 2015 (90)
	c.1279_1284del	p.(Val427_Ala428del)		Kitamoto et al., 2016 (60)
	c.1281_1286del	p.(Ala428_Val429del)		Dutta et al., 2014 (91)
	c.1264_1275delinsATCACT	p.(Val422_Leu425delinsIleThr)		Nanba et al., 2018 (81)
	c.367G>C	p.(Gly123Arg)	Exon 2	Backman et al., 2019 (85)
CACNA1H	c.4289T>C	p.(Ile1430Thr)	Exon 22	Nanba et al., 2020 (67)
CLCN2	c.71G>A	p.(Gly24Asp)	Exon 2	Dutta et al., 2019 (65)
	c.64-2_74del	p.(Met22fs)		Rege et al., 2020 (66)

*[1] RefSeq reference transcript: NM_000890.5 (KCNJ5)/NM_001128839.3 (CACNA1D)/NM_000701.8 (ATP1A1)/NM_001001344.2 (ATP2B3)/NM_004366.6 (CLCN2)/NM_021098.3 (CACNA1H); * Nucleotide change not provided by authors.*

encodes the α 1D subunit of the L-type voltage-gated Ca^{2+} channel $Ca_v1.3$. The identified variants promote an activation of the Ca^{+2} channel at lower depolarization potentials, resulting in increased Ca^{+2} influx (25). Subsequently, two more cases were reported with *de novo* heterozygous *CACNA1D* variants, leading to a severe developmental disorder also associated with developmental delay, intellectual impairment, neurological symptoms (including seizures), and endocrine symptoms, evident as PA and/or congenital hyperinsulinemic hypoglycemia (44, 45).

Recently, rare heterozygous missense germline variants in the phosphodiesterase 2A (*PDE2A*) and 3B (*PDE3B*) genes were identified in 3 out of 11 patients with PA caused by bilateral hyperplasia (51). In addition, PDE2A was a marker of zona glomerulosa and aldosterone-producing hyperplastic areas and micronodules. *In vitro* functional studies supported the involvement of PDE2A and PDE3B in the pathogenesis of bilateral PA. PKA activity in frozen tissue was significantly higher in adrenals from patients harboring *PDE2A* and *PDE3B* variants. Interestingly, inactivating *PDE2A* and *PDE3B* variants increased SGK1 and SCNN1G/ENaCg at mRNA or protein levels (51).

SGK1 (serum and glucocorticoid inducible kinase-isoform 1) belongs to a large family of serine-threonine kinases. SGK1 is expressed in numerous tissues and plays a major role in transmembrane ionic transport, being established as an important regulator of Na^+ transporters (52). Aldosterone is the most notorious hormonal regulator of SGK1 expression. After binding to the cytosolic mineralocorticoid receptor, aldosterone promotes the transcription of SGK1, which regulates a variety of ion transporters, such as ENaC (epithelial sodium channel). SGK1 reduces ENaC ubiquitination and degradation, as well as its cellular internalization (53). Therefore, *PDE2A* and *PDE3B* variants can induce aldosterone signaling by increasing SGK1/SCNN1G(ENag) (51). In addition, an increase in SGK1 activity also stimulates hypercoagulability, fibrosis and inflammation processes (54).

UNILATERAL PRIMARY ALDOSTERONISM

Aldosteronomas are a major cause of unilateral PA, associated with somatic variants in *KCNJ5*, *CACNA1D*, *ATP1A1*, *ATP2B3*, *CLCN2*, *CACNA1H* and *CTNNB1* genes (**Table 3**). These genes

drive autonomous aldosterone production and/or directly contribute for tumorigenesis (68). In 2011, Choi et al. identified recurrent *KCNJ5* gain-of-function variants in aldosteronomas, namely p.(Gly151Arg) and p.(Leu168Arg), that affects residues at the channel ion selectivity filter (23) (**Table 4** and **Figure 1B**).

KCNJ5 is the most frequently affected gene in aldosteronomas (>40%), with even higher prevalence in Japanese and/or Eastern Asian cohorts (65-69% approximately). Characteristically, *KCNJ5* mutant aldosteronomas are more frequent in female (>70%) and younger patients, with larger tumor size. Higher preoperative aldosterone and reduced potassium levels were also identified in these patients, which could contribute to early-onset disease, severity and earlier diagnosis (23, 24, 55, 74, 92).

In 2013 after *KCNJ5* discovery, somatic *CACNA1D* gain-of-function variants were reported in aldosteronomas, with a prevalence of around 10%. *CACNA1D* encodes the α1 subunit $Ca_v1.3$ of a voltage dependent L-type (long-lasting) calcium channel and its pathogenic variants affect conserved residues within the channel activation gate (**Table 3**). Compared to wild-type, mutated $Ca_v1.3$ reaches activation in less depolarized membrane potentials, causing abnormal Ca^+ influx, *CYP11B2* expression, and aldosterone production (**Table 4** and **Figure 1B**). In contrast with *KCNJ5* related aldosteronomas, *CACNA1D* tumors are significantly smaller and more frequent in older male patients (25, 56, 61).

In 2013, Beuschlein et al. identified somatic variants in genes encoding ATPases, *ATP1A1* and *ATP2B3* in aldosteronomas (59). Missense and in-frame deletion variants in *ATP1A1*, which encodes Na^+/K^+ ATPase α subunit, impair pump activity and significantly reduce affinity for potassium, resulting in inappropriate membrane depolarization (**Table 4** and **Figure 1B**). *ATP2B3* encodes a Ca^+ ATPase in which loss-of-function alleles (in-frame deletions) lead to a loss of physiological pump function, responsible for sodium and possibly calcium ions leaking into the cell, inducing membrane depolarization, and contributing to increased calcium concentrations. The combined prevalence of somatic variants in ATPases is around 7% and, until now, no ATPase pathogenic variants were found as germline or surrounding aldosteronoma tissue. Additionally, ATPase mutant aldosteronomas showed a high prevalence among older male patients (61, 93).

As found in other adrenocortical tumors, somatic gain of function variants in *CTNNB1* gene, encoding β catenin, also have been reported in around 5% of aldosteronomas (**Tables 3**, **4**). Affected adrenals had an aberrant β catenin accumulation in the Wnt cell-differentiation pathway and overexpression of luteinizing hormone/choriogonadotropin receptor (LHCGR) and gonadotropin-releasing hormone receptor (GnRHR) (63, 94, 95). Patients harboring aldosteronomas with *CTNNB1* variants are more frequently females (60-70%) and older individuals, with no significant differences in preoperative aldosterone levels, tumor size and frequency familial hypertension compared with those with *KCNJ5* variants (64). Unfortunately, the underlying mechanism leading to *CYP11B2* overexpression due to *CTNNB1* mutations remains unclear. Berthon et al. (96) showed that β-catenin plays an essential role in the control of basal and Angi II-induced aldosterone secretion, by activating *AT1R*, *CYP21* and *CYP11B2* transcription (96).

Due to recent advances in high throughput sequencing, few somatic variants have been recently identified in 2 genes only so far related to FH (*CLCN2* and *CACNA1H*): the missense p.(Gly24Asp) (*CLCN2*), previously reported in FH 2 (28, 65); the splice junction loss c.64-2_74del (*CLCN2*) (65), and more recently, the missense p.(Ile1430Thr) (*CACNA1H*) (**Table 4**) (67).

The knowledge about adrenal lesions associated with PA and the detection rate of somatic variant have been significantly changed since the development of highly specific monoclonal antibodies against CYP11B1 and CYP11B2 (97). Under normal conditions, CYP11B2 was sporadically detected in the zona glomerulosa, whereas CYP11B1 was entirely detected in the zonae fasciculata-reticularis (98). In younger individuals, immunohistochemistry from normal adrenals reveals a continuous CYP11B2 expression throughout the ZG layer, but this pattern changes in adults and CYP11B2 expression becomes discontinuing in ZG (98, 99). Next, Nanba et al. demonstrated that CYP11B2 immunostaining was a powerful tool for histopathological identification of adrenal lesions associated with aldosterone overproduction (100).

Fernandes Rosa et al. performed the most comprehensive study in a cohort of 474 aldosteronomas from the European Network for the Study of Adrenal Tumors, reaching a detection rate of somatic variants of 54%, although *CTNNB1* sequencing was not included in this study (56). Two other studies, which included *CTNNB1* sequencing, demonstrated similar findings: Wu et al. studied 219 aldosteronomas, detecting somatic variants in 58.4% of them (101), and Vilela et al. reported a discovery rate of approximately 50% (102).

Recent studies using immunohistochemistry-guided approach to determine the exact source of abnormal aldosterone production led to the identification of pathogenic somatic variants in around 90% of screened aldosteronomas (81, 82, 88, 103). The lower prevalence of somatic variants found in aldosteronomas in previous studies using conventional approaches, not taking in account CYP11B2 expression, is

TABLE 5 | New histopathological nomenclature (HISTALDO) of aldosterone-producing adrenal lesions in patients with unilateral primary aldosteronism.

Aldosterone-producing Lesions (HISTALDO)	Size	HE visible	Histology
Aldosterone-producing adenoma (APA)	> 10mm	Yes	classical
Aldosterone-producing nodule (APN)	< 10mm	Yes	classical*
Aldosterone-producing micronodule (APM)	Microscopic	No	non-classical
Aldosterone-producing diffuse hyperplasia	Continuous layer of ZG cells	Yes	non-classical

**Non-classical in multinodular forms; HE, hematoxylin-eosin; ZG, zona glomerulosa.*

explained due to the macroscopical selection of non-aldosterone-producing adrenal lesions (81). A recent review confirmed these previous findings, showing a higher detection rate of somatic variants with CYP11B2-guided extraction (85%) when compared to the classical approach with DNA extraction from fresh frozen tissue (54%) (57). Overall, the variant-negative ratio decreased from 46% to 15%. Gene-specific detection rate also increased from 34% to 56% in *KCNJ5*, 8% to 10% in *CACNA1D*, 8% to 12% in *ATP1A1* and 4% to 5% in *ATP2B3* (57).

Moreover, the CYP11B2-guided high throughput sequencing method has revealed a wide complexity of aldosterone-producing lesions in patients with PA (81, 82, 88, 103, 104). In multinodular cases, tumors from the same adrenal might harbor different recurrent somatic variants, suggesting independent triggers for the somatic events (82, 105). Interestingly, aldosterone production may occur from multiple sources: multiple aldosteronomas in the same adrenal gland, dominant non-producing adenoma with satellite CYP11B2 positive non-dominant nodules, and clusters of autonomous aldosterone-producing cells (APCCs) without apparent neoplasia (55, 81, 88, 106, 107).

APCCs are common in normal adrenals and accumulate with age, becoming more often detectable in morphologically normal adult adrenals (108). Somatic pathogenic variants in *CACNA1D, ATP1A1* and *ATP2B3* were found in 35% to 76% of the APCCs, with *CACNA1D* being the most mutated gene (108, 109). Interestingly, the spectrum of affected gene in APCCs is different from aldosteronomas. APCCs may a key player to the understanding of the physiology and pathophysiology of aldosterone production. It has been hypothesized that aldosteronomas can derive from APCCs with autonomous aldosterone production (harboring somatic in aldosterone-driver genes) (15, 99, 108).

Recently, the international histopathology consensus for unilateral PA (HISTALDO) classified the aldosterone-producing lesions (110). (**Table 5**). Aldosteronoma was defined as a well circumscribed CYP11B2-positive solitary neoplasm (≥ 10 mm diameter) composed of clear or compact eosinophilic cells or both cell types. Aldosterone-producing nodule is a CYP11B2-positive lesion (<10 mm diameter) morphologically visible with hematoxylin-eosin staining ("microaldosteronoma"). In this consensus, the nomenclature for APPC was changed to aldosterone-producing micronodules (APMs). APMs are defined as CYP11B2-positive lesion (<10 mm diameter) composed of ZG cells located beneath adrenal capsule. APMs are indistinguishable from normal zona glomerulosa (ZG) cells in hematoxylin-eosin staining (108, 110). In CYP11B2 staining, APMs have a strong uniform immunoreactivity for CYP11B2, without evident neoplasia or hyperplasia (108, 110).

These advances in PA histopathology allowed the definition of classical and non-classical histopathological features associated with PA (**Table 5**) (110). The classical histology is defined by the presence of a solitary aldosteronoma or APN. In contrast, "non-classical" histology is characterized by adrenals with multiple APNs or APMs (or multiple APMs and multiple APNs together) or aldosterone-producing diffuse hyperplasia (110, 111). In summary, non-classical histology is defined by the absence of a dominant aldosterone-producing lesion (such as a solitary aldosteronoma or APN). Interestingly, the mutational spectrum is different between classical and non-classical histology. *KCNJ5* somatic variants are predominant among aldosteronomas (classical histology), whereas *CACNA1D* is the most frequent mutated gene in APMs (81, 111).

IMPACT ON CLINICAL OUTCOME

The impact of genetic and clinical variables in outcome in PA patients have been more properly evaluated after the Primary Aldosteronism Surgical Outcome (PASO) study, which established criteria for clinical and biochemical success in unilateral PA patients after adrenalectomy (112). PASO criteria classified PA patients after adrenalectomy according to the biochemical outcome and clinical success. Complete biochemical success is defined by correction of hypokalemia when present pre-surgery and normalization of the aldosterone-to-renin ratio, and partial biochemical success as a correction of hypokalemia when present pre-surgery and a raised aldosterone-to-renin ratio, but with at least 50% decrease in baseline plasma aldosterone concentration compared to pre-surgical levels. Regarding blood pressure control, complete clinical success is defined as blood pressure <140x90 mmHg without anti-hypertensive medications after 6 months of follow-up, whereas partial clinical success as a reduction in the number or dose of anti-hypertensive medications when compared to pre-surgery (98).

Recently, non-classical histopathological lesions associated with aldosterone excess were found in 25% of the cases in a German cohort of unilateral PA (111). On the other side, APMs were found in only 5% (7 out of 137) of the cases in a Chinese cohort of unilateral PA (113). Therefore, additional studies from patients with different genetic backgrounds are essential to define the prevalence of classical and non-classical unilateral PA among different cohorts.

Of great importance in clinical practice, postsurgical complete biochemical success after adrenalectomy was correlated with histological features in a German cohort of unilateral PA. The rate of biochemical cure of PA was 98% in patients with the classical histopathology (solitary aldosteronoma or APN) compared with 67% in the patients with unilateral PA caused by non-classical histopathology (111). These findings suggested the presence of a baseline abnormal aldosterone production from the contralateral gland in patients with non-classical unilateral PA (**Table 5**).

KCNJ5 somatic pathogenic variants have been associated with complete clinical success in cohorts of unilateral PA from Australia, West Norway, Japan and Brazil (80, 102, 114, 115). In a Brazilian cohort of PA, complete clinical success based in PASO criteria was more frequent in patients with aldosteronomas harboring *KCNJ5* pathogenic variants than in those with pathogenic variants in other

driver genes (102). However, it should be emphasized that these previous studies did not conduct a genetic investigation based on CYP11B2 staining.

Interestingly, *KCNJ5* pathogenic variants have been more frequently detected in aldosteronomas (classical histopathology), which is associated with a higher chance of postsurgical complete biochemical success (57). Recently, somatic *KCNJ5* pathogenic variants were not associated with clinical and biochemical outcome in a small group of 38 aldosteronomas with genetic investigation guided by CYP11B2 staining. However, the influence of *KCNJ5* status in the outcome of PA patients cannot be ruled out and should be further evaluated in larger cohorts of unilateral PA with genetic investigation guided by CYP11B2 staining. Furthermore, the impact of somatic *KCNJ5* pathogenic variants on clinical outcome might depend on the frequency of classical histopathology among unilateral PA cases.

PERSPECTIVES

Genetics of unilateral PA has remarkably improved in the last decade. However, most cases of bilateral hyperplasia remain without genetic etiology (15). Of great importance, a new histopathological classification has been recently proposed for aldosterone-producing lesions in unilateral PA (110). Besides the impact on the comprehension of PA pathophysiology, the histopathological features have influence in the outcome after unilateral adrenalectomy. *KCNJ5* mutational status and classical histopathology of unilateral PA (aldosteronoma) have emerged as relevant predictors of clinical and biochemical outcome, respectively (102, 111). Further studies will be important to characterize the spectrum of classical and non-classical unilateral PA among cohorts from different genetic backgrounds.

AUTHOR CONTRIBUTIONS

LS and AG participated on acquisition, analysis and interpretation of data, and drafted the manuscript. MA designed, drafted and revising critically the manuscript. All authors contributed to the article and approved the submitted version.

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Metabolic syndrome and cardiovascular morbidity in patients with congenital adrenal hyperplasia

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Since the introduction of glucocorticoid (GC) replacement therapy, congenital adrenal hyperplasia (CAH) is no longer a fatal disease. The development of neonatal screening programs and the amelioration of GC treatment strategies have improved significantly life expectancy in CAH patients. Thanks to these achievements, CAH patients are now in their adulthood, but an increased incidence of cardiovascular risk factors has been reported compared to general population in this stage of life. The aim of CAH treatment is to both prevent adrenal insufficiency and suppress androgen excess; in this delicate balance, under- as well as overtreatment might be equally harmful to long-term cardiovascular health. This work examines the prevalence of metabolic features and cardiovascular events, their correlation with hormone levels and GC replacement regimen in CAH patients and focuses on precocious markers to early detect patients at higher risk and new potential treatment approaches.

KEYWORDS

congenital adrenal hyperplasia (CAH)–21-alpha hydroxylase deficiency, cardiovascular risk, metabolic syndrome, diabetes mellitus, obesity, glucocorticoid therapy

Introduction

Congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency (21-OHD) is an autosomal recessive disorder characterized by impaired cortisol secretion and androgen excess (1). 21-OHD is by far the commonest cause of CAH, accounting for around 95% of cases. On the basis of residual enzymatic activity, 21-OHD is classified into classic (CCAH) and non-classic CAH (NCAH), with the latter usually diagnosed later in life. Depending on the presence or absence of aldosterone deficiency, CCAH is classified into salt wasting (SW) and simple virilising (SV) forms, respectively. The mainstay of CAH treatment, especially for CCAH, is glucocorticoid (GC) and mineralocorticoid (MC) replacement to avoid adrenal crisis and manage androgen

excess. The prevention of long-term metabolic and cardiovascular (CV) complications is based on the delicate balance between these two therapeutic objectives (2). In fact, both under- and over-treatment can be equally detrimental for cardio-metabolic health, Figure 1. In this short review, we summarized available data on cardiometabolic health in CAH patients, examined the prevalence of predisposing factors and the mechanisms that promote their development.

Obesity

Obesity is an important independent CV risk factor and is the most frequent component of metabolic syndrome in both children and adults with CAH. Several studies reported a high incidence of obesity, ranging between 30 and 40%, in patients with either classic and non-classic CAH (3–8). Underlying causes include intrinsic susceptibility to adipose tissue accumulation, adipokines imbalance and high leptin levels (4, 9). Notably, in individuals with CCAH, the tendency to precocious adiposity rebound is present in childhood and may persist in adulthood (10, 11). Additionally, CAH patients are prone to increases in visceral adipose tissue, a clear-cut risk factor for CV diseases. Recently, increased visceral adipose tissue (VAT)/subcutaneous adipose tissue (SAT) ratios were found in adolescents with CAH using computed tomography scan (12), although controversial data were found findings by other studies using dual x-ray absorptiometry (13–15).

A possible explanation for the higher adiposity may be due to lower catecholamine levels in CAH, with a consequent reduced lipolysis, especially in the classic form (4). Indeed, in a large cohort of 203 adults patients, higher BMI's were found in females with CCAH compared to those with NCCAH (3).

Moreover, it was hypothesized that increased BMI could be related to both type and dose of GC therapy, with dexamethasone displaying the worst metabolic profile (16). Despite this, current Guidelines do not recommend one singular form of GC over another in adult patients with CAH (2). Interestingly, in the study by Falhammar et al. which included adult patients, most had isolated obesity in the absence of other CV risk factor (17). The influence of genotype on metabolic imbalance was also studied, but no correlation was established between the degree of enzymatic deficiency and obesity (18). In such a complex scenario, lifestyle and family history should always be considered as potential contributors to obesity; Torky et al. indeed showed a significant association between the presence of obesity in adult CAH patients and maternal obesity (19). Since obesity represents the main risk factor for the development of other metabolic comorbidities, lifestyle modification should be always encouraged in these patients.

Arterial hypertension

It is widely recognized that GC excess can increase arterial blood pressure through different mechanisms, including MC mimetic activity, alterations in peripheral and renovascular resistance, reduced nitric oxide system and vascular remodelling

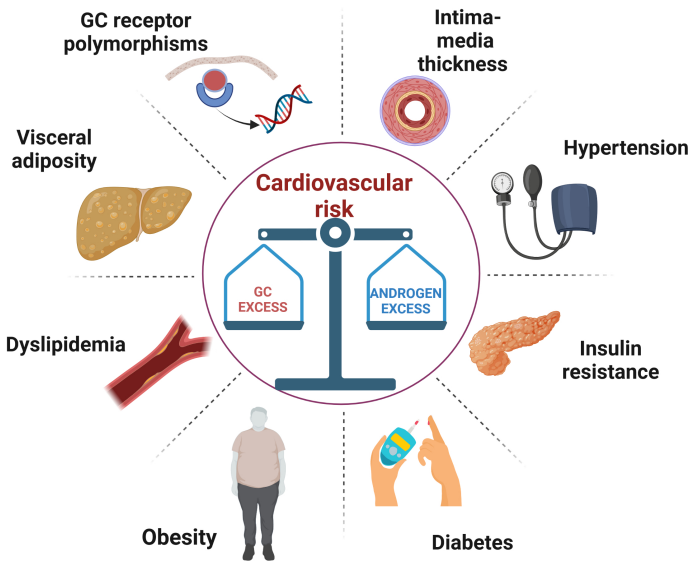

FIGURE 1
A schematic illustration of the metabolic and vascular complications caused by the difficult balance between glucocorticoid overtreatment and androgen excess in congenital adrenal hyperplasia. GC, glucocorticoid.

(20). As a direct consequence of chronic steroid therapy, hypertension is frequently observed in CAH, possibly related to supra-physiological doses of GC although not all studies are concordant (21). An increased 24h ambulatory blood pressure was confirmed in patients with CAH compared to a control group matched for age, sex and BMI (22). Despite this, few studies have assessed the association between GC dose and hypertension in CAH (17, 23–26). Regarding the type of GC used, a direct correlation was observed between blood pressure and dexamethasone treatment in a U.K. cohort of patients (27). Recently, the role of GC in arterial hypertension in adult CAH has been validated by the strong association between blood pressure and suppressed androstenedione level, reflective of excessive GC dose (19). Interestingly, the same study reported a direct correlation between MC dose and arterial blood pressure in childhood (19). Indeed, the contribution of MC replacement cannot be overlooked (4, 17, 19, 28–30) as it is associated with cardiac hypertrophy, vascular remodelling and glomerulosclerosis irrespective of blood pressure levels (31). Fludrocortisone is mainly used in SW-CAH and increases blood pressure through its direct binding with the mineralocorticoid receptor (MR) (32, 33). Periodical assessment of volume status, electrolytes and/or renin activity is suggested to avoid excessive MC replacement (2). As observed in different studies, the need for MCs varies during one's lifetime (18, 30, 34); the physiological resistance to MCs is more pronounced during early childhood due to a lower MC sensitivity in the kidneys, and then decreases with age. Salt supplementation in infancy was associated with lower GC and MC requirement, but no differences were reported in anthropometric parameters; however, blood pressure and body weight tended to increase after the first year of life in both salt-treated and non-salt-treated children with SW form (35).

Hypertension secondary to relative MC overtreatment was probably due to a delay in fludrocortisone down-titration required during long-term follow-up (4, 9, 19, 28, 29). In a recent study Subbarayan et al. confirmed the correlation between fludrocortisone dose and diastolic blood pressure (29); interestingly, they reported an overall reduced incidence of hypertension in CAH compared to previous studies (36, 37). This change probably reflects the use of lower dosing schedules than those previously given (29). Contrastingly, some studies showed no increases in associated hypertension, probably related to both GC and MC under-treatment, as supported by the marked elevation in ACTH and plasmatic renin levels found in most of the patients included (38). A recent cross-sectional study found a direct correlation between 17-hydroxyprogesterone and diastolic blood pressure in children and adolescents with CCAH, suggesting that poorly control disease can pose the basis for cardiovascular risk as well (39).

Vascular disease

Intima media thickness (IMT) is a widely recognized sign of arterial impairment and a predictor of future coronary artery disease and stroke (40). An increased IMT at different vascular districts (common carotid, abdominal aorta, carotid bulb, femoral arteries) was observed in adult patients with CCAH (26); however, this study did not demonstrate progression from increased IMT to atherosclerotic plaque formation. This finding was also observed in younger patients cohort (41–44), especially in hypertensive patients and in those with non-dipping profile (45); i.e. Özdemir et al. found a higher IMT and reduced aortic and carotid distensibility in patients compared to controls, suggesting the presence of early subclinical atherosclerosis (41).

This effect was also present subjects with NCCAH that exhibited higher IMT values in all main arterial districts, as observed for CCAH forms (42); moreover, it was demonstrated, in both CCAH and NCCAH, that abdominal aorta IMT, as well as common carotid artery, were positively correlated with cumulative GC doses and triglyceride serum levels and negatively with androstenedione (an indirectly confirmation of GC treatment excess).

Furthermore, some evidence indicates that IMT could be related to androgen excess (46, 47) too. Carotid IMT was associated with increased androgens (17OH-progesterone and androstenedione) in CAH adolescents and young adults (46). To further stress the importance of adequate GC therapy and androgen control, a recent study showed no increase in IMT in CAH patients, thanks to optimal androgen control in most of the cohort (7). Signs of endothelial dysfunction, measured by flow-mediated dilatation (FMD) and glyceryl trinitrate-mediated dilatation (GTN) were observed in adolescents with CAH, and the impairment was comparable to that of the obese control group (48). Furthermore, novel markers of inflammatory response such as neopterin, a catabolic product of guanosine triphosphate secreted by activated macrophages that might promote vascular dysfunction and plaque formation, were found to be increased in CCAH (49).

Despite these findings, an echocardiographic study found a preserved myocardial function in young CAH adults, although higher GC doses were associated with detrimental effects on cardiac hypertrophy, left ventricular and aortic dilation and subclinical atherosclerosis (50). An increased epicardial fat thickness was observed in children with CAH, strictly related to both BMI and waist circumference. This alteration indicates a "low-grade inflammatory status" that pave the way to the atherosclerotic process later in life (47).

Insulin resistance and Diabetes Mellitus (DM)

Patients with CAH have an increased risk of developing insulin resistance (IR) and subsequently diabetes mellitus (DM) due to chronic GC therapy (3, 4, 17, 27, 34, 38, 51). Mechanisms behind GC-induced DM include among others, IR, increased

hepatic gluconeogenesis and reduced insulin secretion (52). IR in CAH seems to be related not only to cumulative dosing, but also to GC type. A cross-sectional study on adult patients pinpointed a higher prevalence of IR in patients on long-term dexamethasone compared to those taking either prednisolone or hydrocortisone (27). Since dexamethasone is usually given at night to suppress early morning ACTH surge, its detrimental effect on glucose metabolism can be driven also by the disruption of physiological cortisol rhythmicity (53). Although IR has rarely been studied in NCCAH, its prevalence seems to be less pronounced compared to CCAH (54, 55). This may be due to higher GC doses used to suppress androgen secretion in the classic form (4).

One recent study examined a relatively small group of young patients (22, of which 13 were still on steroid therapy) using the hyperinsulinemic-euglycemic clamp, showed a higher rate of IR in both lean and obese patients with NCCAH compared to controls. Interestingly, the compensatory hyperinsulinemia was mainly sustained by a reduction of hepatic insulin clearance rather than a net increased insulin secretion (55). As previously observed for classic form, IR was associated to long-term GC therapy and especially with long-acting GC like dexamethasone (51).

Besides GC, other risk factors such as familiar history of diabetes and obesity can disrupt this delicate balance and boost the development of DM.

Androgen levels should be encountered amongst risk factors as well. To be precise, androgens have different effect on IR depending on gender; low testosterone levels promote metabolic syndrome in men (56), whilst hyperandrogenism is associated with IR, obesity and metabolic syndrome in women, as extensively demonstrated in polycystic ovarian syndrome (57). The negative impact of androgen excess in CAH was confirmed in a cohort of GC-naïve Chinese female with SV-CCAH; IR and adverse metabolic markers were significantly higher in patients compared to controls and directly related to testosterone levels (58). Two other studies supported the role of androgens in IR development in women with CAH and found that IR was more commonly associated with poor androgen control due to low GC replacement (5, 7). Therefore, supraphysiological GC treatment may not be the sole reason for impaired insulin sensitivity, especially in female patients, where androgen excess could play a relevant role.

Despite a host of risk factors, only the Swedish CAH registry found an increased prevalence of DM, especially in female patients, compared to general population (17). As for other comorbidities, it is plausible that the true extent of DM in CAH may be underestimated by the relatively young ages of CAH patients studied so far, and limited lifetime follow-up.

Type of GC and CV risk

Unlike in childhood where hydrocortisone is the GC of choice, in adults there is little consensus on the most appropriate GC replacement regimen. Once final height is achieved, patients are frequently shifted to long-acting GCs to allow once daily dosing and consequent better treatment compliance (2). A recent meta-analysis supported a negative impact of dexamethasone on metabolic parameters compared to either prednisone or hydrocortisone (16). These detrimental effects may be at least partially due to reverse circadian dosing, with long-acting synthetic GC given at bedtime. Therefore, other GCs might be preferred over dexamethasone in the presence of metabolic comorbidities. Dual-release hydrocortisone demonstrated an improvement in glucose metabolism in patients in adrenal insufficiency compared to standard hydrocortisone replacement; however, androgen control was labile in the 6 CAH patients included and there are no data on long-term effects on CV risk (59). More promising results were provided by the use of a delayed-release hydrocortisone formulation that guaranteed a stricter adrenal androgen control with lower steroid dose (60). However, further data are required to determine whether these encouraging results can be translated into long-term cardiovascular health.

Androgen secretion

Androgen excess is one of the hallmarks of CAH, and its control is frequently challenging. Both hypoandrogenismin males and hyperandrogenism in females can lead to adverse metabolic effects thereby increasing CV risk (23, 61, 62).

In the study of 203 patients by Arlt et al., androgens levels were poorly controlled, with most patients having either elevated or suppressed androgens, whereas only 36% presented with normal androstenedione levels (3).

Similar results were found in another study, with only half of the whole cohort having androgens level within normal range (4). New treatment approaches targeting the CRF type 1 receptor at pituitary levels (crinecerfont and tildacerfont) showed promise in reducing androgen biomarkers; ongoing studies are needed to demonstrate long-term improvement in metabolic profile (63).

Dyslipidaemia

Dyslipidaemia does not seem to be significant in CAH patients, as shown by several studies (23, 38, 44, 48). In a cohort of 244 patients only 2% of children and 6% of adults had elevated cholesterol, but with concomitant increase in HDL (4). Very few studies showed alterations in blood lipid profile; Arlt et al. found high cholesterol levels in 46% of adult patients (3) whereas a recent American study of children and young adults showed an increased incidence of low HDL (57.9%) and high triglycerides (42.1%), although this cohort was biased by a higher rate of obesity compared to other European series (19).

The same study suggested a detrimental role of GCs on lipid profile in children, with higher total cholesterol and LDL in those with tighter disease control. Similarly, androstenedione suppression and excessive MC therapy were risk factors for low HDL and high LDL, respectively, in adult patients (19). However, as demonstrated by Zhang et al. on 30 untreated females with SV form, higher TG and lower HDL-cholesterol were associated with androgens excess (58). By the same token, dyslipidaemia is a common finding even in NCCAH (3). Surprisingly, when compared to a controls matched for sex, age and BMI, patients with CAH tended to have higher HDL-cholesterol and adiponectin (22).

It should be mentioned that most available studies involved young patients, thus the true estimate of lipid impairment might be under-representative.

The role of GC receptors polymorphism in CAH patients

Apart from cumulative GC dose, the effect of systemic therapy can be modulated at receptor level; polymorphisms of the GC receptor gene (*NR3C1*) can be either associated with adverse or beneficial metabolic and cardiovascular profiles in general population. The ER22/23EK haplotype is associated with reduced GC sensitivity, thus to a more favorable metabolic profile, whilst the N363S and *BclI* restriction fragment length polymorphism (RFLP) cause GC hypersensitivity (64). As a result, patients harboring the N363S mutation, have a higher incidence of type 2 DM, obesity and CV diseases (65, 66). Similarly, individuals with the *BclI* mutated population has higher prevalence of obesity and hypertension (67). GC polymorphisms have been studied in CAH patients as well. Heterozygotes for the *BclI* haplotype present with higher BMI, waist circumference and higher systolic blood pressure compared to wild-type subjects (68), even though the frequency of this polymorphism was less common in patients with CAH than in general population (69).

The A3669G-polymorphism was instead inconsistently associated with adverse lipid profile in paediatric CAH patients (68, 70). *NR3C1* genotyping might play an important role in predicting individual response to GC therapy and identify patients at high risk for GC-related complications.

Cardiovascular events

Most of the studies on metabolic risk include children or young adults, thus longitudinal studies on subject aged greater than 30 years are scant. A Swedish cohort of 588 patients with CAH of all ages showed an increased risk of CV disease (OR 2.7) compared to reference population; however, no significant differences for cardiac arrest, heart failure and atrial fibrillation were observed. An increase incidence of stroke was reported only for female with NCCAH. Instead, acute coronary syndrome was significantly increased in males with SW-CAH, especially in those with null genotype (17). Also increased thromboembolic events, mostly in female patients, was reported, potentially related to obesity and GC-induced hypercoagulability. However, the contribution of GC therapy to these events was not assessed (17). The same authors found that CV events were the second leading cause of mortality, although half of these patients had a concomitant infection that might have resulted in an overestimation of the CV-related death due to a concomitant adrenal crisis (71).

Interestingly, in the UK cohort only a minority of adults affected by CAH are regularly follow-up by an endocrinologist; this lack of disease-monitoring might negatively impact on patients' well-being and contribute to their mortality rate (3).

Cardiovascular mortality

To date, few studies have assessed disease mortality in adults. Fahlhammar et al. found a higher mortality rates in patients with CAH, the main causes of death were adrenal crisis (42%), CV disease (32%), cancer (16%) and suicide (10%) (71). However, it should be mentioned that false inflation of mortality rates may have been due to increased perinatal mortality prior to introduction of neonatal screening. After excluding of patients who died within the first year of life in whom the correct diagnosis was probably missed, the mortality rate remained higher only for SW-CCAH form and in affected females. A U.K. study found a greater all cause-mortality risk in CAH patients; the mean age at death was 50 years but the underlying causes were not reported (72). Overall, there has been an increase in survival over time reflecting the improvements in diagnostic criteria and GC treatment; the mean age of death in the Swedish CAH population has increased from 21 years during the 1980s to 57 years in 2010 (71).

These data were also confirmed in a recent German study which found an increased mortality in the period between 1973 and 2004 (even after introduction of general screening), mainly related to inadequate GC stress-dose administration, but no further deaths thereafter, supporting the importance of patient education in preventing adrenal crisis (73).

Cardiovascular health in uncommon forms of CAH

Other genetic forms account together for the remaining 5% of all cases of CAH. According to the defective enzyme, the resulting changes include a deficiency of steroids downstream

from the block and an accumulation of precursors upstream that can be shunted to other pathways, Figure 2 (74).

Patients with 11β-hydroxylase deficiency (11-OHD) have deficient aldosterone and cortisol production but elevated androgens and MC precursors (75). Arterial hypertension is found in up to two thirds of patients with classic 11-OHD (76–78) and it could appear in childhood or even only later in life (79, 80); non-classic forms are often normotensive at diagnosis (81). Usually hypertension is mild, but severe cases that required bilateral adrenalectomy, have also been described (82–85). Moreover, malignant hypertension can lead to left ventricular hypertrophy, retinopathy, ischemic heart disease, nephropathy, cerebrovascular events and also death (77, 78, 84–86). These complications were mainly reported in poorly controlled patients as indirectly documented by the severity of virilization (78). Other comorbidities have been rarely described in 11-OHD; obesity is less frequent in 11-OHD compared to 21-OHD (78, 81, 87–89), as observed in a *Cyp11b1* null mouse model, which reflects the absence of direct GC effect on adipose tissue (90). Insulin resistance was found in 10.7% of the population analyzed (78) potentially related to MC excess and hypokalemia (90), but overt diabetes mellitus has been rarely reported (91).

Although there are no studies that directly assessed mortality in 11-OHD, these patients seem to have an increased risk of CV events but fewer adrenal crisis compared to patients with CCAH (77, 92, 93); however, they can develop adrenal insufficiency if GC therapy is withdrawn, because 11-deoxycorticosterone (DOC) alone is not sufficient to prevent adrenal crisis during stressful events (94).

The 17α-hydroxylase deficiency (17-OHD) is the second most common CAH in Brazil; besides MC excess, patients display androgen and estrogen deficiency but they do not manifest adrenal insufficiency thanks to corticosterone excess (74). In a Brazilian cohort of 24 patients with 17-OHD two hypertensive young patients of 34 and 27 years had a history of stroke (95). However, a possible contribution of lifestyle or other genetic factors was advocated as a potential confounding factor (95, 96).

Studies on patients with 3β-hydroxysteroid dehydrogenase type II deficiency (3β-HSD) included neonatal or children, thus there are no data on CV risk in adolescents/adults (97).

The lipoid form (LCAH) is the second most common type of CAH in Japan and Korea (98). The largest study including 57 patients, 43 with classical form, (aged 0.0–47.5 years) did not report CV events (99). To date, there are no longitudinal studies on CV risk in LCAH patients with classical form (100). Similarly, the prevalence of CV risk factors in the milder non-classic form of LCAH (NCLAH) are lacking (100). There is just one case report of an overweight 26 years old female patient with NCLAH

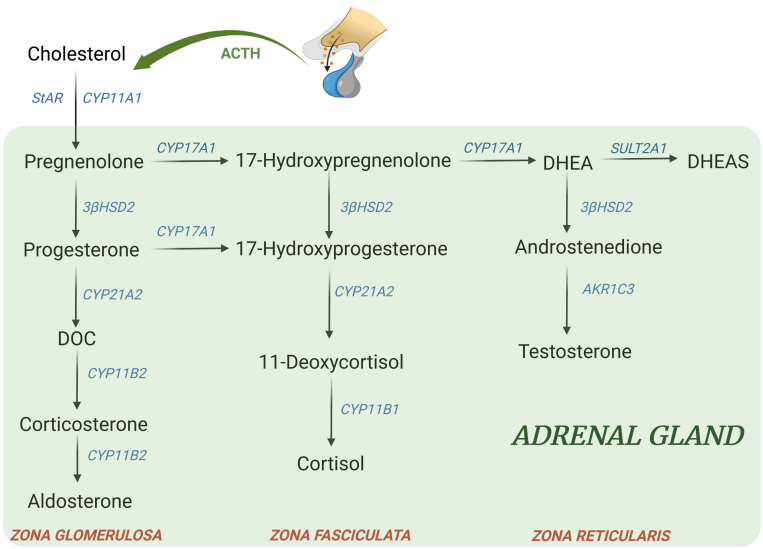

FIGURE 2
Major adrenal steroid synthesis pathways. Uncommon CAH forms include: - 11β-hydroxylase deficiency (11-OHD): block at CYP11B1 → ↓ cortisol; ↑ DOC, ↑ 17-hydroxyprogesterone, androgens, ↑ 11-deoxycortisol - 17α-hydroxylase deficiency (17-OHD): block at CYP17A1 → ↓ 17-hydroxyprogeserone, ↓ androgens; ↑ DOC, ↑ corticosterone - 3β-hydroxysteroid dehydrogenase type II deficiency (3β-HSD): block at 3β-HSD → ↓ aldosterone, ↓ androgens in male; ↑ 17hydroxypregnenolone, ↑ 17-hydroxyprogesterone, ↑ DHEA - Lipoid form (LCAH)→ block at StAR level → ↓ all steroids - P450 oxidoreductase deficiency (PORD): multiple partial blocks of CYP17A1 and CYP21A2→ ↓ androgens; ↑ progesterone, ↑ 17-hydroxyprogesterone, ↑ corticosterone (74). DOC, 11-deoxycorticosterone; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate.

who had concomitant borderline blood pressure but normal HbA1c and lipid profile (101).

Conclusions

Diagnostic and treatment developments over the years have normalized life-expectancy in patients with CAH. Despite this, series on aging CAH patients are still limited in literature. To date, most available studies pointed to an increase cardio-metabolic risk in CAH patients. Features of metabolic syndrome can appear early in childhood and should be rapidly addressed to reduce future CV events. GC overtreatment seems to have a pivotal role in promoting weight gain, which represents the main independent risk factor for the development of metabolic syndrome and directly contributes to hypertension, IR and hypercoagulability. Long-acting GCs may be associated with adverse metabolic profile, but current data are not sufficient to draw conclusions on the best GC schedule for adult patients. Similarly, excessive MC replacement, particularly if not properly down-titrated in adulthood, can contribute to the increased CV risk. On the other hand, androgen excess can be equally harmful and negatively impact on several metabolic aspects, as observed in NCCAH. As a general rule, patients with CAH should receive the lowest possible GC dose to prevent adrenal insufficiency and signs and symptoms of hyperandrogenism. A periodic screening for arterial hypertension and metabolic comorbidities should be performed since early infancy to avoid future CV events. Similar conclusions can be drawn also for rarest forms of CAH, although the CV risk profile of these patients is less characterized due to the low number of studies available. Ongoing and future studies will clarify whether new compounds might promote reduced GC overexposure and guarantee a more stable disease control resulting in a more favorable metabolic profile. Overall, available data pointed to an increased mortality in CAH patients, probably biased by older studies when neonatal screening was not available and GC replacement not appropriate. Conversely, CV morbidity and mortality might be significantly underestimated, since most studies analyze adolescents or young adults, while CV events usually occur later in life. Future perspective can include GR polymorphisms genotyping to identify patients at higher CV risk and allow a personalized GC treatment to avoid long-term adverse consequences.

Author contributions

MB and CS contributed to conception of the study. PM and ML performed bibliographic research. MB, PM, and ML were involved in drafting the manuscript. PM and FC were in charge of figures. MB, FC, and CS were involved in manuscript revision. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Adrenal bleeding due to pheochromocytoma - A call for algorithm

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Background: Adrenal hemorrhage is a rare, usually life-threating complication. The most common neoplasm resulting in spontaneous adrenal bleeding is pheochromocytoma and it accounts for nearly 50% of cases. Currently, the recommendations for the diagnosis and management of patients with adrenal bleeding due to pheochromocytoma are unavailable.

Materials and methods: We performed a database search for all pheochromocytoma patients, diagnosed and treated from 2005 to 2021 in tertiary endocrinology center. 206 patients were identified, 183 with complete data were included in the analysis. We investigated clinicopathological characteristics, treatment and outcomes of hemorrhagic pheochromocytoma cases and characterize our approach to perioperative diagnosis and medical management. Finally our experiences and data from previously published articles concerning adrenal hemorrhage were analyzed to propose a diagnostic and therapeutic algorithm for hemorrhagic pheochromocytomas.

Results: In the whole group, seven patients (4 men and 3 women) with adrenal bleeding were found, (3.8%). Median patient's age was 49 years (range: 36-78 years). The most common manifestation of adrenal bleeding was acute abdominal pain (5/7). Two patients developed shock. Hormonal assessment was performed in five patients, based on 24-hour urinary fractionated metanephrines with urinary 3-methoxytyramine. Normetanephrine was elevated in all patients, metanephrine and 3-methoxytyramine - in four cases (4/5). Most patients (6/7) had symptoms suggesting pheochromocytoma before hemorrhage – most commonly paroxysmal hypertension (4/7). One patient died, before the diagnosis of adrenal bleeding was made. Diagnostic imaging performed in six out of seven patients revealed adrenal tumor, with median largest diameter equal to 7.4 cm (range: 5-11 cm). Five patients had

elective surgery, in one case an urgent surgery was performed. In all cases the diagnosis of pheochromocytoma was confirmed in postoperative histopathology or in autopsy. The perioperative survival rate was 85.7%.

Conclusions: Diagnosis of pheochromocytoma should be always considered in patients with adrenal bleeding, especially with accompanying abdominal pain, hemodynamic shock and previous history of pheochromocytoma-associated symptoms. Lack of proper diagnosis of pheochromocytoma before surgery is associated with an additional perioperative risk. To improve the decision making in this life-threatening clinical situation, based on our results and literature data, we proposed a diagnostic and treatment algorithm.

KEYWORDS

pheochromocytoma, hemorrhage, adrenal, bleeding, diagnosis, treatment

Introduction

Spontaneous adrenal hemorrhage is a rare, potentially life-threatening condition. According to literature, the most common neoplasm resulting in spontaneous adrenal bleeding is pheochromocytoma, which accounts for nearly 50% of cases (1). The mortality rate of a ruptured pheochromocytoma is reported to be about 30% (2, 3). Hypovolemia from hemorrhagic shock, heart failure as a result of catecholamine excess, postoperative hypotension or pulmonary oedema are leading causes of perioperative mortality (2, 4). Although most cases of hemorrhage within a pheochromocytoma are spontaneous, sometimes episodes of bleeding could be precipitated by preceding anticoagulant therapy (5), trauma (6) or thrombolysis (7). Lately, the first case of hemorrhage in a pheochromocytoma in the course of SARS-CoV-2 infection was described (8). Moreover, adrenal hematoma could sometimes mimic neoplasm of the adrenal gland (1, 9, 10).

Most publications dedicated to a hemorrhagic pheochromocytoma are case reports, with only several series published to date, including four literature reviews and one case series (1–3, 11, 12).

Therefore we reviewed the records of patients with hemorrhagic pheochromocytoma treated in our department. Our objective was to investigate clinicopathological characteristics, treatment and outcomes of hemorrhagic pheochromocytoma cases and characterize our approach to perioperative diagnosis and medical management. Based on our results and literature data we proposed a diagnostic and treatment algorithm.

Materials and methods

We performed a database search for pheochromocytoma patients, diagnosed and treated in tertiary endocrinology unit from 2005 to 2021. 206 consecutive patients with pheochromocytoma were identified. Subsequently, 23 cases were excluded due to incomplete medical data (histopathological or imaging) necessary to rule out potential adrenal bleeding. Of the remaining 183 patients with histologically confirmed pheochromocytoma, seven cases with adrenal bleeding confirmed in histopathological examination were found (3.8% of cases). Clinical manifestation, imaging, hormonal status and histopathological results were analyzed. All imaging records of patients suspected for adrenal bleeding were reassessed by one radiologist, skilled in adrenal gland pathology (AG).

24-hour urinary fractionated metanephrines and 3-metoxytyramine were measured using high performance liquid chromatography with electrochemical detection.

Due to the need of urgent hormonal assessment in most cases, only 3 patients have followed the proper dietary restrictions regarding catecholamine-rich products withdrawal before and during 24-hour urine collection.

The summary of patient's concomitant medications and diet are summarized in Table 1.

Statistical analysis

Demographic and clinical characteristics were analyzed by the use of frequency tables for categorical variables and by

TABLE 1 Summary of clinical and pathological characteristics of the patients.

	7	6	5	4	3	2	1	Number of the patient
Median value: 49 yrs	64	49	36	48	36	68	78	Age (yrs)
M:W=4:3	W	W	W	M	M	M	M	Sex
Most common – abdominal pain 5/7 (71.4%)	Abdominal pain	Chest pain, flank pain, paroxysmal hypertension, takotsubo cardiomyopathy	Flank pain, chest pain, tachycardia, takotsubo cardiomyopathy	Abdominal pain, flank pain, headache, nausea and vomiting, paroxysmal hypertension	Abdominal pain	Abdominal pain, nausea and vomiting, paroxysmal hypertension followed by hypotension, seizures with short-lasting apnoea, tachycardia	Abdominal pain, nausea and vomiting, hypotension, MOF***	Symptoms of bleeding
Yes -2/7 (28.6%)	No	No	No	No	No	Yes	Yes	State of shock?
Yes– 3/7 (42.9%)	Yes	No	Yes	No	No	Yes	No	Anemia?
Yes - 3/7 (42.9%)	No	No	No	Yes	No	Yes	Yes	Hyper-glycemia?
No – 5/7 (71.4%)	No	No	No	No	No	No	Yes – aspirin and clopidogrel	Platelet-inhibiting medication or anti-coagulants?
Yes – 6/7 (85.7%)	Yes (persistent hypertension)	Yes (paroxysmal hypertension, pallor, anxiety, tachycardia, headache,diaphoresis)	Yes (paroxysmal hypertension).	Yes (paroxysmal hypertension).	Yes (headaches, paroxysmal hypertension, tremor, pallor, tachycardia, anxiety)	Yes (tachycardia, dyspnoea on exertion).	No	Symptoms suggestive of pheochromocytoma before hemorrhage?
Median value 5 months	48 months	3 months	1 month	24 months	5 months	0,5 month	6 months	Time between onset of symptoms and diagnosis of pheochromocytoma (months)
Yes- 6/7 (85.7%)	Yes– at the time of adrenal hemorrhage	Yes – Sixteen months before the hemorrhage	Yes– at the time of adrenal hemorrhage	Yes– at the time of adrenal hemorrhage	Yes – two months before the hemorrhage	Yes – at the time of adrenal hemorrhage	No	Pheochromocytoma suspected preoperatively?
Right – 6/7 (85.7%)	Right	Right	Right	Right	Right	Right	Left	Affected side
Intra-tumoral – 6/7 (85.7%)	Intra-tumoral	Intra-tumoral	Intra-tumoral	Intra-tumoral	Intra-tumoral	Intra-peritoneal and Retro-peritoneal	Intra-tumoral	Location of bleeding
Median value 7.4cm	6	7.5	9.5	7.4	6.2	11	5#	Maximum diameter of the tumour (cm)
Median value 1929.9ug/24h	785.8 (2.3 fold)	348.6 (1.02 fold)	9738.8 (28.6 fold) – ongoing hemorrhage	8759.4 (25.7 fold)	1929.9 (5.7 fold)	N/A	N/A	MN^ (ug/ 24h) Range: 0-341 ug/24h

(Continued)

TABLE 1 Continued

7	6	5	4	3	2	1	Number of the patient
		355.6 (1.0 fold) – 3 days later					
844.4 (1.9 fold)	4679.9 (10.6 fold)	29927.7 (68 fold) – ongoing hemorrhage 1312.6 (3.0 fold) – 3 days later	6034.9 (13.7 fold)	3647.7 (8.3 fold)	N/A	N/A	NMN^^ (ug/ 24h) Range: 0-440 ug/24h
30.58	404.7	1190.6 – ongoing hemorrhage 539.1 (2.5 fold) – 3 days later	1764.9	505.1	N/A	N/A	3-MT^^^ (ug/ 24h) Range: 0-220 ug/24h
Yes, 14 days	No	No	Yes, 3 days	Yes, 14 days	Hormonal assessment not done	Hormonal assessment not done	Catecholamine -rich food restrictions before assessment?
Perindopril Indapamide Carvedilol Omeprazole Iron supplements	Acetylsalicylic acid Amlodipine Atorvastatin Pantoprazole	Enoxaparin Pantoprazole Iron supplements Acetylsalicylic acid Furosemide	Human insulin Enoxaparin Perindopril Atorvastatin Metoprolol	No medications	Hormonal assessment not done	Hormonal assessment not done	Concomitant medications used at the moment of hormonal assessment
Yes 2 miligrams Of doxazosin	Yes 70 miligram Of phenoxybenzamine	Yes 60 miligrams Of phenoxybenzamine	Yes 40 miligrams Of phenoxybenzamine	Yes 12 miligrams Of doxazosin	Yes 6 miligrams Of doxazosin	No	Alpha blockers administration
Elective	Elective	Elective	Elective	Elective	Urgent	No surgery	Type of surgery
Administration of norepinephrineduring surgery	Uneventful	Red blood cell transfusion	Uneventful	Administration of urapidil during surgery	Red blood cell transfusion	No surgery	Perioperative course
5	4	N/A	1	3	2	N/A	PASS score
<1%	2%	N/A	N/A	<1%	3,4%	N/A	Ki-67
No	No	No	No	No	No	Yes	Death?
77 months	89 months	60 months	156 months	36 months	11 months	Patient died before the diagnosis	Follow-up period

*M: Man **W: Woman *** Multiorgan failure # tumour size on autopsy.
^MN – metanephrine ^^ NMN – normetanephrine ^^^3-MT – 3-methoxytyramine.
N/A – not available.

calculation of the median and range for continuous variables. Length of follow-up was presented as range and median value in months, from the time of curative surgery until the last follow-up appointment. The mean values were calculated with standard deviation. The software Statistica 13 made by StatSoft Polska in 2017 was used.

Consort diagram for the study population is presented on Figure 1.

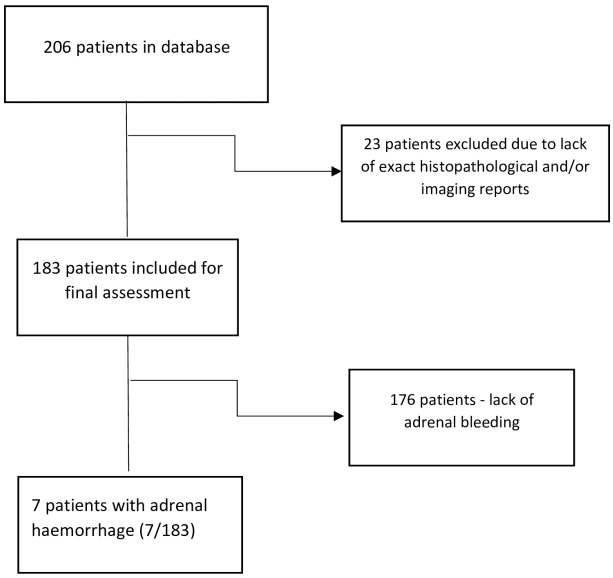

FIGURE 1
Consort diagram for the final study population.

Results

Clinical presentation

In the whole cohort, seven patients with adrenal bleeding were found, comprising 3.8% of pheochromocytoma cases (four men and three women). Median patient age was 49 years (range: 36-78 years). All patients had spontaneous adrenal hemorrhage in the absence of recent abdominal trauma. One patient had a history of platelet-inhibiting treatment. The most common manifestation of adrenal bleeding was acute abdominal pain (5 out of 7 patients).

Other symptoms accompanying ongoing hemorrhage were: nausea and vomiting (3/7 patients), paroxysmal hypertension (3/7 patients), flank pain (3/7 patients), chest pain (2/7 patients), tachycardia (2/7 patients), seizures with short-lasting apnoea (1/7 patients), headache (1/7 patients). Two patients developed shock, in one case resulted in multiple organ failure (MOF). In both patients with severe chest pain, takotsubo cardiomyopathy was diagnosed.

Most patients (6/7 cases) had symptoms suggestive of a pheochromocytoma prior to adrenal hemorrhage – most commonly paroxysmal hypertension (4/7). The median time period between onset of symptoms and diagnosis of pheochromocytoma was 5 months (range: 0.5-48 months).

Nevertheless, in four patients diagnosis of pheochromocytoma was made at the time of adrenal hemorrhage, based on severe clinical manifestation, hormonal status and/or imaging. One patient died, before the diagnosis of adrenal bleeding was established. In two patients pheochromocytoma was suspected before the episode of hemorrhage: two months and sixteen months, respectively.

Imaging

Six out of seven patients had diagnostic imaging: All patients underwent contrast – enhancement computed tomography (CT), with one angio-CT scan. One patient had additional magnetic resonance imaging (MRI) done. Median largest diameter of the lesions was 7.4 cm (range: 5-11 cm). The images revealed different stages and severity of bleeding. The most common features on CT scans were solid-cystic appearance of the lesions, with strong enhancement of the solid component (four patients) and forms of thick-walled hemorrhagic cysts (four patients).

The summary of CT and MRI results are presented in Table 2. The CT and MRI images are shown on Figures 2–5.

Laboratory assessment

Hormonal assessment concerning pheochromocytoma was performed in five patients, based on 24-hour urinary fractionated metanephrines with urinary 3-methoxytyramine.Normetanephrine was elevated in all patients (from 1.9 to 68-fold above the upper limit). Metanephrine concentration was substantially increased in 4 cases (from 2.3 to 28.6-fold above the upper limit) - in one case it

TABLE 2 Summary of the results of Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) in six out of seven patients.

Patient's Number	Computed Tomography	Magnetic Resonance Imaging
1	Not done	Not done
2	Angio-CT: - Right adrenal mass with heterogenous enhancement. - Strong peripheral enhancement. - Suspicion of rupture of central part of the tumour with contrast extravasation to intraperitoneal and retroperitoneal space. - Tumour size: 110x65x80 millimetres.	Not done
3	- Thick – walled hemorrhagic cyst of right adrenal gland. - Strong capsule contrast enhancement. - Central area of fluid attenuation suggestive of recent hemorrhage. - Tumour size:62x60x50 millimetres.	Not done
4	- Thick – walled hemorrhagic cyst of right adrenal gland. - Strong capsule contrast enhancement. - Tumour size: 68x48x74milimetres.	Not done
5	- Right adrenal lesion with heterogenous density. - No contrast enhancement – suggestive of hematoma. - Tumour size: 80x65x95milimetres.	- Right adrenal oval mass. - Low signal intensity on T1-weighted images. - T1 hyper -intense peripheral area, corresponding to blood. - Heterogenous, mostly hyperintense signal on T2-weighted images with fluid-fluid level. - No typical enhancement after contrast administration. - Tumour size: 84x77x96 millimetres.
6	- Right adrenal mass with solid-cystic appearance. - Strong peripheral enhancement (of the solid component) - Tumour size: 75x70x69 millimetres.	Not done
7	- Right adrenal lesion with solid-cystic appearance. - Central area of fluid attenuation, with fluid-fluid level in the posterior part. - Strong peripheral enhancement (of the solid component). - Tumour size: 62x56x56 millimetres.	Not done
Summary	Solid-cystic appearance with strong enhancement of the solid component:4/6 (66.7%) Thick-walled hemorrhagic cyst with strong enhancement of the solid component:3/6 (50%) Thick-walled hemorrhagic cyst with no contrast enhancement: 1/6 (16.7%) Fluid-fluid level:2/6 (33.3%) Contrast extravasation to retro/intraperitoneal space: 1/6 (16.7%) Heterogenous density 2/6 (33.3%)	

only slightly exceeded the normal range. In all patients, at least one metabolite of catecholamines – metanephrine or normetanephrine - was significantly elevated (more than twice the upper reference limit). Both metanephrines were increased in 4 out of 5 patients subjected to hormonal assessment. 3-metoxythyramine was elevated in 4 patients. The median levels of metanephrine, normetanephrine and 3-methoxytyramine were 1929.9 ug/24h (range: 348.6-9738.8), 4679.7 ug/24h (range: 844.4-29927.7) and 505.1 ug/24h (range: 30.58-1764.9), respectively. Two patients had no hormonal tests – one died before the diagnosis of adrenal hemorrhage was made, in the other, an urgent surgery was not preceded by evaluation of hormonal activity. However, the radiological images suggested pheochromocytoma and typical pharmacological treatment was implemented. Anemia was observed in three out of seven cases, similar to hyperglycemia, which was noted also in 3/7 patients.

Treatment

In six out of seven patients pheochromocytoma was suspected preoperatively, based on clinical manifestation, hormonal status or imaging. One patient had been admitted to hospital in hemorrhagic shock and multiple organ failure and had died before an etiology of adrenal bleeding was revealed. The diagnosis of hemorrhagic pheochromocytoma was made on autopsy. In five cases, the elective surgery was performed, preceded by a two- week pharmacological treatment with alpha-receptor blockers. In one case, due to patient's prolonged hemodynamic instability despite supportive care, accompanied by rapid decrease of hemoglobin level and radiological suspicion of intraperitoneal hemorrhage, four-day alpha-receptor blockage was administered, followed by the urgent surgery. None of the patients has transcatheter arterial embolization (TAE) procedure performed before the surgery. In perioperative

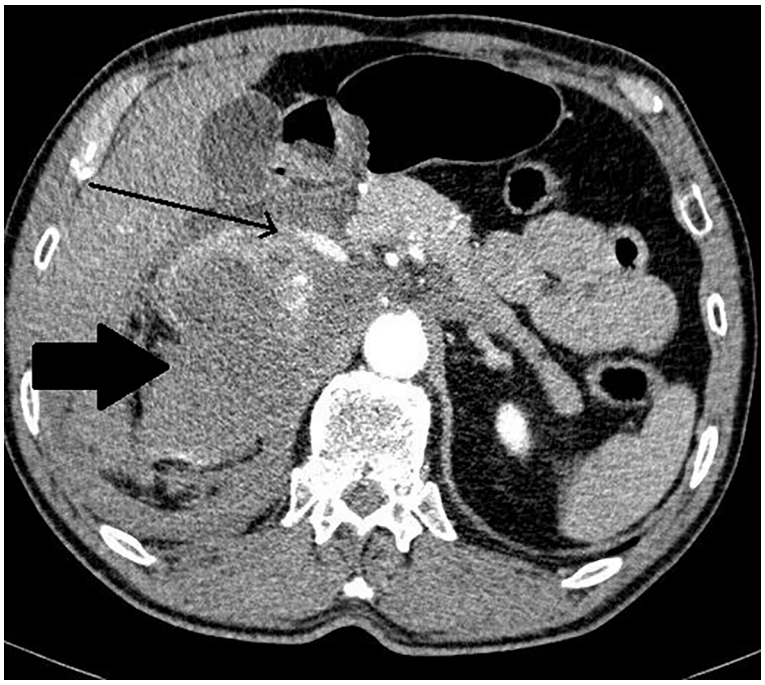

FIGURE 2
Patient number 2: angio-CT, arterial phase, axial image- right adrenal mass with heterogenous enhancement (thick arrow), suspicion of rupture of central part of the tumour with contrast extravasation (thin arrow). Right adrenal gland is not separately visualized. Left adrenal gland visible, with physiological contrast enhancement.

care two patients had indications to red blood cell transfusion. In one case administration of urapidil was necessary due to significant hypertension during surgery. One patient required intravenous infusion of norepinephrine intraoperatively.

Histopathological examination

In all cases the diagnosis of pheochromocytoma was histopathologically confirmed by immunohistochemistry - typical positive staining: synaptophysin and chromogranin and the presence of S100+ sustentacular cells in neoplasm. In all patients massive hemorrhagic changes in the tumor's tissue was confirmed. PASS score was defined precisely in 5 patients. In three cases, it was no higher than 3. In two remaining patients, PASS score was 5 and 4, respectively. Because of massive hemorrhage, in two patients it couldn't be determined. Histopathological images are shown on Figures 6–8.

Outcome and follow – up

In our group, the survival rate was 85.7%. Only one patient died, before the initial diagnosis of adrenal bleeding, shortly after admission to the department. The median follow up period was 68.5 months (Range: 11-156 months). All six patients who survived, remained at good condition, without any evidence of recurrence on last follow-up visit.

The clinicopathological characteristics of the patients are summarized in Table 1.

Discussion

Pheochromocytoma is the most common neoplasm responsible for spontaneous adrenal hemorrhage (1). In our data we found the clinically significant hemorrhage in 3.8% of patients with pheochromocytoma.

According to the literature, the most common symptom of hemorrhagic pheochromocytoma is abdominal pain of acute onset, which is in accordance with our work (2, 3, 11). The pain is presumably connected with local compression of surrounding viscera and adjacent structures by the lesion but it might be also caused by catecholamine over-secretion, accompanied by stimulation of alpha-adrenergic receptors, constriction of intestinal vascular smooth muscle and contraction of the ileocolic sphincter (3, 10, 13).

The status of shock in patients with hemorrhagic pheochromocytoma can be the result of massive bleeding, but also a sudden fall in the blood catecholamine level or excessive

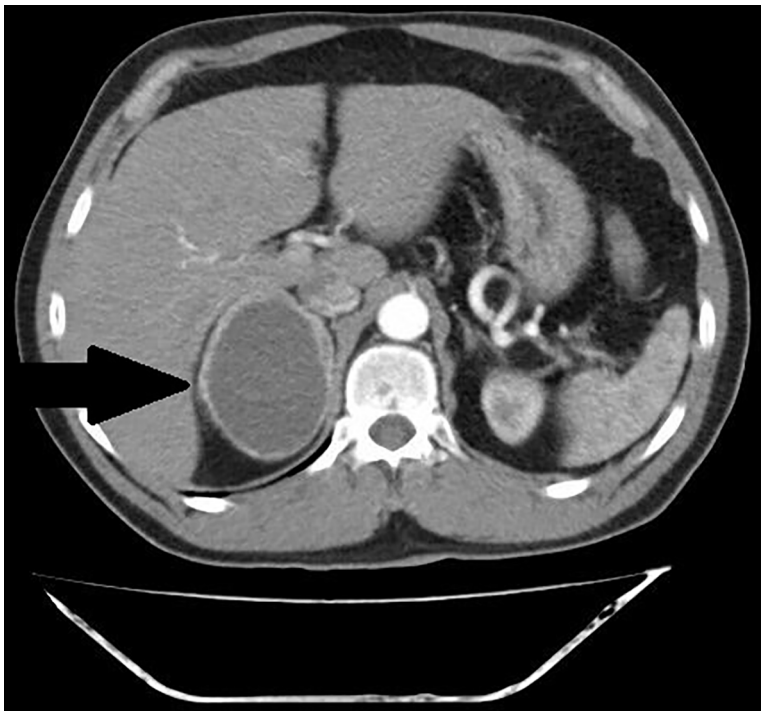

FIGURE 3
Patient number 4: CT of the abdomen, arterial phase, axial image- thick – walled hemorrhagic cyst of right adrenal gland with strong capsule-contrast enhancement (thick arrow).

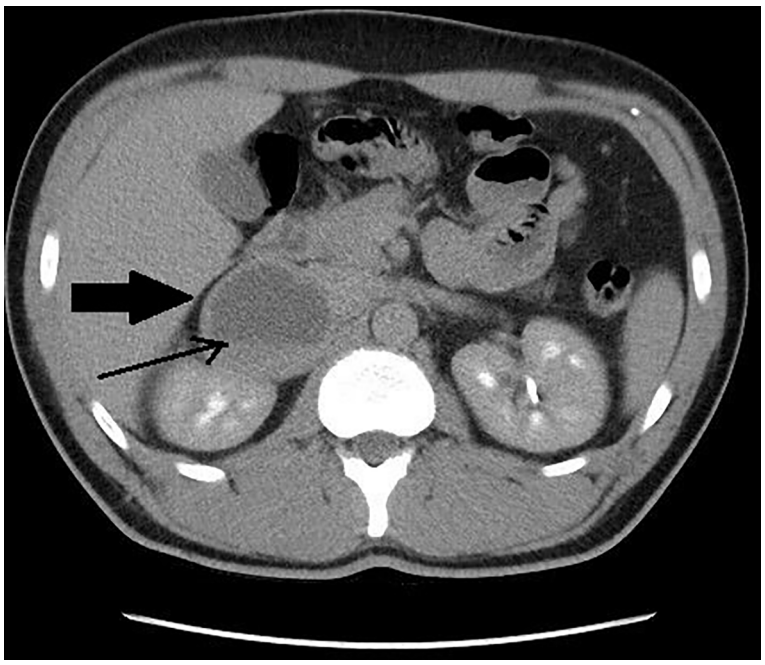

FIGURE 4
Patient number 7: CT of the abdomen, venous phase, axial image – right adrenal lesion with solid-cystic appearance (thick arrow), central area of fluid attenuation, with fluid-fluid level (thin arrow).

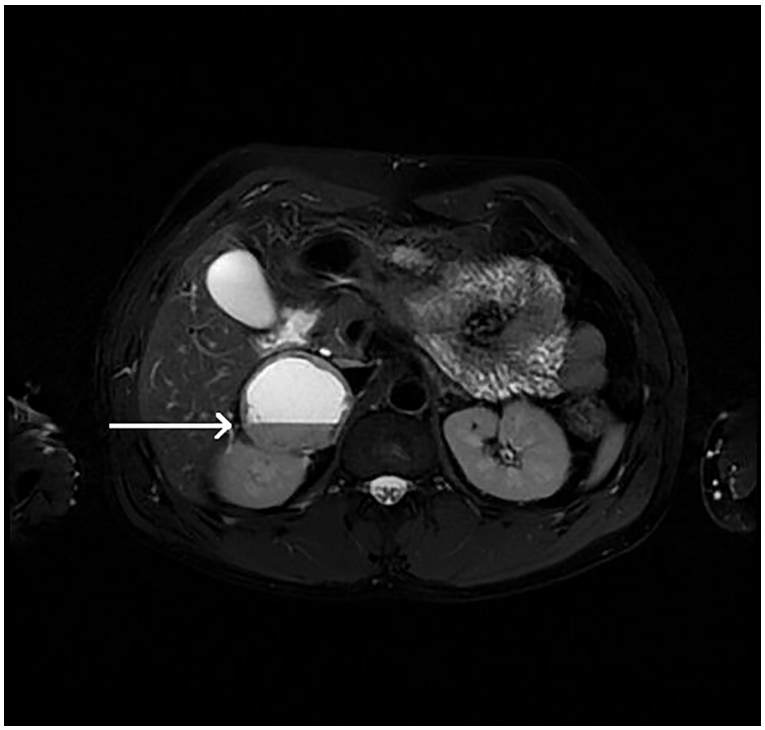

FIGURE 5
Patient number 5: MRI of the abdomen, T2-weighted axial image – right adrenal lesion with mostly hyperintense signal with fluid-fluid level (thin arrow).

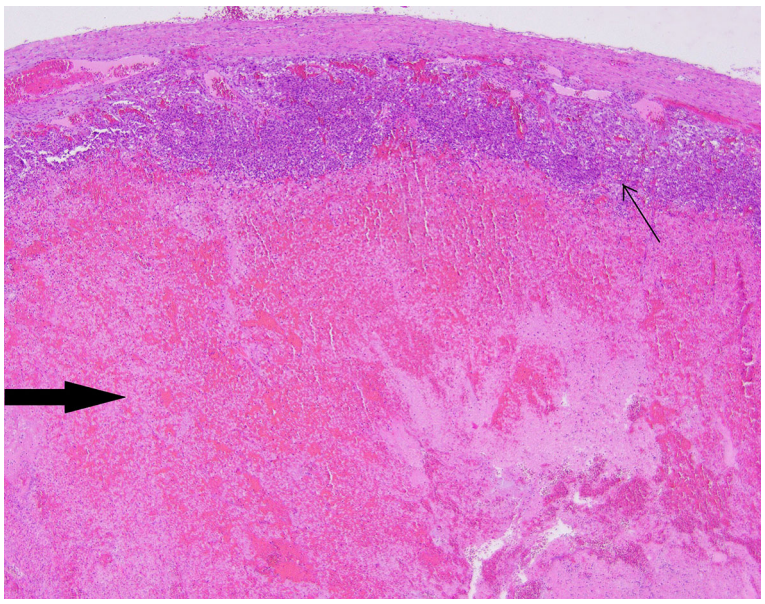

FIGURE 6
Patient number 4: extensive, diffuse hemorrhage in the central part of the pheochromocytoma (thick arrow), the neoplasm's tissue is present as subcapsular narrow rim (thin arrow).

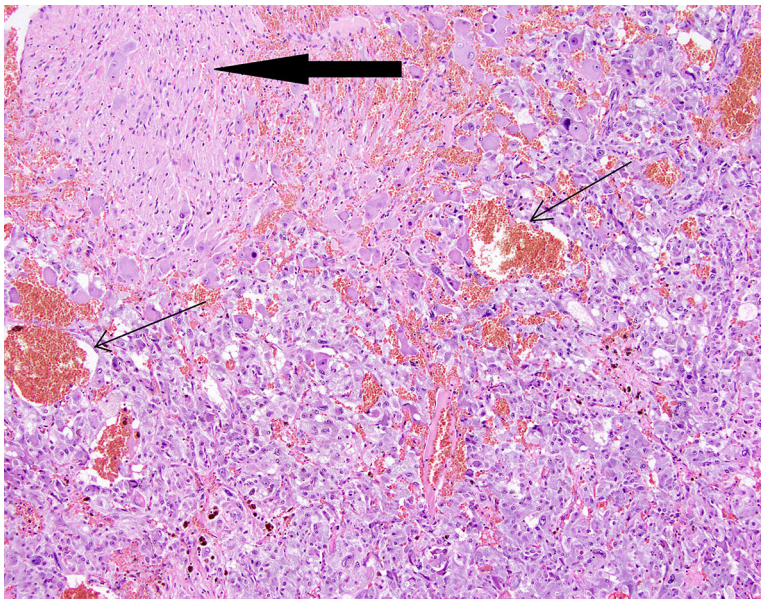

FIGURE 7
Patient number 3: composite pheochromocytoma and ganglioneuroma (ganglioneuroma component - thick arrow). Hemorrhages are present within pheochromocytoma tissue (thin arrows).

release of catecholamines, followed by cardiogenic shock (2, 4, 14). In our group, two out of seven patients developed shock. In one case it was accompanied by massive intra – and retro-peritoneal bleeding and anemia, which suggested a hemorrhagic shock as an important element of patient's hemodynamic destabilization. In second case, multiorgan failure, hyperglyaemia and acidosis without evident anemia raised suspicion that catecholamine crisis could play a key role in patient's deterioration.

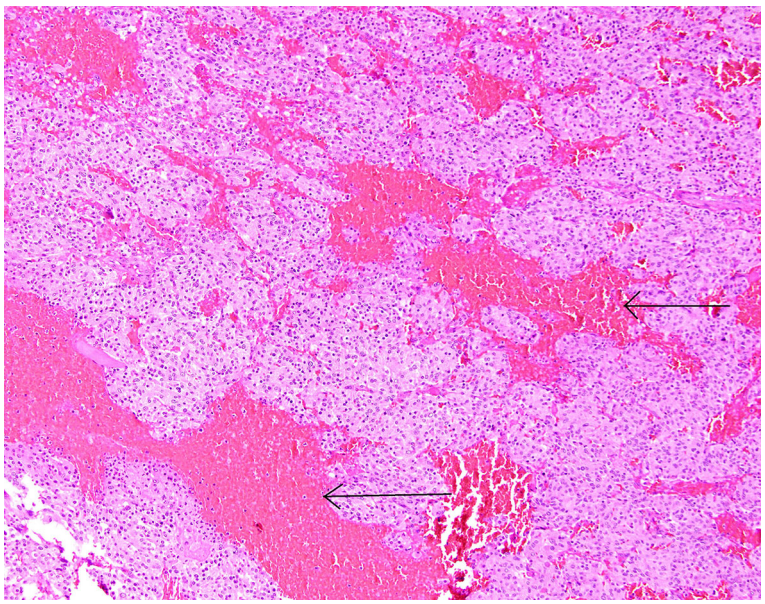

FIGURE 8
Patient number 2: central part of the pheochromocytoma with irregular, diffuse hemorrhages between tumour nests (thin arrows).

Catecholamines oversecretion often leads to tachyarrhytmias, of which severe and refractory sinus tachycardia, atrial fibrillation and ventricular tachycardia seem to be the most common (15).

The rare complication of catecholaminergic crisis, which could accompany hemorrhagic pheochromocytoma is Takotsubo cardiomyopathy, characterized by transient left ventricular systolic dysfunction (16, 17). In our group, Takotsubo cardiomyopathy was diagnosed in two patients.

As spontaneous adrenal hemorrhage is an insidious medical condition, it might remain unrecognized with lethal consequences (18, 19). Similar course of the disease was reported in one of our patients, who died few hours after admission, without a diagnostic statement.

On CT and MRI scans, the attenuation value or signal intensity of an adrenal hematoma depends on the stage of the bleeding. On CT, adrenal bleeding presents heterogenous, hyperdense appearance, which decreases over time (20–23). Contrast-enhanced CT may demonstrate active contrast extravasation in the setting of acute bleeding (21). A homogenous density greater than 50 Hounsfield Units (HU) is characteristic for hematoma, especially in cases with acute hemorrhage (22).

On MRI, the acute stage of adrenal bleeding is characterized by isointense or slightly low signal intensity on T1-weighted images and markedly low signal intensity on T2-weighted images, whereas in the early subacute phase hyperintense T1- and hypointense T2- weighted images are observed (20, 21). The high signal intensity appears at the periphery of the hematoma on T1-weighted images about 7 days after onset of the hemorrhage (23). Moreover, in the late subacute stage T2-weighted images become hyperintense (20, 21). In comparison to that, hyperintense signal on T2-weighted images with avid enhancement after contrast administration is characteristic for pheochromocytoma (20, 21, 24).

The radiographic findings similar to hemorrhagic adrenal neoplasm can be observed in patients with adrenal pseudocysts (hematomas), thus making the differential diagnosis notably difficult, especially in terms of a clot in a hemorrhagic cyst mimicking a solid component or a cyst presenting thick and irregular walls. Undoubtedly, it should be taken into consideration in biochemically negative patients (9, 23). Intravenous contrast injection on MRI or CT could help in stating a correct diagnosis since the presence of enhancement in adrenal mass raises the suspicion for an underlying tumor (20, 21, 23, 24). Interval imaging to assess possible resolution of hematoma could be helpful in patients without biochemical evidence of a functional tumor to exclude neoplasm (1). In some cases, sedimentation level could be seen in adrenal lesions as a sign of a previous hemorrhage (25).

In one of our patients, on MRI examination, low-signal intensity with peripheral hyperintense signal on T1-weighted images and heterogenous, mostly hyperintense signal on T2-weighted images were shown. Uncharacteristically, there was no typical contrast enhancement after both iodinated contrast (on CT) and gadolinium contrast (on MRI) administration in this patient. It could be explained by massive hemorrhage filling in and damaging the tumor's tissue, which was confirmed histopathologically. In this regard, the appearance of the non-contrast enhancing pheochromocytoma in our series was quite different from the cases of hemorrhagic pheochromocytomas reported by the others (20, 21, 23).

All patients with hemorrhagic adrenal mass should undergo hormonal assessment to exclude hormonally active tumors. Both catecholamine and cortisol levels should be evaluated (1, 26).

The preferred tests for biochemical diagnosis of pheochromocytoma is measurement of plasma or urinary free metanephrines with the use of high performance liquid chromatography, especially with mass spectrometry. In patients at low risk for a PPGL, the assessment of plasma free metanephrines has similar diagnostic accuracy as those in urine, however in patients, for whom biochemical proof of PPGL is the objective, the measurement of plasma free metanephrines is recommended. The measurement of urinary fractionated metanephrines might have higher ratio of false-positive results, since their levels are more affected by the diet (27–32). Increments in any plasma metabolite in excess of twice the upper reference limit or increases in two metabolites indicate a high likelihood of pheochromocytoma (27). To optimize the diagnostic performance, use of personalized age-specific reference intervals for plasma normetanephrine and gender-specific reference intervals for urinary metanephrines is recommended (31) .

Due to physiological stress, levels of catecholamines can be elevated in patients with adrenal hemorrhage, even without pathologically confirmed pheochromocytoma, which may lead to misdiagnosis.

Previous work showed that in cases of adrenal bleeding other than underlying pheochromocytoma, concentrations of urinary fractionated metanephrines only slightly exceeded normal laboratory range (50-100% above the upper limit) in about 30% of cases, with predominant normetanephrine level increase – moreover, the patients did not present symptoms indicative of an excess of catecholamines (33). Kyoda et al. revealed normal or slight elevation to no more than 3-fold the upper limit of catecholamines or their metabolites in the plasma and 24-hour urine in patients with hemorrhagic pseudotumor (9).

We also reviewed all the articles published from 2005 to 2022 in PubMed, dedicated to adrenal bleeding with known results of urinary or plasma catecholamines or metanephrines (8, 10, 12, 14, 16, 17, 24, 34–61). The analysis showed that catecholamines or metanephrines (plasma or urine) were elevated in 38.9% of patients with adrenal bleeding without pheochromocytoma. In most cases only normetanephrine/noradrenaline exceeded the normal range. In one patient slight increase in urinary adrenaline was observed. The urinary or plasma normetanephrine levels were increased to less than 3-fold the normal upper limit (maximum 2.1-fold the

upper normal limit for plasma normetanephrine, 2.9-fold for urinary normetanephrine), meanwhile urinary or plasma noradrenaline levels were increased no higher than 1.6 -fold the upper limit of normal range. Contrarily to that, in patients with hemorrhage due to a pheochromocytoma, in most cases both urinary or plasma catecholamines/metanephrines were elevated (21/24, 87.5%). In patients with assessed levels of urinary or plasma metanephrines, at least one metabolite exceeded 2-fold the upper normal limit. Based on that, we might conclude, that adrenal bleeding in non-pheochromocytoma patients results in slightly, non-specific elevation of normetanephrine/noradrenaline to no more than 3-fold the upper limit of normal in about 30-40% of cases. In patients with hemorrhagic pheochromocytomas, most often both catecholamines (or their derivatives) are elevated.

The results are shown in **Table 3** and on **Figures 9**, **10**.

Emergency surgery with regard to hemorrhagic pheochromocytoma has a high mortality rate of 40-45% (1, 3, 11, 22). Operation without proper pharmacological treatment jeopardizes patient's safety due to high risk of catecholamine crisis and postoperative hypotension (4, 56). However, in some groups of patients, i.e. with massive intraperitoneal bleeding, ineffective preoperative management or embolization, emergency exploratory laparotomy becomes the only treatment option (2, 4, 11, 59).

Alpha-adrenergic receptor (AR) blockers are the first-choice medications in preoperative management of pheochromocytoma. Phenoxybenzamine and doxazosin are the most commonly used. The main differences between those two drugs concern selectivity

TABLE 3 Levels of catecholamines/metanephrines in patients with hemorrhagic pheochromocytoma and adrenal bleeding without pheochromocytoma based on the literature (8, 10, 12, 14, 16, 17, 24, 34–61).

	Hemorrhagic pheochromocytoma (our cases included)- No of patients: 24 (8, 12, 14, 16, 17, 24, 34, 35, 37–39, 41–43, 53, 55, 56, 59–61)	**Hemorrhagic non-pheochromocytoma:- angiomyolipoma –No of patients: 1 (54)- adrenal pseudocysts – No of patients:17 (10, 36, 40, 44–52, 57–58)**
Urinary/plasma metanephrines/ catecholamines within normal range	4.2% (1/24)	61.1% (11/18)
Only urinary/plasma **normetanephrine/ noradrenaline** elevated	8.3% (2/24)	33.3% (6/18)
Both urinary/plasma metanephrines/ catecholamines elevated	87.5% (21/24)	5.6% (1/18)
Mean urinary normetanephrine elevation (fold the upper normal limit)+/- SD	18.8 ± 26.3 (range: 1.6-68)	1.89 ± 0.6 (range:1.27-2.9)
Mean urinary metanephrine elevation (fold the upper normal limit)+/- SD	15.0 ± 14.1 (range:1.02-47.8)	Not applicable
Mean plasma normetanephrine elevation (fold the upper normal limit)+/- SD	12.16 ± 10.7 (range 1.3-27.3)	2.1
Mean plasma metanephrine elevation (fold the upper normal limit)+/- SD	16.52 ± 15.6 (range: 1.7-28.6)	Not applicable
Mean urinary noradrenaline elevation (fold the upper normal limit)+/- SD	55.61 ± 112.1 (range:1.1-379.58)	1.4 ± 0.2(range 1.1-1.6)
Mean urinary adrenaline elevation (fold the upper normal limit)+/- SD	67.34 ± 69.0 (range:1.7-176.5)	1.2
Mean plasma noradrenaline elevation (fold the upper normal limit)+/- SD	92.65 ± 175.8 (range:3.6-484.4)	1.1 ± 0
Mean plasma adrenaline elevation (fold the upper normal limit)+/- SD	105.64 ± 177.9 (range:5.8-460.0)	Not applicable

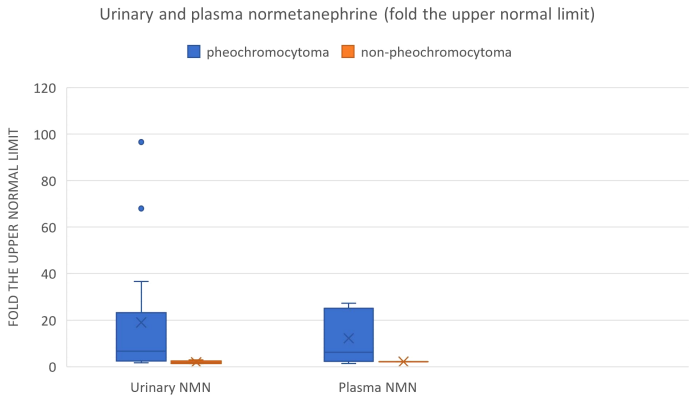

FIGURE 9
Urinary and plasma normetanephrine levels in patients with hemorrhagic pheochromocytoma and adrenal bleeding without pheochromocytoma based on the literature (3, 10, 12, 14, 20, 25–29, 30–39, 40–49, 50–54.).

and affinity of drug-receptor interactions. Phenoxybenzamine as the non-selective, non-competitive alpha-adrenergic receptor blocker binds irreversibly with both alpha 1 and alpha 2 – AR, which results in strong, long-acting inhibition. Doxazosin competitively inhibits only alpha 1-AR. For this reason, treatment with phenoxybenzamine gives more effective AR-receptors blockade and better control of hypertension, at the cost of higher risk of postoperative hypotension and other side effects, like reflex tachycardia, oedema and nasal congestion, comparing to doxazosin. Selective alpha-AR antagonist is more likely to be used in combination with additional antihypertensive drugs, i.e. calcium channel blockers (62, 63).

In our work, three patients were treated with phenoxybenzamine, in three patients doxazosin was administered , as phenoxybenzamine was no longer broadly available in Poland. In one patient, who had been prepared with 12 milligrams of doxazosin in a maximum dose, administration of urapidil was needed during surgery due to significant hypertension.

According to literature, transcatheter arterial embolization (TAE) preoperatively, particularly in a case of a ruptured pheochromocytoma, in patients who don't respond to red blood cell transfusions and initial conservative management, might be preferred initial therapy (1, 3, 7, 11, 53, 59). It can help to stabilize the patient's state, perform biochemical testing and administer pharmacological treatment before undergoing operation, which improves survival (3, 11, 59). Another possible benefit of trans-arterial embolization preceding elective surgery is consolidation of the hematoma and tumor shrinkage (3, 53). There are only three cases evaluating catecholamine levels around TAE – two of them reported

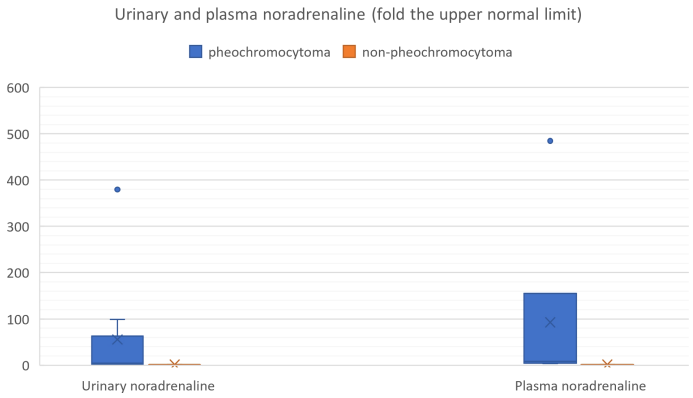

FIGURE 10
Urinary and plasma noradrenaline levels in patients with hemorrhagic pheochromocytoma and adrenal bleeding without pheochromocytoma based on the literature (3, 10, 12, 14, 20, 25–29, 30–39, 40–49, 50–54).

post-TAE peak of circulating catecholamines, resulted in hypertension, nausea, epigastric pain or constipation (11, 64). Therefore, careful observation of patient's vital signs and symptoms is necessary after TAE procedures. Marti et al. included embolization in proposed treatment algorithm for patients with hemorrhagic adrenal neoplasms (1).

In the literature, there are only several studies comprising cases of pheochromocytoma hemorrhage (1–3, 11, 12). There were summarized, together with our work, in Table 4. In comparison to our group, previously reported cases were characterized by higher proportion of emergency surgery (47-58.3% vs 14.3% in our work) and worse survival outcomes (27-41.7% of patients died vs 14.3% in our group). It could be explained by less number of patients with diagnosis of pheochromocytoma stated preoperatively but also more cases of severe, intraperitoneal and retroperitoneal bleeding (21-100% of intraperitoneal bleeding and 50-55% of retroperitoneal bleeding vs both – 14.3% in our work). Kobayashi showed that failed preoperative diagnosis of hemorrhagic pheochromocytoma was an independent factor for poor prognosis. Moreover, there was a strong correlation between correct preoperative diagnosis and elective surgery. Hemodynamic instability had a significant influence on the correct diagnosis of a pheochromocytoma (2). In recent years, improvement of survival rates despite the comparable proportion of more severe hemorrhage can be seen (11). It seems to be connected with better availability of imaging studies, development of new procedures in adrenal bleeding, including TAE and emphasis on preoperative management and preferentially elective type of surgery.

However, in the article summarizing 12 cases of intraperitoneal hemorrhage due to pheochromocytoma, conservative management was connected with 100% mortality (3/3 patients), contrarily to the survival rate of 71.4% (5/7) in patients treated by emergency surgery. Those results therefore stressed the need for more

TABLE 4 Clinicopathological characteristics and treatment results of hemorrhagic pheochromocytoma in our study and previously published series.

	Our work	Kobayashi T. et al. *(2)* 2005	Habib M. et al. *(3)* 2010	Marti J.L et al. *(1)* 2011	Hanna J.S et al. [*(12)*] 2011	Edo N. et al. *(11)* 2018
Number of patients	7	50	53	64	12	74
Median age (years)/ (range)	49 (36-78)	50 (15-80)	50.1 (15-80)	50	51.5(31-76)	50.1 (15-84)
Gender (men:women)	4:3	25:25	27:26	35:29	7:5	41:33
Acute abdominal pain	5 (71.4%)	40 (80%)	42 (79%)	N/A	10 (83.3%)	58 (78%)
Shock	2 (28.6%)	29 (58%)	30 (57%)	N/A	2 (16.7%)	38 (51%)
Prior history of tumor associated symptoms	6(85.7%)	21 (42%)	N/A	N/A	N/A	N/A
suspicion of pheochromocytoma before operation	6 (85.7%)	20 (40%)	N/A	N/A	N/A	N/A
Tumor side (right:left:bilateral)	R' -8 (85.7%) L"-1 (14.3%) B"'-0	R-27 (54%) L-22 (44%) B-1 (2%)	R-30 (56%) L-22 (42%) B-1 (2%)	R-32 (50%) L-31 (48%) B-1 (2%)	N/A	N/A
Median size of the tumour (cm)	7.4	N/A	N/A	7	N/A	N/A
Intratumoral hemorrhage	6 (85.7%)	12 (24%)	13 (25%)	N/A	0	18 (24%)
Intraperitoneal hemorrhage	1 (14.3%)	13 (26%)	13 (24%)	N/A	12 (100%)	15 (21%)
Retroperitoneal hemorrhage	1 (14.3%)	25 (50%)	27 (51%)	N/A	0	41 (55%)
Surgery	6 (85.7%)	41 (82%)	41 (77%)	51 (80%)	9 (75%)	62 (84%)
Emergency surgery	1 (14.3%)#	29 (58%)	29 (55%)	N/A	7 (58.3%)	35 (47%)
Elective surgery	5 (71.4%)	12 (24%)	12 (23%)	N/A	2 (16.7%)	27 (37%)
Surgery not performed	1 (14.3%)	9 (18%)	9 (17%)	9 (14%)	3 (25%)	12 (16%)
TAE*	0	N/A	3 (5%)	4 (6%)	N/A	7 (10%)
Survived	6 (85.7%)	33 (66%)	36 (68%)	N/A	7 (58.3%)	54 (73%)
Died	1 (14.3%)	17 (34%)	17 (32%)	N/A	5 (41.7%)	20 (27%)

N/A – not available.
'R – right.
"L – left.
"' B – bilateral.
* TAE – trans-arterial embolization.
# including one case with urgent surgery after four-day alpha-receptor blockage.

aggressive and faster surgical interventions in case of massive intraperitoneal hemorrhage in pheochromocytoma patients (12). Successful hemodynamic stabilization by TAE procedures in patients with ruptured pheochromocytomas accompanied by intraperitoneal hemorrhage has been also described (34, 35).

None of the previous series has focused on histopathological results in a hemorrhagic pheochromocytoma. Nevertheless, in the literature, reported cases of adrenal bleeding in pheochromocytomas with high PASS score, metastases and recurrences can be found (53, 55, 59, 64). Moreover, Xin-Gao et al. showed that intratumoral hemorrhage was abundantly detected in histologically high-graded pheochromocytomas (65). In our group, the PASS score was no higher than 3 in three cases, but it exceeded 3 in two remaining patients. Nevertheless, in two patients it couldn't be determined. Moreover in four out of five patients with known hormonal status, the level of dopamine metabolite, 3-metoxythyramine was substantially elevated. It is known that dopamine-producing tumors are more likely to present aggressive course of the disease and metastasize (66). Therefore, we suggest that in patients with hemorrhagic pheochromocytoma, careful follow-up is of great importance, since due to a massive bleeding, histopathological assessment is especially difficult and carries a risk of a PASS score underestimation and underprediction of other histopathological factors suggesting malignant course of the disease.

The potential risk factors for the pheochromocytoma hemorrhage are difficult to establish, since the most cases are diagnosed at the moment of ongoing adrenal bleeding, which may affect levels of circulating catecholamines and tumor size. The previous cases with repetitive assessment of catecholamines showed that their levels can fluctuate after the episode of adrenal bleeding within pheochromocytoma, probably because of tumor tissue damage and necrosis (60, 67). In one of our patients, concentration of urinary metanephrines decreased significantly three days after the hemorrhage (see Table 1, Patient Number 5). Catecholamine levels can be also influenced by pharmacological treatment with vasopressors (17). Some authors suggest, that the mechanism of hemorrhage may be explained by excessive amount of circulating catecholamines leading to vasoconstriction of the draining venules and extensive necrosis of pheochromocytoma, with subsequent decrease of catecholamines secretion, increase in blood flow into the tumor and high intratumoral pressure. It can result in rupture of the thin walled adrenal venules and tumor capsule. Moreover, anatomical characteristics of the adrenal gland vessels may also contribute to an increase in intratumoral pressure because the blood outflow vessels in the adrenal glands are much narrower than inflow vessels and the muscle bundles surrounding the central and external adrenal veins are arranged eccentrically, leading to turbulent flow within the vein (1, 3, 18, 23, 38, 59, 60).

In addition, AR-blockers have been reported as a potential cause of tumor rupture in pheochromocytoma (60).

In the group of 64 patients with hemorrhagic pheochromocytoma, described by Marti et al. median tumor size was 7 cm, which is similar to our observations (7.4 cm in our group). Based on the earlier studies (1–3) and our research, adrenal bleeding seems to be predominantly observed in pheochromocytomas localized in right adrenal glands. In two recent series, it was reported more often in men than women (1, 11), but some works, as well as our observations, did not confirm the predominance of men (2, 3, 12). The median age at diagnosis of hemorrhage in reported series, including ours, was about 50 years (1–3, 11, 12).

The major limitations of our study is its retrospective design. Moreover, a non-surgical character of our department, might result in bias in patient selection with respect to mortality, treatment strategies, and timing of surgery. Some patients with more severe bleeding and hemodynamic instability might have be transferred directly to surgical departments, which could explain low rate of intra- and retro-peritoneal bleeding in our patients and high percentage of preoperatively diagnosed pheochromocytoma. It could also lead to an underestimation of a number of hemorrhagic cases among all pheochromocytoma patients.

Based on the current available literature and our own experience, we suggest an algorithm for the diagnosis and treatment of adrenal hemorrhage in the course of pheochromocytoma (1–4, 7, 11, 12, 14, 15, 22, 23, 26, 27, 35, 38, 40, 43, 62, 68). It is shown on Figure 11. We have also summarized the medical management in hemorrhagic pheochromocytoma:

If hemorrhagic pheochromocytoma is suspected, perform urgent CT scan or MRI of the abdomen. It can help to assess the severity of bleeding and reveal underlying adrenal mass. Patients with unstable hemodynamic conditions due to catecholamine-induced arrythmias (rapid and recurrent heart rate [HR] and blood pressure [BP] changes, prolonged systolic BP <80 or > 160mmHg or HR < 50 or > 120 beats/min, a cardiovascular history of catecholamine-related complications, a need for intravenous antihypertensive or antiarrhythmic agents) or hemorrhagic shock should be treated in intensive care units (15). For the latter, fluid resuscitation and red blood cell (RBC) transfusion are the mainstay of treatment. In hemodynamically stable cases, perform hormonal assessment of cortisol and catecholamines. Increments in either metanephrine or normetanephrine in excess of twice the upper reference limit (for normetanephrine especially at least 3-fold) or increases in two metabolites indicate a high likelihood of pheochromocytoma (27). All hemodynamically stable patients should be qualified to elective surgery preceded by pharmacological management. Start the preoperative treatment of pheochromocytoma patients with alpha-adrenoceptors antagonists 7-14 days before the surgery. Beta-adrenoceptor blocking agents can be added in case of tachyarrhytmias, only after adequate pretreatment with alpha-adrenoceptors blockers (not earlier than 2-3 days after alpha-blockade). The β -blockers implementation without preceding α-blockers treatment may result in hypertensive crisis due to

FIGURE 11
Algorithm for the diagnosis and treatment of adrenal hemorrhage in the course of pheochromocytoma. *mainly hemorrhagic pseudotumour, metastases, myelolipoma, primary adrenocortical carcinoma ** The upper limit of normal *** metanephrine****normetanephrine

inhibition of β2-adrenoceptor mediated vasodilatation with coexistence of unopposed α -adrenoceptor-mediated vasoconstriction (27, 68). Cardioselective β1-adrenoceptors blockers are preferable. Labetalol and Carvedilol, with a higher potency for β- than α-adrenergic receptors should be avoided in monotherapy in pheochromocytoma patients (15, 27, 68). Calcium channel blockers are good option for the patients with uncontrolled blood pressure despite α -adrenoceptor blockers use, in case of α-adrenoceptor blockers intolerance and severe side effects or in patients with normal blood pressure or only intermittent hypertension to avoid α -adrenoceptor induced hypotension. (27, 62, 69). In such cases, ivabradine can also be used (15). In case of hypotension, unrelated to blood loss, consider potential β2-receptor overstimulation and administer a non-selective β2-adrenoceptor blocking agents (propranolol) or phenylephrine. For all patients adequate fluid replacement (with intravenous saline infusion in the evening before operation) and high-sodium intake prior to surgery is also important (62). Suggested optimal presurgical heart rate and blood pressure targets are: seated blood pressure <130/80mmHg and an upright systolic blood pressure > 90mmHg; heart rate 60-70 and 70-80 bpm in seated and upright position, respectively (15). For the patients with hypertensive crisis, intravenous administration of phentolamine sodium, nitroprusside, nicardipine or urapidil is recommended (69). In hemodynamically unstable patients despite conservative management, perform trans-arterial embolization (TAE) of adrenal arteries, followed by careful monitoring of patient's vital signs. Patients unsuccessfully treated by TAE procedures, hemodynamically unstable despite conservative management, in case of unavailable TAE or with massive intraperitoneal bleeding should be qualified to emergency surgery.

After the operation of hemorrhagic pheochromocytoma, careful life-long follow-up of the patients seems to be the most appropriate.

Conclusions

Adrenal bleeding is a rare, life-threatening complication of pheochromocytoma, which constitutes a diagnostic and therapeutic challenge. Proper diagnosis is essential for adequate preparation for surgery. Clinical symptoms including abdominal pain, accompanying by hemodynamic shock and previous history of pheochromocytoma-associated symptoms should alert about potential risk of adrenal bleeding. The emergency surgery in patients with adrenal bleeding from ruptured pheochromocytoma can be connected with significantly higher morbidity and mortality. Stabilizing arterial embolization could be an effective initial therapy in patients with hemodynamic instability, giving the opportunity for better preoperative management. In patients with hemorrhagic pheochromocytoma, careful postoperative follow-up is of great importance, since due to a massive bleeding, histopathological assessment may be less credible.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The study was approved by Jagiellonian University Ethical Committee (approval number:1072.6120.218.2020). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

ER, data collection, data analysis, manuscript drafting, manuscript editing and approval, study coordination. JK, data analysis, manuscript editing and approval. AG and MU-B, data collection, analysis, manuscript editing, and approval. ML, data collection, manuscript editing, and approval. MO, manuscript drafting, editing, and approval. EP-M and AH-D, manuscript editing and approval, study coordination. AG-J, data collection, data analysis, manuscript drafting, editing and approval, and study coordination. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Disorders of the adrenal cortex: Genetic and molecular aspects

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Adrenal cortex produces glucocorticoids, mineralocorticoids and adrenal androgens which are essential for life, supporting balance, immune response and sexual maturation. Adrenocortical tumors and hyperplasias are a heterogenous group of adrenal disorders and they can be either sporadic or familial. Adrenocortical cancer is a rare and aggressive malignancy, and it is associated with poor prognosis. With the advance of next-generation sequencing technologies and improvement of genomic data analysis over the past decade, various genetic defects, either from germline or somatic origin, have been unraveled, improving diagnosis and treatment of numerous genetic disorders, including adrenocortical diseases. This review gives an overview of disorders associated with the adrenal cortex, the genetic factors of these disorders and their molecular implications.

KEYWORDS

adrenal cortex, genetics, adrenal tumors, aldosterone secretion, cortisol secretion

1. Introduction

Adrenal glands are the major regulators of body homeostasis and endocrine stress response (1). They are small yellowish structures located on the upper poles of the kidneys, in the retroperitoneal area on the lateral edge of the vertebral column. They are found within perirenal fat and are surrounded by the renal fascia. The left adrenal gland is crescent-shaped, while the right is triangular. The weight of each gland in a healthy adult is 8-10 g and the average dimensions are 5.0x3.0x0.6 cm. They are highly vascular and receive their blood supply from 3 arteries: the superior, middle and inferior adrenal arteries. These arise from the inferior phrenic artery, abdominal aorta and renal arteries, respectively (2). The adrenal arteries form a capsular arteriolar plexus, which supplies the adrenal glands. With respect to venous drainage, the right adrenal has a single vein that drains directly to inferior vena cava, whereas the longer left adrenal vein drains into the renal vein (3).

The adrenal glands are comprised of two distinct parts, the cortex and the medulla. The medulla found in the center of the adrenal gland is composed of chromaffin cells and it is dependent on tissue interactions with the adrenal cortex (4). The cortex forms the outer part and is responsible for up to 90% of the adrenal weight. The adult adrenal cortex plays a vital role in normal physiology, being the site of steroid hormone production (3, 5). It consists of three morphologically and functionally distinct compartments. The outer zona glomerulosa (ZG) makes up about 15% of the cortex and produces aldosterone, a mineralocorticoid that controls blood pressure by regulating intravascular volume through sodium and water retention (6, 7). Beneath the ZG, is zona fasciculata (ZF), that comprises a major part of the adrenal gland and is the site of glucocorticoid synthesis. This is where cortisol is produced, a hormone with important effects on the immune system, metabolism and cardiovascular system. The innermost zone is zona reticularis (ZR), which produces adrenal androgens including androstenedione, dehydroepiandrosterone (DHEA), as well as its sulfate, DHEAS (8).

Adrenal cortex cellular function is finely regulated by complex mechanism that involve paracrine and endocrine responses. Dysregulation of signaling pathways in the adrenal cortex is associated with the development of adrenal tumors, some are benign and most rarely, malignant tumors (9).

The advance of new technologies in the field of genetics made possible to determine variations and structures at a genone-wide level (10). Next generation sequencing (NGS) became available at the beginning of the 21st century lowering the costs of DNA sequencing beyond what is possible with standard dye-terminator methods (11). In the clinical context, NGS has greatly improved the discovery of disease associated variants, facilitating not only faster and precise diagnosis but also risk factor prediction for complex disorders (12). For example, a recent study analyzed gene expressions in cortisol-producing adenomas (CPA) with *PRKACA* mutation and compared to *GNAS* and *CTNNB1* mutant CPAs. NGS analysis revealed differences between *PRKACA* mutant and *GNAS* and *CTNNB1* mutant CPAs, such as increased cortisol production in *PRKACA* mutant CPAs (13). This study allows better understanding of pathways involved in CPA and also may direct a more precise treatment approach for those individuals who harbors CPAs. Another study made use of whole-exome sequencing to determine the proportion of cells exhibiting the disease-causing variant *KCNJ5* p.G151R in an individual already diagnosed with bilateral adrenal hyperplasia (BAH). The results indicated a very low-level mosaicism (less than 0.5%) in the germline DNA, while all adrenocortical cells tested from 11 different nodules harbored the disease-causing variant. This finding has implication in patient prognosis and, family risk prediction (14). In this review, we intend to highlight the genomic and molecular aspects of adrenocortical tumors and its implication in patient survival.

2. Hormone secretion

The precursor of all adrenal steroid hormones is cholesterol, which is found in circulating low-density lipoprotein (LDL) particles. Briefly, LDL particles are taken up by adrenal cells *via* LDL-receptor mediated endocytosis (15, 16). The vesicles formed during this process subsequently fuse with lysozymes, where hydrolysis generates free cholesterol. Alternatively, cholesterol can either be uptaken from circulating HDL cholesterol *via* the scavenger receptor class B type 1 (SR-B1), or produced de novo from the acetyl coenzyme A (CoA) (17). Cellular cholesterol that is in excess is stored in the form of cholesteryl esters (CEs); the conversion of cholesterol to CEs is catalyzed by the enzyme CoA-acetyltransferase (ACAT) (18). In the adrenal glands, CEs act as the cholesterol 'storage' for the production of steroid hormones (18).

2.1 Hypothalamic-pituitary-adrenal (Hpa) axis: Glucocorticoid secretion

The secretion of glucocorticoids is regulated by the HPA axis. Their synthesis is stimulated by ACTH, which is released into the bloodstream by the anterior pituitary as part of a 241-amino acid precursor, POMC. In turn, ACTH production is regulated by corticotropin-releasing hormone (CRH), which is released by the neuroendocrine neurons in the paraventricular nucleus of the hypothalamus. Secretion of CRH is dependent on circadian rhythm, as well as various stressors (fever, hypotension, hypoglycemia) acting on the hypothalamus. The HPA axis is a negative feedback system, in which cortisol acts as a direct inhibitor of the synthesis of both ACTH and CRH.

2.2 Renin-angiotensin-aldosterone system (RAAS): Mineralocorticoid secretion

Secretion of mineralocorticoids is regulated mainly by the RAAS and potassium, while it also responds acutely to ACTH (19, 20). The juxtaglomerular (JG) cells in the afferent arterioles of the kidney contain prorenin, which is inactive. When JG cells are activated (in response to intravascular volume depletion, or decreased sodium in the distal convoluted tubule or β-activation) prorenin is cleaved to renin (18, 21). Once renin is released in the blood it acts on angiotensinogen, which is synthesized in the liver and is converted to angiotensin I (Ang I) in the kidney by renin. Ang I is then converted to angiotensin II (Ang II) by the angiotensin converting enzyme (ACE) in the lungs. Ang II and potassium increase the expression of aldosterone synthase (*CYP11B2*), while they also stimulate aldosterone production and glomerulosa cell proliferation (22).

In turn, aldosterone acts on mineralocorticoid receptors in kidney cells, from the distal convoluted tubule to the cortical collecting tubule. The result of its action is increased sodium reabsorption and excretion of potassium and hydrogen ions.

2.3 Adrenal androgen secretion

ZR cells produce androgens, the most important of which are DHEA and DHEAS (23). These are weak precursors that are converted to testosterone and estrogens (such as estradiol) in the peripheral tissues (24). It is established that steroidogenesis is under the control of ACTH which stimulates the transport of intracellular cholesterol into the adrenal cortex (25).

3. Disorders of growth of the adrenal cortex

3.1 Adrenal hyperplasia

3.1.1 Congenital adrenal hyperplasia (CAH)

CAH is a group of autosomal recessive disorders of the adrenal cortex caused by enzymatic deficiencies in the adrenal steroidogenesis pathway (26, 27). Depending on the degree of residual enzymatic activity, various forms of CAH have been described in the literature, including the most severe form (classic salt-wasting variant), followed by the classic simple virilizing form as well as milder forms (non-classical variant).

3.1.1.1 21OH deficiency

More than 90% of CAH cases are due to deficiency in 21-hydroxylase (*CYP21A2*) (Online Mendelian Inheritance in Man [OMIM] #201910 (28). The gene encoding 21OH, *CYP21A2*, is located on chromosome 6p21.3, within the human leukocyte antigen (HLA) major histocompatibility complex locus (29). *CYP21A2* and *CYP21A1P*, a homologous pseudogene, are approximately 30kb apart. Because of the high degree of sequence homology between these duplicate genes, meiotic recombination events are common in this region. Almost 95% of *CYP21A2* disease causing mutations are *CYP21A1P*-derived variants or deletions used due to recombination events (30, 31). Defects in 21OH result in impaired production of aldosterone and cortisol and elevated precursors, most notably 17-hydroxyprogesterone (17OHP), elevated levels of 17OHP are used for the diagnosis of CAH. In addition, excess of androgens occurs due to constitutive adrenal androgen synthesis, and results in virilization.

The most severe form of 21OH deficiency is due to variants that inactivate *CYP21A2* completely. Without neonatal screening, the phenotype in these cases manifests within the first 2 weeks of life with a life-threatening adrenal crisis (32). In the non-classic cases, the adrenal crisis is prevented. This is because some enzyme activity is preserved, and as a result aldosterone and cortisol production are not completely abolished (28, 33). The non-classic cases are thus characterized by symptoms attributed to the androgen excess: premature puberty, hirsutism and irregular menses. In some cases, patients may present with few or no symptoms and are identified by family genetic studies for other reasons (34). Females with non-classic CAH usually present with similar symptoms as those with polycystic ovary syndrome (PCOS), including hyperandrogenism (clinical or biochemical), and menstrual abnormalities (33, 35–37), and thus is difficult to differentiate between the two, leading to misdiagnosis of non-classic CAH as PCOS in some cases (38–40). Thus, it is suggested that patients undergo measurement of 17OH-progesterone levels followed by ACTH-stimulation test (41, 42).

3.1.1.2 11βOH deficiency

Approximately 8% of CAH cases are due to 11β-hydroxylase (*CYP11B1*) deficiency (43). *CYP11B1*, encoded by *CYP11B1*, is an enzyme regulated by ACTH, which catalyzes the conversion of 11-deoxycortisol to cortisol in the zona fasciculata. Patients with impaired 11-hydroxylation present with decreased corticosterone and cortisol synthesis, accumulation of the precursor deoxycorticosterone, and overproduction of adrenal androgens. Although deoxycorticosterone is a weak mineralocorticoid, in elevated concentrations it mimics the action of aldosterone, suppressing the renin-angiotensin axis, increasing blood pressure, and sometimes causing hypokalemia (43).

3.1.1.3 17OH deficiency

Deficiency of 17α-hydroxylase (*CYP17A1*) is rare, and severely damaging variants in *CYP17A1* result in absent cortisol as well as androgens, causing puberty failure and sexual infantilism (44). *CYP17A1* is expressed in the ZF and the ZR, but not in the ZG. Both 46,XY and 46,XX patients with 17OH deficiency have female external genitalia, and present at puberty as phenotypically female. They have hypergonadotropic hypogonadism without secondary sexual characteristics, and low-renin hypertension.

3.1.1.4 *3βHSD2* deficiency

There exist two isoforms of 3β-hydroxysteroid dehydrogenase: 3βHSD1 and *3βHSD2*, encoded by *HSD3B1* (the homologous type I gene) and *HSD3B2*, respectively. *HSD3B1* is expressed in the placental and peripheral tissues (breast, prostate and skin), while *HSD3B2* is expressed exclusively in the adrenals and gonads (45). *3βHSD2* deficiency is characterized by deficiency of both glucocorticoids and mineralocorticoids, as well as by dehydroepiandrosterone (DHEA) overproduction. DHEA is converted to testosterone by extra-adrenal 3βHSD1, and patients present in infancy with

underdeveloped 46,XY genitalia and – rarely – 46,XX virilization (46).

3.1.1.5 Lipoid congenital adrenal hyperplasia

The most severe defect of steroidogenesis is lipoid congenital adrenal hyperplasia (LCAH). LCAH is caused by defects in the steroidogenic acute regulatory protein (StAR) and is characterized by deficiency of all steroid hormones. StAR regulates the transfer of cholesterol from the outer to the inner mitochondrial membrane, a vital step in the initiation of steroidogenesis. As a result, cholesterol cannot be mobilized. Adrenal lipid droplets subsequently accumulate and are seen on the autopsy, thus the name of the disorder. In both 46,XY and 46,XX patients, it presents with female external genitalia and an adrenal crisis in the neonatal period (47).

Regarding the current treatment for CAH, there is no consensus yet, therefore, it still remains a challenge. It usually includes glucocorticoid and mineralocorticoid replacement therapy (48).

3.2. Adrenocortical tumors

Adrenocortical tumors (ACTs) can be sporadic or familial, unilateral or bilateral, and non-secreting or secreting. The latter secretes various adrenal steroid hormones; the exact hormone varies depending on the tumor type. Unilateral ACTs are common, and approximately 10% of the general population appears to have an adrenal cortical lesion (49). They are often discovered incidentally when evaluating for another disease and are thus called incidentalomas (50, 51). Once discovered, they are evaluated by abdominal computed tomography (CT). The vast majority of them are benign adrenocortical adenomas (ACAs). Some ACAs are non-secreting, while others can secrete cortisol and cause Cushing syndrome (5-47% of cases), or aldosterone and cause Conn syndrome (1.6-3.3%) (50, 52). The rest of ACTs are adrenocortical carcinomas (ACCs), which are rare (prevalence 4-12 cases per million).

3.2.1 Benign cortisol-producing adrenocortical tumors

Cushing's syndrome (CS) has an estimated incidence of 39-79 per million people per year in various populations, with a female-to-male ratio of 3:1 (53–56). Data from various studies suggest that there is an increased prevalence in people with early-onset osteoporosis, type 2 diabetes, or hypertension, but the precise estimates vary (57–60).

CS is characterized by cortisol overproduction. The cause of 80% of endogenous CS cases is over-secretion of ACTH by a pituitary corticotroph adenoma or – less frequently – by a neuroendocrine tumor (61–63). In rare cases, neuroendocrine tumors such as pheochromocytoma and medullary thyroid carcinoma produce corticotropin-releasing hormone (CRH), which then results in pituitary ACTH over-secretion (61–63). In 20% of the cases, CS is ACTH-independent, and the cause is the primary overproduction of cortisol by the adrenal glands. In such cases, the most frequent underlying pathology is a cortisol-producing adenoma, while adrenocortical carcinomas and bilateral adrenal hyperplasia are responsible for less than10% of the cases (64). Bilateral adrenal hyperplasia in particular may be either isolated, or part of a syndrome, and can be divided into two entities based on the size of the nodules: primary bilateral macronodular adrenal hyperplasia (PBMAH), which is characterized by several nodules (diameter >10mm) (65), and two micronodular forms. The latter are primary pigmented micronodular adrenal hyperplasia (PPNAD) and isolated micronodular adrenocortical disease (iMAD) (diameter <10mm) (61–63, 66).

The cAMP/PKA pathway is the main regulator of cortisol production (67). PKA (protein kinase A) consists of two regulatory subunits and two catalytic subunits that – under normal conditions – are bound together. In adrenocortical cells, the pathway is activated by the binding of ACTH to *MC2R*, a G protein-coupled receptor (GPCR). This triggers an increase in cAMP levels, which binds to the PKA regulatory subunits causing the release from the catalytic subunits. The catalytic subunits, then translocate to the nucleus, where they phosphorylate, and thus activate, transcription factors that promote cortisol synthesis (Figure 1) (68).

3.2.2 Primary bilateral macronodular adrenal hyperplasia (PBMAH)

PBMAH is usually diagnosed in patients at 40-65 years old that present CS and low levels of ACTH, or - more recently - when investigating an adrenal incidentaloma. Many terms have been used over the years to describe PBMAH. Such terms include primary macronodular adrenal hyperplasia (PMAH), autonomous macronodular adrenal hyperplasia (AMAH), bilateral macronodular adrenal hyperplasia (BMAH), and 'huge' or 'giant' macronodular adrenal disease. Another term, ACTH-independent massive bilateral adrenal disease (AIMBAD), has also been used in the past, but in later studies the secretion of cortisol appeared to be regulated by corticotropin and thus, this term is not used anymore (69).

In general, PBMAH presents with bilateral macronodules and enlargement of the adrenal glands. In the majority of cases (77-87%), the macronodules exhibit ectopic or excessive expression of G-protein coupled receptors, including luteinizing hormone/choriogonadotropin (LH/hCG) responsible for Cushing syndrome during pregnancy and after menopause (70), glucose-dependent insulinotropic peptide (GIP) that is responsible for food-dependent Cushing syndrome (71), serotonin 5HT, catecholamines, Ang II, glucagon and vasopressin (65, 72–77). The binding of these receptors to their ligands mimics the result of

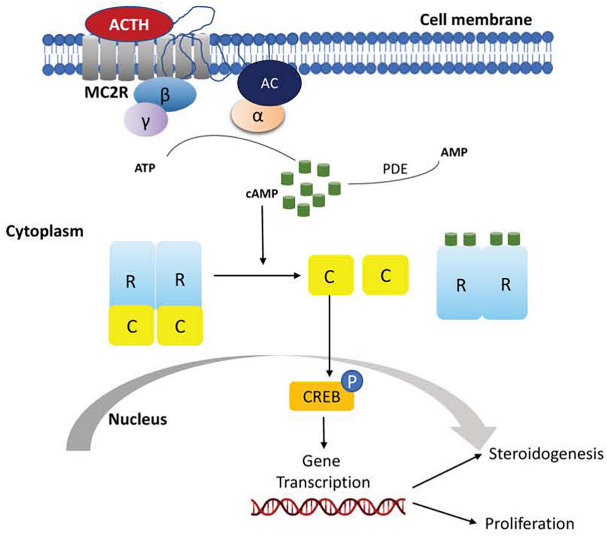

FIGURE 1
Schematic representation of activation of the cyclic adenosine monophosphate (cAMP) signaling pathway in normal adrenocortical cells.

ACTH binding to *MC2R* leading to activation of cAMP/PKA pathway and thus excessive cortisol secretion (78). So far, the exact molecular mechanism of the ectopic receptor expression has not been completely elucidated (79).

Genetic variants resulting in increased activity of the cAMP/PKA pathway *via* a variety of mechanisms have been reported in patients with PBMAH. For example, variants in *PDE11A*, that encodes phosphodiesterase type 11A, have a prevalence of 24-28%, whereas inactivating germline variants in *PDE8B*, encoding phosphodiesterase type 8B, and *PRKACA* copy number gains, have also been encountered (80–83). Another component of the cAMP/PKA pathway associated with PBMAH is the Gα subunit, encoded by GNAS*1* (84). Activating variants in GNAS*1* cause McCune-Albright syndrome, which is associated with 'café au lait' spots, polyostotic fibrous dysplasia, precocious puberty and hyperfunction of multiple endocrine glands (84). These GNAS*1* variants are somatic and lead to continuous activation of the cAMP/PKA pathway and thus, cortisol-producing adenomas (85). Finally, in a case report of an isolated case of bilateral adrenal hyperplasia, the synergistic action of two variants (p.C21R and p.S247G) on the same allele of *MC2R* (encoding the melanocortin 2 receptor/ACTH receptor) resulted in autonomous cortisol secretion *via* constitutive activation of the cAMP/PKA pathway (86). This case is remarkable in that if those defects had happened in isolation, they would have led to receptor inactivation (86).

Rarely, PBMAH can occur as part of genetic tumor predisposition syndromes such as familial adenomatous polyposis (*APC*), multiple endocrine neoplasia type 1 (*MEN1*) and hereditary leiomyomatosis (*FH*) (83, 87–89). It is important to mention that these genetic alterations are associated with other tumors and are responsible only for a limited number of PBMAH cases. Additionally, PBMAH can be associated with aromatase expression leading to elevated estrogens, independently of sex (79).

More recently, germline variants in the armadillo repeat-containing 5 (*ARMC5*) have been found to implicate in the pathogenesis of PBMAH, with their prevalence estimated between 21%-26% (90, 91). *ARMC5* is a cytosolic protein that had no enzymatic activity, and its function depends on interactions with other proteins (92). *ARMC5* is located on chromosome 16p11.2. In mice, as well as *in vitro*, *ARMC5* has been shown to play an important role in regulating steroidogenesis, proliferation, apoptosis, T-cell differentiation and immune responses (92–95). Most of the patients harboring *ARMC5* variants had adrenal CS, with the hypersecretion of cortisol being more severe compared to that seen in patients that had *ARMC5* variants that were predicted to be benign or did not have *ARMC5* variants. In addition, damaging variants or deletions in *ARMC5* were identified in several families with PBMAH (96, 97), whereas an association of *ARMC5* with primary hyperaldosteronism was also reported in 2015 (98).

In a small number of cases, somatic variants in genes participating in other biological processes have been described. These include two chromatin regulator genes, *DOTIL* (that encodes a histone H3 lysine methyl-transferase) and *HDAC9* (that encodes a histone deacetylase) (99). In addition, a study of two siblings from a family that segregated PBMAH implicated the Endothelin Receptor type A *EDNRA*, which encodes a G-coupled protein. However, this association remains to be confirmed in follow-up studies (100).

A meaningful update of the 2022 WHO classification of adrenocortical tumors was recently summarized by Mete and co-workers early this year. As a result of the advance on next generation sequencing studies, it is possible to recognize that PBMAH is caused by germline variants in one out of many susceptible genes, with a second hit in the somatic cells. These findings strongly suggest a neoplastic instead of hyperplastic condition. To avoid a misnomer for the disease, the 2022 WHO classification changed the nomenclature of primary bilateral macronodular adrenal hyperplasia to bilateral macronodular adrenocortical disease (101).

3.2.3 Primary pigmented nodular adrenal disease (PPNAD)

PPNAD is most commonly diagnosed in children and young adults and is a rare cause of ACTH-independent hypercortisolism. It is most commonly part of Carney complex (CNC), an autosomal dominant tumor predisposition syndrome. CNC presents with various endocrine tumors including pituitary adenomas, thyroid benign tumors, and testicular Sertoli cell-calcified tumors, as well as non-endocrine tumors, most commonly pigmented skin lesions, skin and cardiac myxomas (102, 103). PPNAD in patients with CNC has a prevalence close to 60% (104, 105).

With respect to the molecular background, CNC is caused by germline inactivating variants in the PRKAR1A gene, located at the 17q24.2-24.3 locus (CNC1 locus). PRKAR1A variants are found in 37% of patients with the sporadic form of the disease and in more than 70% in the typical familial forms, with almost 100% penetrance (105, 106). PRKAR1A encodes the regulatory subunit type 1α (R1α) of PKA. As a result, inactivating PRKAR1A variants result in aberrant activation of the cAMP/PKA pathway. To date, approximately 140 pathogenic variants have been reported (https://PRKAR1A.nichd.nih.gov/hmdb/mutations.html). The majority of them are located in exons 2,3,5,7, and 8 while about 20% are located in intronic sequences and affect splicing (105, 106). Most of the variants are small deletions and insertions, base substitutions or combined rearrangements involving up to 15bp (107). In almost all cases (90%), the genetic alteration leads to a premature stop codon. Subsequently, the transcripts containing the premature stop codon are degraded by nonsense mediated decay (NMD). As a result, the amount of RIα protein produced is half of the normal amount (107–109). Large chromosomal deletions involving the PRKAR1A gene, even though rare, have also been identified (110, 111). Occasionally, the pathogenic variant (missense, short in-frame insertions/deletions and splice variants) may lead to the production of an abnormal protein that is incapable of responding appropriately to cAMP levels or properly bind to the PKA catalytic subunits (108, 112).

In some families without variants in PRKAR1A, the causative gene has not been identified yet; however, genetic linkage analysis of tumors has shown that there is another affected locus on chromosome 2p16 (CNC2 locus) (113, 114). The majority of those cases have been diagnosed with CNC later in life (115). In addition, a single patient with CNC that presented with abnormal skin pigmentation, acromegaly and myxomas, was found to harbor copy number gains of locus containing the *PRKACB* gene (116). Moreover, a recent study with 353 CNC patients and/or PPNAD, showed that the majority of patients with isolated PPNAD harbored a germline c.709-7del6 variant (105).

During the past years, variants affecting the phosphodiesterase genes *PDE11A* and *PDE8B* have emerged as putative causes of PPNAD. The loci harboring these genes had the most significant associations in a genome-wide association study performed on individuals lacking genetic defects in *GNAS* or PRKAR1A, while the locus harboring *PDE11A* showed the largest loss-of-heterozygosity in tumor samples (117). In addition, targeted sequencing of *PDE11A* revealed that patients with CNC that also had PPNAD and/or testicular large cell calcifying Sertoli cell tumors were more likely to have variants in *PDE11A*, compared to patients without these tumors (118). All of these patients also had germline variants in PRKAR1A, raising the possibility that *PDE11A* variants act as genetic modifiers that elevate the risk for PPNAD and/or LCCSCT in CNC. With regards to *PDE8B*, a single nucleotide variant (c.914A>C/p.His305Pro) was detected in a 2 year old female patient diagnosed with PPNAD; the variant was inherited from the patient's father. This variant was subsequently shown to lead to decreased *PDE8B* activity *in vitro* (119).

It is worth noting that variants in *PDE11A* and *PDE8B* have been found in other types of adrenocortical tumors as well. In addition to PBMAH tumors (described above), haploinsufficiency of *PDE11A* has been implicated in ACA and ACC (80), and in vitro studies have demonstrated that putative PBMAH-causing variants compromised the enzymatic activity of *PDE11A* (81). Furthermore, in a cohort of patients with adrenocortical tumors without variants in PRKAR1A, *GNAS*, or *PDE11A*, 7 patients harbored variants in *PDE8B* (82). Two of these variants were then experimentally shown to decrease protein activity (82).

Recently, genes encoding for the catalytic subunits of PKA have also been implicated in micronodular PBMAH. A study of two patients with familial PBMAH, and of three patients with sporadic iMAD, identified germline copy number gains of the locus that harbors *PRKACA*, which encodes the Ca catalytic subunit. Tumor samples from these patients revealed elevated basal as well as cAMP-stimulated PKA activity (120, 121). In addition, copy number gains involving the *PRKACB* locus (which encodes the Cβ catalytic subunit), were reported in a patient with CNC that presented with myxomas, acromegaly, and abnormal skin pigmentation. The patient was found to have increased cAMP-induced kinase activity in lymphocytes, resembling what is seen in CNC patients with PRKAR1A variants. Moreover, increased Cβ levels were found in several cell types as well as in breast myxoma cells (116).

Finally, the wingless-type-(Wnt)-β-catenin pathway has also been suggested to play a role in micronodular BAH. Somatic variants in the β -catenin gene (*CTNNB1*) were found in two (11%) patients with PPNAD in a previous study, with one of these patients also harboring a PRKAR1A variant. These variants were encountered in larger adrenocortical adenomas that arose within the context of PPNAD, and were absent from the surrounding hyperplastic adrenocortical tissue (122). A different study landed further support to the involvement of the Wnt-β-catenin pathway, by showing accumulation of β-catenin in PPNAD tissues, as well as activating somatic *CTNNB1* variants in macronodules, but not in micronodules or the contralateral adrenal gland (123).

3.2.4 Cortisol-producing adrenocortical adenomas (ACA)

Cortisol-producing adenomas exhibit overactivation of the cAMP/PKA signaling pathway. The most prevalent CPA-causing defect is alteration of the catalytic α-subunit of PKA (*PRKACA*) and the most common variant has been reported to be p.Leu206Arg (124). Other rare variants have been described (120, 125, 126), all localized in a region of *PRKACA* that affects its interaction with the regulatory subunit 1α. Activating variants in *PRKACA* lead to continuous activation of PKA by abolishing the interaction between its catalytic and regulatory subunits. In addition, they can lead to hyperphosphorylation of certain substrates, thereby altering substrate specificity (127). An activating somatic variant in *PRKACB* has also recently been reported in a patient with CPA; *in vitro* studies showed that this variant confers higher sensitivity to cAMP (128).

Furthermore, somatic inactivating variants in *GNAS* and PRKAR1A have been found in sporadic adrenocortical tumors (126, 129–133). The genetic alterations in both genes lead to increased signaling *via* the cAMP/PKA pathway, however they activate different downstream effectors. Adrenal lesions that harbored variants in *GNAS* or PRKAR1A had overactivation of the p53 and MAPK signaling pathways, respectively. In PRKAR1A-mutant tumors, genes related to Wnt-signaling pathway (*CCND1*, *CTNNB1*, *LEF1*, *LRP5*, *WISP1* and *WNT3*) were overexpressed, while in *GNAS*-mutant tumors, there was increased expression of extracellular matrix receptor interaction and focal adhesion pathways (NFKB, *NFKBIA* and *TNFRSF1A*) (134).

3.2.5. Aldosterone-producing benign adrenocortical neoplasms

Excess of aldosterone production characterizes a group of adrenal cortical disorders including aldosterone-producing adenomas, adrenal cortical hyperplasia, familial hyperaldosteronism (*FH*, <1%) and rarely, carcinomas (<1%) (135–137).

3.2.6 Benign adrenocortical tumors producing aldosterone

Primary aldosteronism (PA) is the most frequent secondary form of hypertension, accounting for approximately 10-20% of patients referred with resistant hypertension and 5% of patients in primary care (138, 139). PA is typically due to unilateral adenomas that produce aldosterone (APA) (65%), or BAH (35%) that leads to autonomous aldosterone production (140). The remaining cases include unilateral hyperplasia (2%), familial hyperaldosteronism (*FH*, <1%) and aldosterone-producing ACC (<1%) (137). The vast majority of PA cases are sporadic and only 6% are familial (141).

3.2.6.1 Inherited forms of PA

Four forms of *FH* (type I-type IV) have been described so far and they are inherited in an autosomal dominant manner (136).

The underlying cause of *FH* I (also known as glucocorticoid-remediable aldosteronism-GRA) is the formation of a chimeric gene, resulting from an unequal fusion of the regulatory regions of *CYP11B1*, which encodes 11β-hydroxylase, and *CYP11B2*, that encodes aldosterone synthase. Both enzymes are responsible for the last steps of cortisol and aldosterone synthesis, respectively (142, 143). Formation of the chimeric gene leads to aldosterone overproduction under the regulation of ACTH (143). Treatment is based on the use of glucocorticoids (144). Genetic testing for the chimeric gene (*CYP11B1*/*CYP11B2*) should be considered for patients who are diagnosed with PA and have a family history of the disease, onset of PA before the age of 20 years, or family history of stroke at a young age (145).

FH II is due to mutations in the *CLCN2* gene, which encodes the chloride channel CIC2. Among other tissues, CIC2 is expressed in the adrenal glands. Gain-of-function variants in *CLCN2* lead to increased Cl– ions efflux, which causes cell membrane depolarization and opening of voltage-gated calcium channels, triggering aldosterone production (146, 147). *FH* II is the most common form of *FH*, with a prevalence of 1.2-6% in patients with PA (141, 148, 149).

FH III is due to coding variants in the G-protein coupled inward rectifying potassium channel 4 (GIRK4), which is encoded by *KCNJ5*. Genetic defects in this gene cause a lack of ion selectivity and increased sodium influx, which results in cell depolarization triggering calcium entry into the cells. This signals an increase in *CYP11B2* expression and increase in aldosterone production. The severity of hyperaldosteronism has been shown to be related to the type of *KCNJ5* variant in some patients (150–152), but not in all of them (153). The majority of patients with germline variants in *KCNJ5* present with polydipsia, polyuria and refractory hypertension during childhood. In these patients, aldosterone hypersecretion is high enough to require bilateral adrenalectomy (145). There is heterogeneity in the age at which patients present the disease,

and in some cases, the symptoms can be controlled with mineralocorticoid-receptor antagonists (MRAs) (150–154).

FH IV is caused by germline variants in *CACNA1H*, which encodes the pore-forming α1 subunit of the T-type calcium channel Cav3.2. These variants cause alterations in calcium current properties, leading to increased intracellular calcium concentration and production of aldosterone. Germline variants in *CACNA1D*, which encodes Cav1.3 (the α1D subunit of the voltage-dependent L-type calcium channel) have also been described in patients with PA; these variants occur exclusively de novo. These patients present with a severe early-onset form of hyperaldosteronism associated with a complex neurological disorder, with the phenotype also including seizures and neurological abnormalities (PASNA) (155).

Finally, germline variants in *PDE2A*, *PDE3B* and *ARMC5* have also been reported in patients with PA (98, 156). The first two genes were associated with PA within the context of BAH; however, these are not considered as genetic causes of *FH* (156).

3.2.6.2 Aldosterone-producing adrenocortical adenomas (APA)

In the past decade, major advances have been made in unraveling the molecular background of APAs (157, 158). Variants have been identified in genes associated with familial forms of APA, including *KCNJ5* and *CACNA1D* as well as in *ATP1A1* and *ATP2B3* (which encode two Na+/K+ and Ca2+ ATPases) (155, 159–161). The most frequent defects are recurrent variants in *KCNJ5*, encountered in more than 40% of APAs in Caucasians, with two particular variants (p.G151R and p.L168R) being responsible for 36% of cases (162). Variants in *KCNJ5* appear to be more frequent in Asian cohorts (162–166) (~70% prevalence), and in women compared to men (63% vs 24% prevalence) (163). Variants in *CACNA1D* are reported in up to 10% of patients with APA, while *ATP1A1* and *ATP2B3* variants are less frequent (167).

Additionally, the Wnt/β-catenin signaling pathway has been shown to play a vital role in the adrenal cortex development and the biosynthesis of aldosterone (168). It has been shown to be continuously active in approximately 70% of APAs (169). Under normal unstimulated conditions, β-catenin is in the cytosol as part of the axin complex along with Caseine Kinase 1β, glycogen synthase kinase 3β and adenomatous polyposis coli (*APC*). Binding of the Wnt ligand to its receptor causes β-catenin to dissociate from the axin complex and translocate to the nucleus where it induces the expression of the transcription factors T cell factor (TCF) and lymphocyte enhancer factor (LEF) (168). Somatic variants in *CTNNB1* gene, encoding β-catenin, have been identified in 2-5% of patients with sporadic APA (155, 170, 171); similarly, to APAs due to *KCNJ5* variants, these APAs are associated with larger adenomas and are more commonly seen in females. Variants in *CTNNB1* have also been reported in two pregnant patients with APA that exhibited increased adrenocortical expression of the gonadotropin releasing hormone (GnRH) and LH/hCG receptors (172). Based on this, an association with pregnancy or menopause was suggested, but this was not confirmed in a follow-up study (172). In rare cases, somatic variants in *PRKACA* and *GNAS* have been described in patients with cortisol and aldosterone co-secreting adenoma as well (173, 174). However, their role in the development of APA remains unclear, because those variants are similar to the ones found in CPAs and ACC (99, 120, 131, 175–177).

3.3 Adrenocortical carcinoma

ACCs are rare tumors derived from the adrenal cortex. They affect both adults and children with an annual incidence of 0.7-2.0 cases per million per year (178, 179). They are responsible for steroid excess in 60-70% of cases (52, 180, 181) and they represent one of the most aggressive class of endocrine tumors with an overall poor prognosis (5-year survival rate <35%) (129). However, the exact 5-year survival rate varies depending on the tumor stage, from 82% for tumors in stage I to 18% for tumors in stage IV (182). Thus, the stage at the time of the diagnosis is a crucial prognostic factor. Approximately 40-60% of patients present with signs and symptoms related to hormone excess (183–185). Another 30-40% present with non-specific symptoms associated with local tumor growth (30-40%), including early satiety, abdominal fullness and flank or abdominal pain (184, 185). The remaining 20-30% of ACCs are discovered incidentally on imaging studies for unrelated medical conditions (Table 1) (187). With regards to the age distribution, ACC seems relatively more common in children than in adults and is often associated with hereditary tumor syndromes (186, 188). In fact, the elucidation of genetic alterations underlying familial syndromes predisposing to ACC has led to the identification of signaling pathways involved in the development of cancer such as Insulin growth factor 2 (IGF-2), Wnt-beta catenin and p53 pathways (189).

TABLE 1 Hormone secretion of functional adrenocortical carcinomas.

Hormone	Symptoms	Incidence
Cortisol (186)	Osteoporosis, early onset hypertension, hyperglycemia/diabetes, facial plethora, muscle weakness	50-80%
Androgen (49, 186)	Acne, hirsutism, menstrual abnormalities, male baldness	40-60%
Estrogen (186)	Testicular atrophy, gynecomastia	1-3%
Aldosterone (157)	Hypertension, muscle weakness	Rare

3.3.1 Molecular basis of ACC: As part of a tumor predisposition syndrome

3.3.1.1 Li-Fraumeni syndrome (LFS)

LFS is an autosomal dominant disorder that predisposes to various types of cancer including brain cancer, leukemia, soft tissue sarcoma and osteosarcoma, premenopausal breast cancer and ACC. LFS accounts for 50-80% of pediatric cases of ACC (190–192). The clinical criteria for the diagnosis of 'classic' LFS include a sarcoma diagnosis before the age of 45, with a first-degree relative diagnosed with any type of cancer before 45 years and another first or second-degree relative with any cancer diagnosis before the age of 45 years or a sarcoma diagnosis at any age (193). Germline variants in *TP53*, the underlying genetic cause of LFS, have been identified in 70% of cases, while de novo variants have been shown to have a prevalence of 7-20% (129, 190, 194). In a cohort of 286 *TP53*+ patients from 107 families, the cumulative cancer incidence was 50% by 31 years for females and by 46 years for males (190). Of those patients, 67% had their first cancer diagnosis before the age of 17 years, while five patients were diagnosed with ACC before the age of 17 years. Of those patients, 50% had a second cancer diagnosis and ACC was present in one of them (190).

3.3.1.2 Beckwith-Wiedemann syndrome (BWS)

BWS is a systemic overgrowth disorder caused by genetic or epigenetic changes that ultimately result in upregulation of insulin-like growth factor 2 (*IGF2*) (195). Loss of heterozygosity of the 11p15 locus, which harbors *IGF2*, is a common finding in childhood ACC (196). BWS is characterized by hemihypertrophy, macrosomia, macroglossia, hyperinsulinism, omphalocele and distinct facial features (197). In addition, in the first 8 years of life, patients with BWS are at increased risk for embryonal tumors including hepatoblastoma, neuroblastoma and Wilms's tumor (197–201). The risk of developing intra-abdominal tumors is approximately 5-10% and thus patients with BWS need to undergo regular screening for early diagnosis and management. ACC is the next most common type of tumor reported in BWS patients; other common benign adrenal pathologies include adrenal cysts and ACAs (200).

3.3.1.3 Multiple endocrine neoplasia 1 (*MEN1*)

MEN1 is inherited in an autosomal dominant manner and is caused by germline heterozygous variants in the *MEN1* gene on chromosome 11q13. Its main manifestations are hyperparathyroidism (95%), entero-pancreatic neuroendocrine tumors (50%) and pituitary adenomas (40%). Associated adrenal lesions, mostly ACA and hyperplasias, are present in 20-55% of *MEN1* cases (202). Most of these adrenal lesions are nonfunctional (87, 203, 204). ACC occurs only in a small fraction of patients with *MEN1* (87, 205, 206). In two cohorts of sporadic ACC, somatic variants of *MEN1* were shown to have a prevalence of 7% (207, 208).

3.3.1.4 Lynch syndrome

ACC has also been reported in cases of Lynch syndrome (hereditary nonpolyposis colorectal cancer, HNPCC) (209–213). Lynch syndrome is an autosomal dominant disorder caused by germline heterozygous coding variants in DNA-mismatch repair genes (*MSH2*, *MSH6*, *MLH1* and PMS2). Patients have a significantly increased risk of cancer, especially colorectal and endometrial cancer, and thus screening for Lynch syndrome is recommended in all patients that are diagnosed with colorectal cancer (209, 214). The prevalence of Lynch syndrome in a large cohort of patients with ACC was reported to be approximately 3% (215).

3.3.2 Other

ACC has been reported in patients with neurofibromatosis type 1, familial adenomatous polyposis, and Werner syndrome (186, 216–223). In addition, ACC has been reported in two cases of patients with CNC (224, 225).

In general, the discovery of genetic syndromes that confer an increased risk for ACC has yielded important clues into the molecular basis of ACC development. For example, the association between FAP and adrenal tumors provided the basis for the insights into the role of β-catenin signaling in adrenal tumors, while the link between ACC and BWS combined with gene expression profiling suggested the IGF-1 receptor as a target for ACC therapy. The latter hypothesis has now been tested in clinical trials (226).

4. Treatment approaches and clinical trials

Patients with unilateral adrenal adenomas that are hormonally active receive treatment with unilateral adrenalectomy (25). Individuals diagnosed with CS are evaluated for the need of adrenalectomy based on the degree of cortisol excess, comorbidities, age and preference of the patient; typically, surgical resection of the is the first-line treatment (50). However, in some cases where hypercortisolemia is resistant to surgery or in cases where surgical treatment is contraindicated due to patient co-morbidities pharmacotherapy is indicated (227). This includes ketoconazole along with metyrapone, mitotane, or etomidate (227, 228). Recently, two new drugs were approved by the FDA, levoketoconazole and osilodrostat, which are both inhibitors of steroidogenic enzyme activity. Non-functioning adrenal tumors are evaluated to verify eligibility for partial adrenalectomy based on size and potential to malignancy (229). In general, surgery should be considered in lesions >4cm in size or those that are hormonally indeterminate, even if imaging characteristics resemble those of a benign lesion (50). Based on the guidelines from the Endocrine Society, the diagnosis of PA in patients with hypertension should include screening (elevated aldosterone-renin ratio) followed by

confirmatory testing (including salt loading, fludrocortisone or captopril administration, which all fail to sufficiently lower aldosterone levels in patients with PA) (140). Regarding ACCs, complete surgical resection remains the only curative treatment in patients with resectable stages I-III (187, 230), while adjuvant therapies are used to decrease recurrence. In addition, Mitotane, a dichlorodiphenyltrichloroethane analog (DTT), is the only medication that is specifically approved for ACC and can be used either as adjuvant or in advanced stages in combination with classic cytotoxic agents (231). Phase I trial studies using cixutumumab, an insulin growth factor receptor (IGF-1R) antibody have showed promising results in terms of lower toxicity and better disease outcome compared to mitotane (232). Moreover, a trial investigating the combination of cixutumumab with the mTOR inhibitor, temsirolimus, showed that almost half of the patients achieved prolonged stable disease and therefore, current treatment options may be improved (233).

There are at least 15 clinical trials currently recruiting patients diagnosed with ACC. Location includes United States, Europe and China. Over 20 studies, all over the world, are recruiting patients diagnosed with hyperaldosteronism, comprising either observational or interventional studies. There are also about 20 studies currently recruiting patients with CS and many other studies are now recruiting for other adrenal cortex-related diseases. Information about current and past trials, as well as institution and collaborators, can be found at clinicaltrials.gov, a resource provided by the United States National Library of Medicine.

5. Conclusion

Here, we summarized the main genetic and molecular aspects of adrenocortical diseases, which are, in some cases, difficult to diagnose. The rapid progress of next generation sequencing (NGS) techniques has opened new horizons to examine and diagnose adrenocortical diseases with unbiased mechanisms. For example, exome sequencing may be used in patients with a clinical phenotype but no identified variant in one of the known causative genes, in order to perform an unbiased scan and potentially identify causative variants in genes previously not associated with the disorders. This not only yields a diagnosis, but also provides new clues into disease pathophysiology. Despite the progress, diagnosis based on genetic screening is still limited to either large centers and/or patients with financial access to these analyses. Therefore, heath care providers still face limitations in offering access to precise medicine to all patients. Considering that a great number of adrenocortical diseases is due to genetic onset, we believe that increasing access to NGS will greatly improve early and precise diagnosis of adrenocortical disorders. Consequently, more accurate treatments will be delivered to individuals that harbor genetic alterations leading to disorders of the adrenal cortex.

Author contributions

GP: Writing - original draft; AM: Writing - review & editing; FF: Conceptualization; Supervision; Writing - review & editing. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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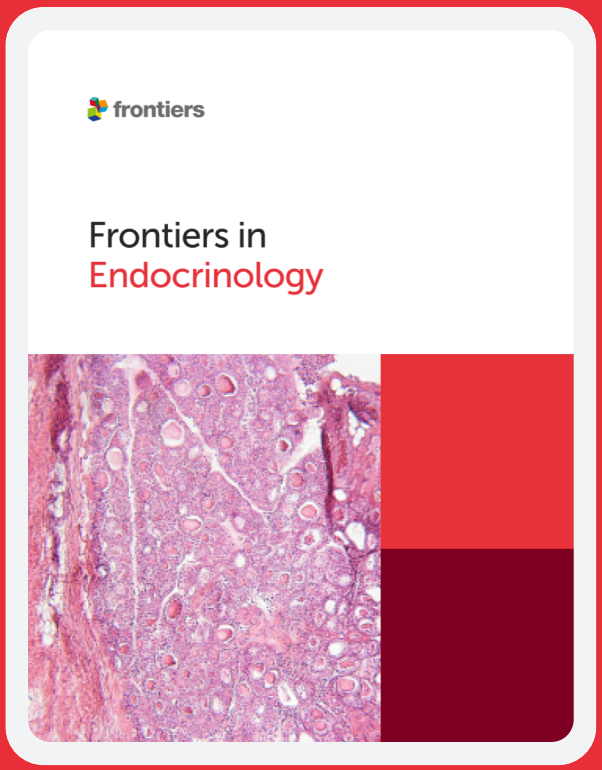

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