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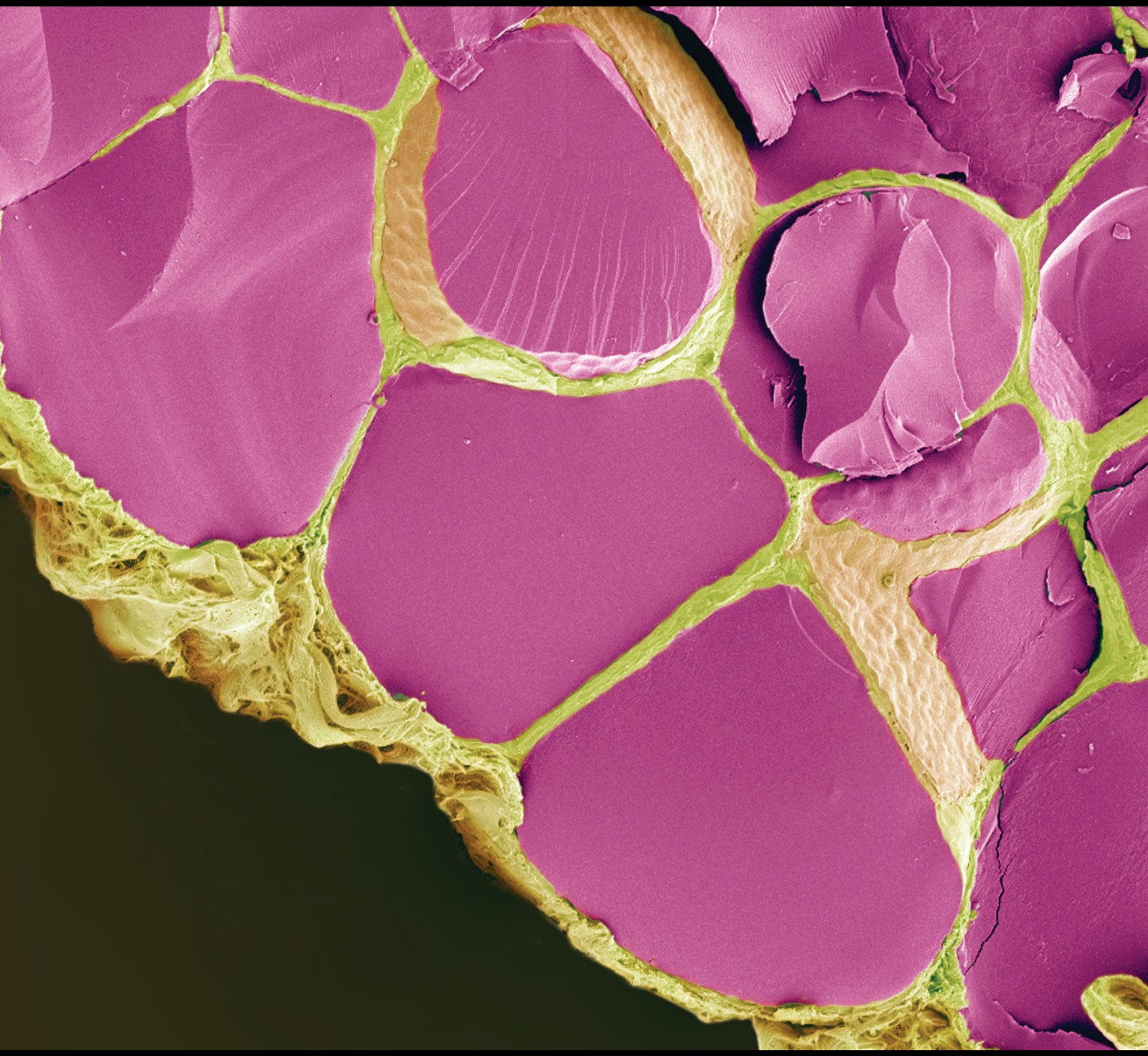
Disease of Adrenal Glands

Guest Editors: Claudio Letizia, Gianluca Iacobellis, Gian Paolo Rossi, and Frederic Castinetti



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Editorial Board

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Editorial
Disease of Adrenal Glands

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The adrenal gland has been historically an object of interest and scientific curiosity. This is also due to its very heterogeneous structure, number of hormones, complex neural innervation, and multiple and different physiological functions. The adrenal gland also entails an outstanding example of paracrine interactions occurring between histogenetically different tissues as the cortex and the medulla.

This special issue is a great opportunity for the reader to learn the latest and emerging findings on the pathophysiology, diagnosis, and treatment of the adrenal glands disorders. The issue provides a variety of excellent articles covering a broad and contemporary spectrum of aspects of the diseases of the adrenal gland.

Of particular interest and novelty is the interplay between hormones of the adrenal glands and other organs, such as the adipose tissue, the endothelium, the bone, and even the brain. In addition to the well-established effects on lipid and glucose metabolism, the hormones of the adrenal glands display a fascinating cross talk with the adipose tissue [1–3]. The interaction between the adrenals and adipokines is extensively discussed by A. Y. Kargi and G. Iacobellis in a comprehensive and updated review paper. The potential of the fat depot surrounding the adrenal tumors to act like a brown adipose tissue (BAT) is a rapidly emerging topic that will certainly deserve further attention and investigation [4]. Interestingly the authors provided a theoretical basis for potential future pharmacological interventions aimed at adrenal hormone targets in the adipose tissue.

Primary aldosteronism is the most common endocrine cause of arterial hypertension. It can cause excess damage to the organs that are target of hypertension and higher cardiometabolic risk [5]. This contention was supported by previous experimental data obtained by Karl Weber's group in rats infused with aldosterone, which exhibited hypercalciuria and raised parathyroid hormone (PTH) levels [6], and, more importantly, by findings in patients with aldosterone-producing adenoma who also showed elevated serum PTH levels that were then normalized by adrenalectomy [7]. The effect of the adrenal hormones on bone metabolism in patients with primary aldosteronism is nicely addressed by L. Petramala et al. The authors sought to test the hypothesis that hyperaldosteronism may influence mineral homeostasis through higher urinary calcium excretion leading eventually to secondary hyperparathyroidism. Of further interest, G. Mazzocchi et al. showed that PTH stimulates aldosterone secretion in a concentration-dependent manner [8], a finding that was complemented by the demonstration of the mineralocorticoid receptor in the human parathyroid cells [9]. It is well established that a substantial amount of sodium is bound to proteoglycans of bone, connective tissue, and cartilage and that the osmotic force created by the high sodium concentration maintains the high water content in the latter tissue, allowing it to withstand high pressures during exercise [10]. In this special issue P. Alonso et al. further expand on the relationships between the adrenal gland and the skeleton by showing a reduction in the bone mineral density

in children with congenital adrenal hyperplasia when bone age rather than chronological age is considered. Altogether these findings further enhance the multitargeting effects of hormones like cortisol and aldosterone and open new avenues for the understanding of the interactions between adrenal and other hormones and tissues originally thought to be totally unrelated. These evidences are rapidly changing the paradigm that hormonal systems work independently.

V. Salpietro et al. extensively discussed the potential neurological manifestations in children with adrenal dysfunction and the compelling need for an early diagnosis.

The causative and prognostic role of neoangiogenesis in patients with pheochromocytoma is analyzed by M. Bialas and colleagues. Neoangiogenesis was evaluated by assessing microvessel density (MVD) and the expression of vascular endothelial growth factors (VEGFs). The study showed that MVD is not able to differentiate between benign and malignant pheochromocytomas. However, the authors proposed that high MVD could be a promising factor for antiangiogenic therapy in malignant cases of pheochromocytoma.

Accurate genetic testing of adrenal glands tumors, such as pheochromocytomas, is the topic of interesting articles by D. A. Rowbotham et al. and R. Martins and M. J. Bugalho. Promising results suggest that mutations in the von Hippel-Lindau (VHL) gene elongin BC protein complex could be important factor for the development of pheochromocytomas.

Genetic analysis is certainly a key factor, but how do we manage cardiovascular disease prevention in adrenal diseases? Cushing's syndrome and even subclinical hypercortisolism are associated with higher cardiovascular risk [11]. But do we have effective tools to predict and possibly prevent this risk? Y.-F. Wang et al. stressed out the need of using the congestive heart failure (C), hypertension (H), age (A), diabetes (D), and stroke (S), the CHADS2 score, to predict major cardiovascular events in patients affected by Cushing's syndrome. Perhaps, other biomarkers, such as imaging of organ-specific fat depot, will emerge for the cardiovascular risk stratification in the adrenal gland diseases [11].

Finally, the best surgical approach and treatment for different clinical conditions and adrenal tumors, such as adrenal benign tumors, incidentalomas, or metastatic adrenal tumors, are also object of discussion in this special issue. There is a consensus that laparoscopic adrenalectomy should be considered as the first choice treatment for the resection of adrenal benign tumors, as suggested by S. Chuan-yu et al, whereas the role of laparoscopic surgery in small incidentaloma is still controversial, as nicely discussed by M. Pędziwiatr et al. The surgical management of adrenal tumors is object of recent debate and investigation. A recent and large multinational observational retrospective population-based study suggested that adrenal-sparing surgery should be the surgical approach of choice for multiple endocrine neoplasia type 2-related phaeochromocytoma to reduce the Addisonian-like complications and consequent lifelong dependency on steroids [12].

In conclusion, the interest in the adrenal gland is now encompassing a broader audience of physicians and researchers. This special issue is aimed at engaging the reader in an exciting reading of the new insights on the adrenal glands and related disorders.

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Frederic Castinetti
Claudio Letizia

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Research Article

Profiling of Somatic Mutations in Phaeochromocytoma and Paraganglioma by Targeted Next Generation Sequencing Analysis

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At least 12 genes (*FH, HIF2A, MAX, NF1, RET, SDHA, SDHB, SDHC, SDHD, SDHAF2, TMEM127,* and *VHL*) have been implicated in inherited predisposition to phaeochromocytoma (PCC), paraganglioma (PGL), or head and neck paraganglioma (HNPGL) and a germline mutation may be detected in more than 30% of cases. Knowledge of somatic mutations contributing to PCC/PGL/HNPGL pathogenesis has received less attention though mutations in *HRAS, HIF2A, NF1, RET,* and *VHL* have been reported. To further elucidate the role of somatic mutation in PCC/PGL/HNPGL tumourigenesis, we employed a next generation sequencing strategy to analyse "mutation hotspots" in 50 human cancer genes. Mutations were identified for *HRAS* (c.37G>C; p.G13R and c.182A>G; p.Q61R) in 7.1% (6/85); for *BRAF* (c.1799T>A; p.V600E) in 1.2% (1/85) of tumours; and for *TP53* (c.1010G>A; p.R337H) in 2.35% (2/85) of cases. Twenty-one tumours harboured mutations in inherited PCC/PGL/HNPGL genes and no *HRAS, BRAF*, or *TP53* mutations occurred in this group. Combining our data with previous reports of *HRAS* mutations in PCC/PGL we find that the mean frequency of *HRAS/BRAF* mutations in sporadic PCC/PGL is 8.9% (24/269) and in PCC/PGL with an inherited gene mutation 0% (0/148) suggesting that *HRAS/BRAF* mutations and inherited PCC/PGL genes mutations might be mutually exclusive. We report the first evidence for *BRAF* mutations in the pathogenesis of PCC/PGL/HNPGL.

1. Introduction

Phaeochromocytoma (PCC) and paraganglioma (PGL) are neuroendocrine tumours deriving from chromaffin cells of the medulla of the adrenal glands or from extra-adrenal chromaffin tissue like ganglia of the sympathetic nervous system, respectively. Approximately 10% of tumours are malignant but the most common presentation results from

the cardiovascular effects of catecholamine hypersecretion that causes hypertension, tachycardia, excessive sweating, and/or anxiety. The majority of PCC/PGL occur sporadically but although only about 10% of cases have a family history, more than a third of cases harbour a germline mutation in one of the 12 inherited PCC/PGL genes (*FH, HIF2A, MAX, NF1, RET, SDHA, SDHB, SDHC, SDHD, SDHAF2, TMEM127,* and *VHL*) that have been shown to be mutated in multiple families and some of these genes are also somatically mutated in sporadic PCC/PGL [1–8]. Germline mutations in most of these genes may also cause head and neck paraganglioma (HNPGL) which are derived from parasympathetic nervous system ganglia and are nonsecretory [9–12]. It appears that disruption of multiple cellular signalling pathways is implicated in PCC/PGL/HNPGL tumourigenesis. Thus known inherited predisposition gene products belong to multiple functional classes including kinase receptor and signalling regulators (*RET* and *NF1*), transcription factors (*MAX*), energy metabolism components (*FH*, *SDH*-subunits *A*, *B*, *C*, and *D*, and cofactor *SDHAF2*), constituents of the cellular response to hypoxia (*VHL* and *HIF2A/EPAS1*), and endosomal signalling (*TMEM127*) [1–8, 13–15]. Nevertheless, gene expression studies have suggested that most PCCs and PGLs can be classified into two distinct groups (cluster 1 and cluster 2) by transcription profiling: cluster 1 includes tumours that harbour mutations in genes linked to the hypoxic gene response (*VHL, HIF2A, SDHA, SDHB, SDHC,* and *SDHD*) and cluster 2 contains tumours harbouring mutations in genes that are involved in the kinase signalling characterized by the activation of the PIK3/AKT/mTOR and RAS/RAF/ERK pathways (*RET, NF1, TMEM127, MAX*, and *HRAS*) [14–18].

The last 10 years have seen considerable progress in identifying the genetic basis of inherited PCC/PGL/HNPGL, but relatively less progress has been made with respect to understanding the somatic mutations that underlie tumour initiation and progression in these tumour types. Somatic mutations in inherited PCC/PGL genes can be detected in ~25–30% of the sporadic tumours (mainly involving *RET, VHL, NF1, MAX*, and *HIF2A* genes). Recently, an exome resequencing study led to the identification of somatic *HRAS* activating mutations in sporadic PCC/PGLs [19]. Subsequently, this finding was confirmed and the overall frequency of somatic *HRAS* mutations has been estimated at about 7% [20]. However, to date *HRAS* analysis in PCC/PGL has mostly been performed by Sanger sequencing and the frequency of *HRAS* mutations might have been underestimated because of the lower sensitivity of Sanger sequencing analysis, compared to next generation sequencing approaches, to detect mosaic mutations [21]. Furthermore, in contrast to many other tumour types, there is little information available on many of the genes most commonly mutated in human neoplasia. We therefore investigated, in tumour samples, the frequency of mutations in critical regions of 50 human cancer genes by next generation sequencing in a large series ($n = 85$) of inherited and sporadic PCC/PGL/HNPGL.

2. Materials and Methods

2.1. Subjects. Tumour material from 85 patients with PCC ($n = 60$), PGL ($n = 5$), or HNPGL ($n = 20$) was collected for analysis. 21 patients were known to harbour a germline inherited gene mutation (*VHL* = 10, *RET* = 3, *NF1* = 1, *SDHB* = 5, *SDHC* = 1, and *SDHD* = 1) and 64 cases were sporadic. All patients gave informed consent and the study was approved by the South Birmingham Ethics Committee.

2.2. Next Generation Sequencing (NGS)

2.2.1. Technical Assessment. To investigate the sensitivity of somatic mutation detection, a dilution series of two well characterized DNA samples was created. A sample bearing *HRAS* (c.81T>C) and one a *PIK3CA* (c.1173A>G) were titrated together to create serial dilutions containing allelic frequencies ranging from 50% to 0.1%. Each dilution was amplified using the Ion AmpliSeq Cancer Hotspot Panel v2 (Life Technologies, UK). Amplicons underwent library preparation according to protocol. Before emulsion PCR each point of the titration curve was identified by using a different Ion Xpress Barcode. Subsequently library was run on 318 chip v2 (Life Technologies, UK) on the Ion PGM (Life Technologies, UK). The output reads from the chip were processed using the Torrent browser suite software (v.4.0.2).

2.2.2. Sample Sequencing. DNA was isolated from both tumour material and peripheral blood using standard methodology. Genomic DNA (gDNA) samples were quality-checked on DNA NanoDrop 1000 considering acceptable absorbance ratio greater than 1.7 for both 260/280 and 230/260 nm. Each sample was then quantified with the Qubit2.0 fluorometer (Life Technologies, UK) by using the Quant-IT dsDNA BR Assay (Life Technologies, UK). For the AmpliSeq Library, 10 ng of gDNA was used for library generation. Libraries were indexed using the Ion Xpress Barcode Adapter Kit and quantified using the Quant-IT dsDNA HS Assay (Life Technologies, UK) on Qubit 2.0. Appropriate dilutions were performed based on amplicon concentration at the 80–125 bp range. Twenty pM of individual indexed amplicon libraries were pooled for emulsion PCR and 16 samples were sequenced on the Ion Torrent PGM platform using the 318 v2 chip (Life Technologies, UK). Mean coverage for each sample was over 1000x. Sequence reads were mapped against the human reference genome (hg19) with the Torrent Mapping Alignment Program (TMAP) using the default software settings. Output was restricted to the targeted regions as defined by the sequence capture design BED file, and SNPs and INDELs were characterized as being significantly different from the reference sequence if the variant to reference base frequency was greater than 5%. All identified variants within a particular sample were saved as variant call format file (VCF version 4.1). VCFs were examined with the online tool "Ingenuity Variant Analysis" (Qiagen) for variant annotation and prediction of variant effects on genes. In addition, BAM files were inspected manually in order to remove likely artefactual variants (i.e., close to homopolymers) and to detect any mutations in

Table 1: Mutations detected in inherited PCC/PGL/HNPGL genes.

Tumour ID	Type of tumour	Gene	Mutation type
P1	PCC	*RET*	c.1900T>C
P2	PCC	*RET*	c.1901G>A
P3	PCC	*RET*	c.2753T>C
P4	PCC	*VHL*	c.241C>T
P5	PCC	*VHL*	c.292T>C
P6	PCC	*VHL*	c.292T>C
P7	PCC	*VHL*	c.292T>C
P8	PCC	*VHL*	c.292T>C
P9	PCC	*VHL*	c.292T>C
P10	PCC	*VHL*	c.292T>C
P11	PCC	*VHL*	c.292T>C
P12	PCC	*VHL*	c.292T>C
P13	PCC	*VHL*	c.374A>C
P14	PCC	*SDHB*	c.470delT

known driver genes, especially insertions and deletions that were not called by the Ion Torrent software.

2.3. Sanger Sequencing. To confirm NGS results, fragments of hotspot codons in *HRAS* (exons 2 and 3), *BRAF* (exon 15), and *TP53* (exon 10) were amplified, in both tumour and constitutional (blood) DNA, by PCR and sequenced with automated Sanger sequencing. Primers sequences and PCR conditions are available on request.

2.4. Statistical Analysis. Patients with mutated *HRAS* and *BRAF* were compared to patients with negative genetic screening.

P values <0.05 were considered statistically significant.

3. Results

3.1. Technical Assessment of Next Generation Sequencing Assay. The analytical sensitivity of the AmpliSeq Hotspot panel was determined using serial dilutions of tumour DNA carrying *PIK3CA* and *HRAS* mutations. This demonstrated that it was possible to reliably detect mutations at 1% allele frequency (data not shown). Sequence coverage was assessed considering the number and distribution of reads that were present in the target DNA regions. Each sample had approximately 317000 mapped reads with a mean read length of 107 bp that generates approximately 23 Mb of sequence with depth of coverage of 1400 reads.

3.2. Detection of Mutations in Inherited PCC/PGL/HNPGL Genes. 21 patients were known to harbour a germline mutation in an inherited PCC/PGL gene (*VHL* = 10, *RET* = 3, *NF1* = 1, *SDHB* = 5, *SDHC* = 1, and *SDHD* = 1). 13 of these mutations (*RET* ($n = 3$) and *VHL* ($n = 10$)) were detected by NGS in 13 tumours (see Table 1) and 8 patients had a clinical or previous molecular diagnosis of a germline inherited PCC/PGL gene mutation (*NF1* ($n = 1$), *SDHB* ($n = 5$), *SDHC* ($n = 1$), and *SDHD* ($n = 1$)) that was not covered by the NGS assay. No mutations in inherited PCC/PGL genes were detected in the 64 sporadic tumours.

3.3. Detection of Activating Mutations in Protooncogenes and Tumour Suppressor Genes. Six tumours (PCC = 6/60, PGL = 0/5, and HNPGL = 0/20) harboured an activating missense mutation in the *HRAS* hotspot region of codons 13 and 61 (c.37G>C; p.G13R = 1 and c.182A>G; p.Q61R = 5), giving an overall frequency of 7.1% (6/85, 95% CI = 2.63%–14.73%). In each case the somatic status of the mutations was confirmed when the detected mutation was absent in matched constitutional DNA (blood) (Table 2). In one PCC tumour (1/85) an activating *BRAF* mutation was identified (c.1799T>A; p.V600E, 1.2%, 95% CI = 0%–6.38%) (Table 2). In two tumours a missense mutation (c.1010G>A; p.R337H, 2/85, 2.4%, 95% CI = 0.29%–8.2%) occurring in the tetramerisation domain of TP53 protein was identified (Table 2).

3.4. Exclusion of Mutations in Protooncogene Hotspots. No mutations were detected at hotspot mutation regions in 11 oncogenes frequently mutated in human cancer (*AKT, MET, PIK3CA, KRAS, NRAS, IDH1, IDH2, NOTCH, SMO, ABL*, and *EGFR*).

3.5. Relationship between Clinical Status and HRAS Mutations. We investigated the relationships between *HRAS* mutation and tumour location and presence or absence of an inherited PCC/PGL/HNPGL gene mutation. The frequency of *HRAS* mutations in PCC, PGL, and HNPGL was 6/60 (10%, 95% CI = 3.76%–20.51%), 0/5 (0%, 95% CI = 0%–52.18%), and 0/20 (0%, 95% CI = 0%–16.84%), respectively. Among 21 tumours from patients with known inherited disease and/or detectable mutation in inherited PCC/PGL/HNPGL genes there were no *HRAS* mutations (0%, 95% CI = 0%–16.11%) whereas a mutation was present in 6/64 (9.4%, 95% CI = 3.52%–19.30%) of patients without a clinical diagnosis of inherited PCC/PGL/HNPGL or a detectable mutation in an inherited PCC/PGL/HNPGL gene ($P = 0.33$).

To further investigate possible relationships between these attributes we combined our data for *HRAS* mutation status with 18 mutations in 353 PCC/PGL/HNPGL from two previously published studies (Tables 3 and 4) [19, 20]. Meta-analysis results showed that the overall prevalence of *HRAS* mutations in the cohort of PCC/PGL is 5.48% (24/438, 95% CI = 3.54%–8.04%) increasing to 8.9% (24/269, 95% CI = 5.80%–12.98%) considering cases without an inherited PCC/PGL gene mutation and to 9.87% (23/233, 95% CI = 6.36%–14.44%) considering only PCC samples without an inherited PCC/PGL gene. In PCC/PGL with an inherited gene mutation the *HRAS* mutation frequency was 0% (0/148, 95% CI = 0%–2.46%) (PCC/PGL unknown mutation versus PCC/PGL known mutation, $P = 0.0001$).

4. Discussion

A wide repertoire of genetic and epigenetic events can be implicated in human neoplasia. Previous studies have

Table 2: Oncogene mutations identified in next generation sequencing analysis of 85 PCC/PGL/HNPGL.

Tumour ID	Clinical diagnosis	Type of tumour	Gene	Codon change	Aminoacid change	Allele frequency
P19	Phaeochromocytoma	PCC	*HRAS*	c.37G>C	G13R	72%
P20	Phaeochromocytoma	PCC	*HRAS*	c.182A>G	Q61R	36%
P21	Phaeochromocytoma	PCC	*HRAS*	c.182A>G	Q61R	27%
P22	Phaeochromocytoma	PCC	*HRAS*	c.182A>G	Q61R	40%
P23	Phaeochromocytoma	PCC	*HRAS*	c.182A>G	Q61R	50%
P24	Phaeochromocytoma	PCC	*HRAS*	c.182A>G	Q61R	26%
P25	**Phaeochromocytoma**	**PCC**	***BRAF***	**c.1799T>A**	**V600E**	**10%**
P26	**Phaeochromocytoma**	**PCC**	***TP53***	**c.1010G>A**	**R337H**	**4%**
P27	**Phaeochromocytoma**	**PCC**	***TP53***	**c.1010G>A**	**R337H**	**21%**

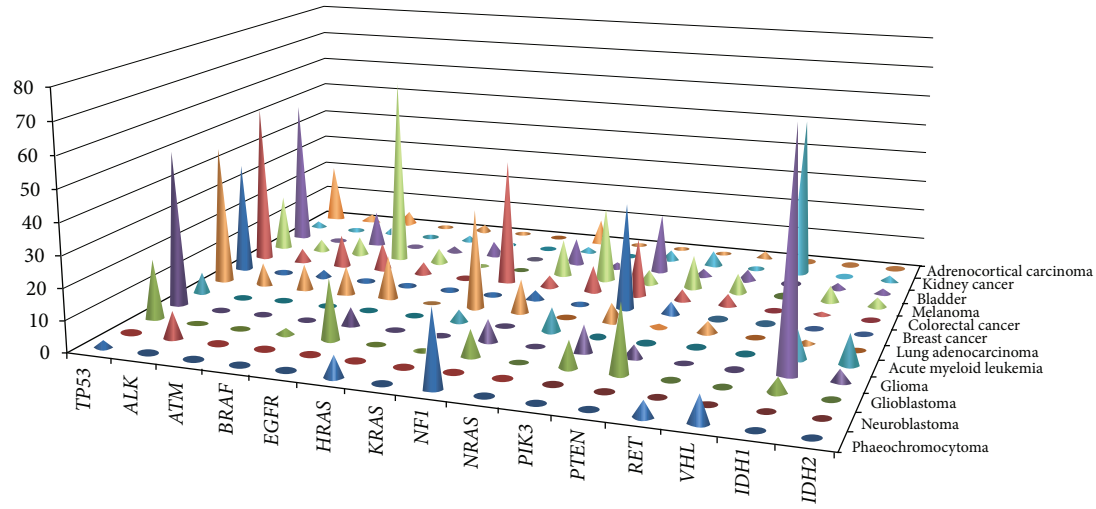

Figure 1: Comparison of somatic variant frequencies in multiple cancer genes in different cancer types.

demonstrated that tumour suppressor gene (TSG) inactivation and oncogene activation in PCC/PGL may result from somatic copy number abnormalities (SCNA), intragenic mutations, and epigenetic silencing of transcription by promoter methylation [22]. Common copy number changes in PCC include loss of chromosomes 1p, 3q, 3p, 11p, 11q, 6q, 17p, and 22 [23–27] and gain of chromosomes 9q, 17q, 19p13.3, and 20q [28, 29]. Epigenetic inactivation of candidate TSGs has been reported relatively frequently in PCC/PGL. Thus promoter methylation of candidate TSGs including *RASSF1A, FLIP, TSP1, DCR1, DCR2, DR4, DR5, CASP8*, and *HIC1* was reported at a frequency of >20% of tumours analysed [30–32]. Until recently, investigations of patterns of somatic mutations in PCC/PGL/HNPGL had concentrated on analysing genes known to be associated with inherited PCC/PGL/HNPGL. Thus *NF1, VHL, RET*, and *MAX* germline and somatic mutations have been reported in 3–25%, 13–9%, 5–5%, and 1–3% of tumours, respectively [33]. Recently activating mutations in *HIF2A* and *HRAS* were reported in a subset of these tumours [6, 7, 19, 20]. Whilst *HIF2A* mutations may be found in multiple tumours from patients without a detectable germline mutation (suggesting low level constitutional mosaicism) or occasionally as a germline mutation [5], to date *HRAS* mutations have only been detected as somatic changes (germline *HRAS* mutations are associated with Costello syndrome but PCC/PGL/HNPGL are apparently not a feature of this disorder) [34]. The frequency of *HRAS* mutations in our cohort was similar to that in other recent studies. *HRAS* mutation hotspots at codons 13 and 61 affect the RAS GTP hydrolysis domain, leading to a constitutive activated state with resistance to upstream inhibitory proteins, such as neurofibromin (*NF1* gene product). This overactive RAS signalling leads to increased activity of downstream effectors, most notably the RAS/RAF/ERK and PI3K/AKT/mTOR signalling pathways linked to increased cell proliferation and tumour formation [14–18]. The identification of *HRAS* mutations as a new pathogenetic driver in sporadic PCC opens up the possibility of new therapeutic approaches—though in most cases surgical removal seems likely to be the treatment of choice. *BRAF* mutations are found in multiple cancer types (Figure 1), notably those that are also associated with mutations in isoforms of RAS (i.e., malignant melanoma, colorectal cancer). Our results demonstrate for the first time a somatic *BRAF* mutation in PCC/PGL/HNPGL samples. The mutation detected is the most common *BRAF* mutation found in human neoplasia and results from a T to A transversion at nucleotide 1799. c.1799T>A (p.V600E) mutant BRAF proteins are characterized by an increased kinase activity and have been demonstrated to induce cellular transformation in *in vitro* studies [35].

Table 3: Meta-analysis of *HRAS* mutations in phaeochromocytoma, paraganglioma, and HNPGL.

Study	Frequency of *HRAS* mutations in phaeochromocytoma					Frequency of *HRAS* mutations in paraganglioma					Frequency of *HRAS* mutations in HNPGL				
	All	Positive samples	Sporadic	Positive samples	Inherited	All	Positive samples	Sporadic	Positive samples	Inherited	All	Positive samples	Sporadic	Positive samples	Inherited
Current study	60	**6**	44	0	16	5	**0**	5	0	0	20	**0**	15	0	5
Crona et al. [19]	72	**3**	50	0	22	9	**1**	6	0	3	1	**0**	1	0	0
Oudijk et al. [20]	216	**14**	140	0	76	55	**0**	24	0	31	0	**0**	0	0	0
Total	348	***23***	234	0	114	69	***1***	35	0	34	21	***0***	16	0	5

Table 4: *HRAS* mutations frequencies in phaeochromocytoma and paraganglioma.

Study	Overall frequency of *HRAS* mutations			Frequency of *HRAS* in PCC/PGL with unknown mutations			Frequency of *HRAS* in PCC with unknown mutations		
		All	Positive samples		All	Positive samples		All	Positive samples
Current study	7,06%	**85**	6	12,24%	**49**	6	13,95%	**43**	6
Crona et al. [19]	4,88%	**82**	4	7,14%	**56**	4	6,00%	**50**	3
Oudijk et al. [20]	5,17%	**271**	14	8,54%	**164**	14	10,00%	**140**	14
Total	5,48%	***438***	24	8,92%	***269***	24	9,87%	***233***	23

Since BRAF is involved in ERK kinase activation in the RAS/RAF/ERK signalling pathway, it will be interesting to further compare the biological behaviour of *HRAS* and *BRAF* mutated tumours in order to determine whether they have similar clinical characteristics and to determine if they will fall into the "cluster 2" group of gene expression patterns. PCC/PGLs with *BRAF* V600E mutations may be predicted to respond to BRAF and MEK inhibitors, such as vemurafenib and trametinib/selumetinib, better than wild type *BRAF* tumours [36–39].

The presence of *BRAF* and *HRAS* mutations in PCC/PGL suggests that activation of the RAS/RAF/ERK signalling pathway can be triggered by mutations at various levels in the pathway. An implementation of the panel used, including more genes of this pathway, could be useful to identify low frequency somatic variants in PCC/PGL. Loss of heterozygosity at 17p13 is frequent in many tumour types including breast [40], colon [41], and hepatocellular cancers [42] and phaeochromocytoma [22], with an occurrence ranging from 30% to 60%. The tumour suppressor gene *TP53* maps in this region and has been demonstrated to be implicated in the tumourigenesis process in different types of cancers. Loss of TP53 function could arise from epigenetic alterations allelic losses and mutational events. Due to its involvement in carcinogenesis, *TP53* has been intensively studied in PCC/PGL but, despite frequent allele loss, *TP53* mutations have been reported rarely [23, 43–45]. However, in our series, NGS results followed by direct DNA sequencing demonstrated the presence in two sporadic PCC samples of a somatic *TP53* gene mutation (c.1010G>A; p.R337H) (Table 2). The R337H substitution has previously been extensively characterised as a founder germline mutation and the altered protein demonstrated to act as a conditional mutant that loses its function only when a small increase in intracellular pH occurs in cells. Initially, the c.1010G>A (R337H) mutation was thought to predispose only to adrenal cortical carcinoma (ACC) (for which the penetrance of the allele has been estimated to be ~10% and the increased risk was estimated at a 20,000-fold increase [46, 47]); however, several studies have highlighted it in association with Li Fraumeni-like syndrome [48], breast cancer [49, 50], choroid plexus carcinoma, and osteosarcoma [51, 52]. To our knowledge, this is the first report on this mutation in sporadic phaeochromocytoma. By using an approach utilising targeted deep sequencing we were able to confidently detect the presence or absence of a large repertoire of hotspot mutations, paying particular attention to a set of them. The absence of mutations in *ALK* and *NRAS* was of particular interest (Figure 1) because mutations in these genes have been described in neuroblastoma (~9.2%, 0.83% of cases, resp.; Figure 1) and some epigenetically inactivated candidate TSGs (i.e., *RASSF1A, FLIP, CASP8*, and *HIC1*) are common to both PCC/PGL and neuroblastoma. *IDH1* and *IDH2* mutations have been described in gliomas, leukaemia, and other malignancies (Figure 1) and may cause methylation abnormalities by a similar mechanism to that associated with *SDH* gene subunit mutations [53]. Therefore despite *IDH1/IDH2* hotspot regions potentially representing candidate genes for somatic inactivation in PCC/PGL/HNPGL, no mutations were identified (Figure 1) [54]. Our findings are consistent with the hypothesis that mutations in *HRAS* and inherited PCC/PGL genes are mutually exclusive driver mutations (though comprehensive analysis of all inherited phaeochromocytoma/PGL genes has not been undertaken in all patients). If this hypothesis is correct, it can be suggested that *HRAS* profiling of PCC/PGL/HNPGL tumour material could aid the management of patients by enabling enhanced stratification of their risk of inherited disease. Furthermore, we suggest that molecular profiling should be expanded to include *BRAF* analysis. Currently histopathology cannot reliably predict the likelihood of malignancy in PCC/PGL/HNPGL whereas the presence of a germline *SDHB* mutation is associated with an increased risk of malignancy [55–57]. The identification, careful characterisation, and follow-up of cohorts of patients with *HRAS/BRAF* mutation positive tumours could enable the natural history of such tumours (e.g., absence of malignant or recurrent disease) and so facilitate personalised management of patients with these tumours.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Adrenal Tumors with Unexpected Outcome: A Review of the Literature

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The finding of an adrenal mass should induce a diagnostic work-up aimed at assessing autonomous hormone production and differentiating between benign and (potentially) malignant lesions. The common differential diagnosis in adrenal incidentaloma consists of (non-)functioning adenoma, pheochromocytoma, myelolipoma, metastasis, and primary carcinoma. There remains a category of lesions that are hormonally inactive and display nonspecific imaging characteristics. We provide a succinct literature review regarding pathologies from this category. Imaging and histological characteristics are discussed, as well as clinical management. In conclusion, an adrenal mass may present a diagnostic challenge. After exclusion of most common diagnoses, it can be difficult to differentiate between possible pathologies based on preoperative diagnostic tests. Surgical resection of possibly harmful tumors is indicated, for example, lesions with malignant potential or risk of spontaneous hemorrhage. Resection of an obviously benign lesion is not necessary, unless problems due to tumor size are expected.

1. Introduction

Clinicians may be confronted with adrenal masses in four different scenarios. The first category comprises patients presenting with endocrinological symptoms suggesting adrenal origin, such as virilization or Cushing's syndrome as seen in selected adrenocortical adenomas and carcinomas. Hypertension, flushes, and headache may be signs of pheochromocytoma or aldosterone-producing adenoma (Conn's syndrome). Secondly, patients may present with nonspecific symptoms that turn out to be caused by an adrenal tumor such as pain, fatigue, weight loss, or the sensation of an abdominal mass. Thirdly, adrenal metastases might be found in the work-up of another malignancy, for example, lung cancer. Finally, an adrenal mass may be found incidentally during evaluation for nonrelated complaints: a so-called adrenal incidentaloma.

The common differential diagnosis includes six entities which account for the large majority of all adrenal masses. This will be discussed first. Secondly, we discuss a remaining category that consists of ten entities that are hormonally inactive and display nonspecific imaging characteristics.

2. Differential Diagnosis

The common differential diagnosis in adrenal incidentaloma consists of nonfunctioning adenoma, functioning adenoma, pheochromocytoma, and adrenocortical carcinoma. Myelolipomas and metastases from various malignancies are also common and should be included [1, 2]. The ranking by likelihood of these diagnoses varies depending on individual presentation. In general, most incidentalomas (70–80%) are benign adenomas which cause no symptoms. However, in 5–20% of patients who have no endocrine signs or symptoms, analysis reveals subclinical hypercortisolism [3–5]. Pheochromocytoma makes up about 1.5–14% of incidentalomas, adrenocortical carcinoma (ACC) is found in 1.2–11%, aldosterone-producing adenoma is found in 1.6–3.3%, and adrenal metastases are found in 1–18% [6, 7].

3. Diagnostic Work-Up

The diagnostic work-up should be aimed at assessing autonomous hormone production and differentiating between benign and (potentially) malignant lesions.

Evaluation of cortisol and (nor)metanephrine secretion should be performed in all patients presenting with an adrenal mass, even in absence of clinical signs of Cushing's syndrome or pheochromocytoma [3, 6, 58, 59]. Also, clinicians should be aware of the possibility of adrenal insufficiency in case of bilateral lesions. Screening for primary hyperaldosteronism by measuring plasma aldosterone concentration and plasma renin activity should be performed if hypertension and/or hypokalemia are present [6, 58]. The most accurate predictor to differentiate between benign and malignant masses is attenuation on unenhanced CT. If the lesion's attenuation value is ≤10 Hounsfield units (HU), malignancy is extremely unlikely [60]. In case of HU > 10, a contrast wash-out sequence should be performed. A washout > 50% after 15 minutes is indicative of adrenal adenoma. Combined use of attenuation measurement and washout values can be used to discriminate adenomas from other adrenal masses with 98% sensitivity and 92% specificity [61].

Percutaneous adrenal biopsy has high false negative rates and there is a risk of complications. Therefore, the only role of percutaneous biopsy in the evaluation of an adrenal mass is confirming metastatic disease in patients with known extra-adrenal malignancy and confirming the diagnosis of ACC when radical resection is deemed not possible [3, 58].

4. Remaining Pathologies: A Mixed Group

There remains a category of lesions that are hormonally inactive and display nonspecific imaging characteristics, which poses a diagnostic challenge. Here we discuss individual entities from this group. A summary of imaging and pathological characteristics of these lesions is provided in Table 1.

Primary adrenal lymphoma (PAL) is a rare finding with less than 200 cases described in the literature. In 70–80% of cases both adrenal glands are affected [8–11, 62]. On imaging studies, PAL typically presents as a large mass in which cystic or hemorrhagic components may be present. Homogeneous and heterogeneous lesions are reported in similar frequencies. Diffuse large-cell B cell lymphoma is the most commonly reported subtype, anaplastic large cell or T-cell lymphoma are only reported sporadically [8, 11–13]. Treatment consists of combination chemotherapy, sometimes preceded by surgery in cases of a large tumor mass [8, 14]. Prognosis depends heavily on treatment response, but a mean overall survival of 15 months has been reported [10, 14].

Liposarcomas account for 45% of all retroperitoneal soft tissue sarcomas. Five histological subtypes are known, of which well-differentiated liposarcomas (WDLS) and dedifferentiated liposarcomas (DDLS) are most commonly found retroperitoneally [16]. DDLS is found as a focal lesion with low attenuation on T1-weighed MRI within a well-delineated, lipogenic, and septated mass that is the WDLS in approximately 10% of all cases [17]. Histologically, the dedifferentiated area is characterized by atypical nonlipogenic stromal cells with hyperchromatic nuclei that are scattered in fibrous septa. With increasing grade of dedifferentiation, cellularity increases and nuclear atypia is more prominent. Despite often severe nuclear deformities, the mitotic rate is not very high [18]. Retroperitoneal liposarcomas are notorious for recurring and prognosis is poor: 5-year overall survival rates differ from 36 to 55% [19–21].

Schwannomas originate from Schwann cells in peripheral nerve sheaths. Approximately 3% of schwannomas are located in the retroperitoneal space, where it may involve the adrenal gland and/or mimic an adrenal mass [23, 24]. All schwannomas display benign behavior, except for a poorly defined proportion of the rare subtype melanotic schwannoma [25]. The appearance of a schwannoma on CT-scan is a round and well-circumscribed mass, hypo- or isointense compared to muscle that enhances after contrast administration [26]. On T1-weighted MRI images, signal intensity is intermediate and similar to muscle. On T2-weighted images, signal intensity is markedly increased [26]. Histologically, a schwannoma can be recognized by the presence of elongated spindle cells, organized in areas of both high and low cellularity, called Antoni A and B tissue [24, 26]. Immunohistochemical staining is positive for neuron-specific enolase (NSE), microfilament proteins, and S-100 protein, the neural protein in Schwann cells [24, 27].

To our knowledge, there are no reports on recurrent retroperitoneal schwannoma after radical resection.

Ganglioneuromas typically arise from primordial neural crest cells present in the adrenal medulla [28–30]. Calcifications may be apparent on CT-scan in 30%–60% of cases. Unenhanced attenuation values are relatively high: >25 HU. Biological behavior is benign in most cases, although malignant transformation is supposedly possible [28].

Idiopathic adrenal haematomas may be discovered as incidentaloma, due to abdominal complaints or due to adrenal insufficiency. Imaging characteristics vary from well-demarcated homogeneous masses to heterogeneous lesions suspect for periadrenal infiltration [33]. Adrenalectomy is often performed in order to obtain a diagnosis.

Adrenal cavernous haemangiomas are very rare and have only been described in individual case reports [36–39].

Table 1: Summary of imaging and pathological characteristics of rare adrenal pathologies.

Diagnosis	Number of cases	Imaging characteristics		Pathological characteristics		Clinical behavior
		CT	MRI	Histology	Immunohistochemistry +	
Primary adrenal lymphoma [8–15]	<200	Mostly hypodense tumors, aspect homo- or heterogeneous, slight to moderate contrast enhancement.	Iso/hypointense in T1 and hyperintense in T2.	Atypical cells, anisokaryosis, hyperchromasia, necrosis.	Most common: CD45/CD20/CD40 (B-cell) CD3/CD30/CD43 (T-cell)	Malignant
Dedifferentiated liposarcoma [16–22]	>500	DDLS: nonlipogenic, heterogeneous node within a well-delineated, lipogenic, and septated mass that is the WDLS	WDLS: >75% fat, nonlipomatous components are prominent thick septa. Nodular nonadipose areas may be present. DDLS within WDLS: low to intermediate on T1 and intermediate to high on T2.	Atypical nonlipogenic stromal cells with hyperchromatic nuclei, scattered in fibrous septa. Cellularity and nuclear atypia increase with dedifferentiation. Mitotic rate typically <8/10HPF.	MDM2, CDK4	Malignant
Schwannoma [23–27]	>500	Round, well-circumscribed, hypo- or iso-intense compared to muscle, enhancement postcontrast.	Intermediate on T1 (isointense to muscle), mared increase on T2.	Elongated spindle cells in areas of both high (Antoni A) and low cellularity (Antoni B).	NSE, microfilament, S100	Benign
Ganglioneuroma [28–32]	>60	Att. > 25 HU, homogeneous aspect, calcifications in 30%–60%.	Hypointense on T1, heterogen. hyperintense on T2.	Ganglion cells, spindle cells, nerve fibres.	NSE, synaptophysin, S100, and CD57.	Benign Rare: transformation to malignant nerve sheath tumor.
Idiopathic adrenal haematoma [33–35]	>10	Variable: homo/heterogeneous depending on lesion's age.	High intensity on T1 in periphery of lesion suggests hemorrhage.	Hemorrhage, necrosis and hemosiderin.	—	Benign
Cavernous haemangioma [36–39]	>60	Heterogeneous, central cystic/necrotic components, calcifications, nodular peripheral enhancement postcontrast.	Homogeneous on T1, high intensity on T2.	Necrosis, cystic components, large vascular spaces, single lining of endothelium.	—	Benign Risk of spontaneous hemorrhage. Rare: transformation to angiosarcoma.

Table 1: Continued.

Diagnosis	Number of cases	Imaging characteristics		Pathological characteristics		Clinical behavior
		CT	MRI	Histology	Immunohistochemistry +	
Angiomyolipoma [40–44]	<10	Heterogeneous: contains fat, possibly small enhancing foci.	—	Adipose tissue, smooth muscle fibres.	HMB45, MART1/MelanA, smooth muscle actin.	Benign Risk of spontaneous hemorrhage.
Epithelioid angiosarcoma [45–49]	>20	Irregular margins, nonhomogeneous density, calcifications.	High intensity on T2.	Vascular spaces lined by endothelial cells with epithelioid features, possibly pleomorphism.	Factor VIII, also CD34 and UEA-1 (less specific).	Malignant
Leiomyosarcoma [50, 51]	<20	Heterogeneous, possibly also liquid components.	—	Spindle-shaped neoplastic cells, nuclear pleomorphism, giant cell formation.	Smooth muscle actin.	Malignant
Cyst [52–57]	>600					
Pseudocyst	39%	Fibrous wall, no endothelial/epithelial lining, dependent on age septations, blood products, fluid-fluid level, or soft tissue component.	Intermediate/high density in T1, marked bright up in T2.	Usually unilocular, no endothelium. Contains brown/reddish fluid. Connective tissue walls calcificated/hyalinized.	—	Benign
Endothelial	45%	Thin wall (≤3.5 mm), smooth borders and pure cystic internal structure. Att. < 20 HU. No contrast enhancement.	—	Smooth endothelial lining, contains clear or milky fluid.	D2-40.	Benign
Epithelial	9%			Lined with cylindrical epithelium.	Calretinin and WT-1.	Benign
Parasitic	7%	Floating membrane or daughter cysts, septal or mural calcifications, coexistent hydatid cysts of other organs.	—	Thick, possibly calcificated walls, parasites within.	—	Benign

DDLS: dedifferentiated liposarcoma, WDLS: well-differentiated liposarcoma, Att: attenuation on unenhanced CT, HU: Hounsfield units, NSE: neuron-specific enolase, UEA-1: Ulex Europaeus Agglutinin 1, WT-1: Wilms tumor protein, and Immunohistochemistry +: Positive immunohistochemical staining.

Recurrence after complete resection is not reported; however malignant transformation to angiosarcoma may be possible.

Adrenal angiomyolipomas are extremely rare with only five cases reported [40–44]. These tumors are classified in the family of perivascular epithelioid tumors (so called PEComas). It may be difficult to differentiate this tumor from (ad)renal carcinomas on imaging studies and even upon histological examination. The presence of both adipose tissue and cells positively staining for muscle and melanoma markers are required for definitive diagnosis.

Adrenal leiomyosarcomas and epithelioid angiosarcomas are also exceptionally rare. Concise histomorphological examination combined with positive staining of specific immunohistochemical markers is necessary to confirm the diagnosis [45, 50]. Invasion of periadrenal tissue and the occurrence of distant metastases are certainly possible, but complete resection in early stage could prevent this from happening [46].

Adrenal cysts form a subcategory which can be divided into pseudocysts, endothelial cysts, epithelial cysts, and parasitic cysts [52]. On CT imaging, differentiation from malignant cystic neoplasms or pseudocysts associated with malignant tumors is not possible [63]. Pseudocysts and endothelial cysts are both considered vascular lesions, the first originating from adrenal hemorrhage and the latter from a preexistent vascular or lymphatic malformation [53, 54]. Adrenal lymphangioma is a subtype of an endothelial cyst. Histologically, the diagnosis can be established by determining the endothelial origin of the cells through immunohistochemical staining (CD31, CD34, D2-40). Epithelial cysts are more difficult to characterize, as the adrenal gland lacks acini where such a cyst should originate from. An alternative explanation suggests embryonic origin, where the cyst would develop from displaced mesothelial tissue [53]. Parasitic cysts are very rare, mostly caused by infection with echinococcus. However, the adrenal glands are involved in less than <0.5% of infected patients [52]. Of note, all adrenal pathologies may display cystic degeneration which should not be confused with these four subtypes of adrenal cysts.

5. Conclusion

An adrenal mass may present a diagnostic challenge. If a diagnosis is not established after exclusion of the most common diagnoses, a category remains that consists of rare entities. It may be difficult or even impossible to differentiate between these pathologies based on preoperative diagnostic tests. Radical surgical resection is indicated in case of possibly harmful tumors, for example, lesions with malignant potential, risk of spontaneous hemorrhage, or increase in size over time. Clinicians should assess these issues using clinical judgment complemented with radiological evaluation of the lesion, aimed at characteristics summarized in the present study. This will result in resection of benign lesions, but this is inevitable given the uncertainty that may remain after complete diagnostic work-up. Surgical resection is not necessary if a lesion is judged to be certainly benign unless the size of the lesion causes problems, for example, due to a mass effect on other abdominal organs.

Conflict of Interests

The authors declare there is no conflict of interests that could be perceived as prejudicing the impartiality of the research reported.

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Research Article

Multiple Components of the VHL Tumor Suppressor Complex Are Frequently Affected by DNA Copy Number Loss in Pheochromocytoma

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Pheochromocytomas (PCC) are rare tumors that arise in chromaffin tissue of the adrenal gland. PCC are frequently inherited through predisposing mutations in genes such as the von Hippel-Lindau (*VHL*) tumor suppressor. VHL is part of the VHL elongin BC protein complex that also includes CUL2/5, TCEB1, TCEB2, and RBX1; in normoxic conditions this complex targets hypoxia-inducible factor 1 alpha (HIF1A) for degradation, thus preventing a hypoxic response. *VHL* inactivation by genetic mechanisms, such as mutation and loss of heterozygosity, inhibits HIF1A degradation, even in the presence of oxygen, and induces a pseudohypoxic response. However, the described <10% VHL mutation rate cannot account for the high frequency of hypoxic response observed. Indeed, little is known about genetic mechanisms disrupting other complex component genes. Here, we show that, in a panel of 171 PCC tumors, 59.6% harbored gene copy number loss (CNL) of at least one complex component. CNL significantly reduced gene expression and was associated with enrichment of gene targets controlled by HIF1. Interestingly, we show that VHL-related renal clear cell carcinoma harbored disruption of *VHL* alone. Our results indicate that VHL elongin BC protein complex components other than VHL could be important for PCC tumorigenesis and merit further investigation.

1. Introduction

Von Hippel-Lindau (VHL) disease is a rare inherited syndrome which predisposes individuals to a variety of malignant and benign tumors including renal cell carcinoma and pheochromocytoma (PCC) [1]. Renal cell carcinomas are cancers of the kidney that account for approximately 102,000 deaths worldwide each year [2, 3]. Renal clear cell carcinoma (RCC), arising in the proximal convoluted tubules of the kidney transport system, is the most common subtype of renal cell carcinomas (comprising about 88% of tumors) and is tightly associated with inactivating mutations of the *VHL* gene [4, 5]. PCC, the other principal VHL-related cancer, is a rare catecholamine-secreting cancer originating in chromaffin cells of the adrenal gland [6–8]. Although these tumors can be benign, the malignancy rate ranges from 10 to 15%. Malignant PCC is identified histologically by the presence of metastasis (commonly to lymph nodes, liver, lungs, and bone). Patients with malignant PCC have a high risk of mortality and morbidity. The overall 5-year survival rate of malignant PCC is 40–77% [9–11]. Therefore, a greater understanding of the biology underlying PCC is needed in order to advance diagnostic testing and prognosis.

In approximately one-third of cases, PCC arises in patients with germ-line mutations in predisposing genes such as *VHL*, *NF-1*, *MEN2/RET*, and *SDH* subunits, *TMEM127*, *MAX*, or *HIF2A*, among others [12–14]. Studies indicate that *VHL* is among the most frequently targeted genes in PCC, mostly affected by genetic mechanisms such as mutations and loss of heterozygosity (LOH) [15–17]. In keeping with Knudson's two-hit hypothesis [18], tumors from patients who

have a germ-line mutation in one *VHL* allele aresusceptible to somatic inactivation of the remaining allele. Indeed, studies show that a somatic "second hit," which can arise through epigenetic or genetic mechanisms, results in a loss of *VHL* gene expression, abnormal VHL protein function, and consequent tumorigenesis [19–21].

VHL is a component of the VHL elongin BC complex—composed of the proteins VHL, CUL2 or CUL5, RBX1, and elongin B/elongin C (elongins B and C are encoded by *TCEB1* and *TCEB2,* resp.). This complex acts as an E3 ubiquitin-ligase and drives the proteasomal degradation of targeted proteins [22, 23]. The hypoxia-inducible factor 1α (HIF1-α, encoded by *HIF1A*), the primary target of this complex, regulates over 80 genes associated with tumor progression, glycolysis, angiogenesis, and metastasis and is negatively regulated by the VHL elongin BC complex [24, 25]. Hypoxia inducing factor 1 (HIF1) is composed of an alpha subunit, which is negatively regulated by the VHL elongin BC complex and a beta subunit, which is constitutively expressed [26]. Under normoxic conditions, the hydroxylation of HIF1-α at two prolyl residues (P402 and P564) by PHD-containing proteins creates a binding site for *VHL* and results in proteasomal degradation of HIF1-α (Figure 1(a)) [27–29]. In hypoxic conditions, PHD-containing proteins no longer hydroxylate HIF1-α and VHL cannot add destabilizing ubiquitin polymers to HIF1-α. HIF1-α can then heterodimerize with HIF1-β and translocate into the nucleus where it binds to hypoxia response elements (HRE) and promotes the expression of genes, such as *PDK1*, *PFKL*, *GLUT1*, and *VEGF*, that mediate the cellular hypoxic response. Genetic alterations affecting *VHL* or other complex components can lead to abnormal stabilization of HIF1-α, resulting in aberrant translocation of HIF1 to the nucleus and ectopic activation of target genes, such as *VEGF*, *PDK*, and *EPO*, to elicit a hypoxic response, even in normoxic conditions (Figure 1(c)) [30–32].

Previous studies of PCC have reported that disruption of the VHL elongin BC protein complex occurs through gene copy number loss, mutation, or epigenetic silencing of the *VHL* gene and that this disruption leads to tumorigenesis through activation of HIF1 targets [16, 20, 33, 34]. The role of the other components of the VHL elongin BC complex is largely uncharacterized. In the present study, we investigated DNA-level alterations—gene copy number losses (CNL) and promoter hypermethylation—affecting other components of the VHL elongin BC protein complex in PCC. We assessed the effects of these alterations at the gene expression level and the impact of complex component disruption on enrichment of HIF1-target expression. Finally, we explored whether similar disruptions were present in another *VHL*-inactivated cancer type, RCC. Our results indicate that, while *VHL* is disrupted in both PCC and RCC, other components of the VHL elongin BC complex, particularly *RBX1* and *CUL5*, are significantly disrupted in PCC and their status might be an important clinical consideration in PCC.

2. Materials and Methods

2.1. Pheochromocytoma DNA Copy Number Data Analysis. Information regarding DNA copy number alterations affecting VHL tumor suppressor complex components (*VHL*, *TCEB1*, *TCEB2*, *RBX1*, *CUL2*, and *CUL5*) was obtained from 171 PCC tumors available through The Cancer Genome Atlas Project (TCGA). Gene dosage alterations were assessed using the Affymetrix SNP6.0 platform at the Broad TCGA Genome Characterization Center [36]. Processed level 3 data was accessed through the UCSC Cancer Genome Browser [37–39]. Briefly, raw copy number data was segmented using a circular binary segmentation algorithm [40] and mapped to hg18 genome assembly. In order to exclude polymorphic variations, a fixed set of common germ-line copy number variant probes were removed prior to segmentation. Coordinates were converted to hg19 using a local repository of galaxy, running the LiftOver utility [41].

Segmented data was loaded into the Integrative Genomics Viewer (IGV) [42, 43], and information regarding the six complex component genes was exported as a.tdm file. DNA copy number alterations were defined as follows: (1) DNA copy number loss (signal intensity log 2 ratio < -0.3), copy number neutral (log 2 ratio between -0.3 and 0.3), or copy number gain (log 2 ratio > 0.3).

2.2. Pheochromocytoma Gene Expression Data Analysis. Gene-level transcription estimates, in the form of RSEM normalized counts, were obtained for the six complex component genes analyzed and were obtained from processed RNA sequencing data derived from 171 tumors and 4 adjacent nonmalignant tissues [44]. Gene expression profiles were generated using the Illumina HiSeq 2000 RNA sequencing platform by the University of North Carolina and TCGA Genome Characterization Center. Individual expression profiles were loaded into IGV, and expression information for each gene was exported. Genes were mapped to the human genome hg19 coordinates using UCSC cgData HUGO probeMap.

2.3. Pheochromocytoma DNA Methylation Data Analysis. Methylation analyses using the Illumina Infinium HumanMethylation450 platform were performed at Johns Hopkins University, University of Southern California, and TCGA genome characterization center. Probes mapping to the six complex component genes were extracted. Only probes mapping to the promoter region, which are most likely to have an effect on gene expression, were selected for further analysis. The ratio of the intensity of the methylated bead type to the combined locus intensity (termed as beta values (βV)) was calculated using BeadStudio software. To assess the difference in probe methylation between PCC and nonmalignant tissue, a delta beta value (dβV) was calculated for each probe: an average βV was calculated for each probe in the nonmalignant cohort, and these values were subtracted from the PCC βVs on a tumor-by-tumor basis.

2.4. Pheochromocytoma and Renal Clear Cell Carcinoma DNA Mutation Analysis. Somatic mutation data using Illumina sequencing platforms were obtained from TCGA. Data was derived from 171 PCC tumors samples that also contained expression and copy number information. *VHL* complex component genes (*CUL2*, *CUL5*, *TCEB1*, *TCEB2*, and *RBX1*)

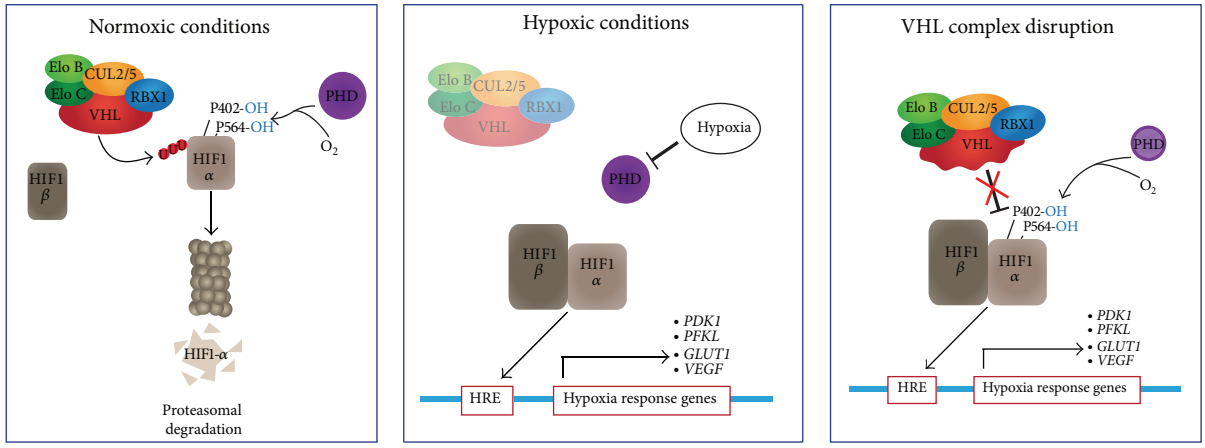

Figure 1: Schematic illustration of the role of the VHL elongin BC complex in the HIF1 pathway in normoxic, hypoxic, and pseudohypoxic conditions. Under normal physiological conditions (a), HIF1-α becomes hydroxylated on two prolyl residues. Hydroxylation of HIF1-α generates a binding site for the VHL elongin BC complex, consisting of elongin B, elongin C, CUL2 or CUL5, RBX1, and VHL, which directs the polyubiquitination of HIF1-α and targets it for proteasomal degradation [35]. In hypoxic conditions (b), PHD proteins no longer hydroxylate HIF1-α and VHL cannot add destabilizing ubiquitin polymers to HIF1-α. HIF1-α can then heterodimerize with HIF1-β and translocates into the nucleus where it binds to hypoxia response elements (HRE) and promotes the expression of genes, such as *PDK1*, *PFKL*, *GLUT1*, and *VEGF*, that mediate the cellular response to hypoxic conditions. Similarly, in some cancer types, such as PCC and RCC (c), a loss of function event (such as DNA sequence mutation or copy number loss) of VHL can result in an upregulation of HIF1-target genes independent of the oxygenation status of the tumor cells.

as well as 3 genes known to be frequently mutated in PCC (*RET, HRAS*, and *NF1*) were classified as having an inactivating mutation if the result was a frame shift insertion, frame shift deletion, splice site mutation, missense mutation, or a nonsense mutation. Somatic mutation data was also analyzed in the same way in a cohort of 417 out of 522 RCC tumors where DNA sequence data was available.

2.5. Renal Clear Cell Carcinoma DNA Copy Number Data Analysis. Copy number data and mutation data for 522 RCC were downloaded from cBioPortal for Cancer Genomics (http://www.cbioportal.org) [45, 46]; of these, 411 samples had concurrent copy number and mutation data. The same criteria used for PCC were applied to define copy number loss and gain. Mutations with a neutral or low mutation assessor score were not considered in mutation frequency calculations.

2.6. Correlation of DNA-Level Alterations with Gene Expression in Pheochromocytoma. In order to assess the effect of DNA-level alteration on gene expression of the VHL tumor suppressor complex components, PCC was divided into up to three groups based on copy number alteration status (copy number loss, copy number neutral, and copy number gain) and expression was compared between groups using GraphPad software v6. For most genes (*VHL*, *CUL5*, *TCEB1*, and *TCEB2*) three group comparisons were performed using a Kruskal-Wallis test. Since the majority of copy number alterations were CNL rather than gain, a two-group comparison was also performed for each of the six genes comparing CNL and neutral copy number using a Mann-Whitney *U* test. *RBX1* did not show copy number gain in any sample; therefore only a Mann-Whitney *U* test was performed for this gene. In all comparisons, a P value < 0.05 was considered significant.

Correlation between gene expression and promoter hypermethylation was assessed through Spearman correlation analysis using GraphPad software v6. Each probe was correlated separately using a gene expression matrix of the 171 PCC samples. An example of the correlation of the probe, cg07288693, located in the promoter region of *RBX1*, is shown in Supplementary Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/546347.

2.7. Gene-Set Enrichment Analysis. In order to assess possible effects of HIF1-target genes due to the disruption of the VHL elongin BC tumor suppressor complex, we evaluated a gene-set enrichment analysis (GSEA) for every sample using the single sample gene-set enrichment analysis (ssGSEA). Briefly, ssGSEA calculates separate enrichment scores (ES) for each pairing of a sample and gene set. Each ssGSEA ES represents the degree to which the genes in a particular gene set are coordinately up- or downregulated within a sample [47]. A rank normalized expression matrix for 171 PCC samples and 20,533 genes was used as input on the ssGSEA implementation in GenePattern public server [48]. ssGSEA was performed using default parameters using the SEMENZA_HIF1_TARGETS gene set available from the Molecular Signatures Database v4.0 (Broad Institute). This gene set contains 36 genes that are transcriptionally regulated by hypoxia-inducible factor 1 (HIF1) [49]. ES for each sample are available in Supplementary Table 2.

3. Results

3.1. Inactivation of VHL Elongin BC Complex Components in Pheochromocytoma. We first examined the mutation status of the *VHL* gene in 241PCC tumors from the Catalogue of Somatic Mutations in Cancer (COSMIC). Consistent with literature reports, 24 out of 241 (10%) cases harbored *VHL* gene mutation [17]. The data from TCGA also showed a very low frequency of *VHL* mutation, at 2%. We next analyzed the copy number status of component genes: *VHL*, *RBX1*, *CUL2*/*CUL5*, *TCEB1*, and *TCEB2*. In a cohort of 171 PCC tumors from The Cancer Genome Atlas (TCGA), the frequency of gene disruption for three of these complex components was remarkably high (*RBX1*, 30.4%; *VHL*, 26.9%; *CUL5*, 21.6%), while two remaining complex components exhibited the modest disruption frequencies in PCC tumors: *TCEB1* (6.4%) and *TCEB2* (2.9%). *CUL2* did not exhibit any gene CNL according to our parameters. Interestingly, copy number gains were infrequent in all complex component genes; no gene displayed gain in more than 5% of cases (Figure 2(a)).

Strikingly, when complex gene disruption was considered cumulatively, 59.6% of PCC harbored genetic loss of at least one of the complex components, while 24.3% harbored disruption of 2 or more complex components (Figure 2(c)). Gene CNL events were not mutually exclusive: 17.5% of PCC had genetic loss of both *VHL* and any other complex component; 33.3% harbored loss of a complex component other than *VHL*, and 8.8% harbored loss of *VHL* alone (Figure 2(b)). These findings highlight the importance of VHL elongin BC protein complex disruption at the genetic level in PCC.

We next investigated whether gene silencing by aberrant DNA methylation affected any of the complex component genes. Gene methylation data was available for 171 PCC tumors and 4 adjacent solid nonmalignant tissues. We found that all but one probe (cg03160045) had a βV of less than 0.14 in both PCC and nonmalignant samples, indicating that complex component genes were not highly methylated. Further, the dβV ("PCC βV" and "nonmalignant βV") were close to zero in all cases, for all probes, suggesting that methylation of complex component genes did not differ between tumor and nonmalignant samples. Interestingly, we found that methylation was significantly negatively correlated with *RBX1* expression (Supplementary Figure 1); however, given the low dβV for all probes, we could not confidently attribute the effects of methylation to expression levels of *RBX1*. Therefore, for the remainder of the analysis, we focused on gene CNL.

3.2. Enrichment of HIF1-α Target Genes in Pheochromocytoma. We next examined whether genetic disruption of other complex component genes might lead to overactivity of HIF1 and aberrant expression of HIF1-target genes. We performed single sample gene-set enrichment analysis (ssGSEA) on the panel of 171 PCC and found that positive HIF1-target gene-set enrichment (Supplementary Table 1) occurred to some degree in all cases (median ES = 6132.4) (Figure 2(c), Supplementary Table 2). Gene CNL of at least one complex component could explain HIF1-target gene enrichment in 59.6% of cases. Since 33.1% of cases harbored genetic loss of VHL components other than *VHL*, and TGCA mutation data indicated a *VHL* mutation rate of only 2%, inactivation of other VHL elongin BC complex components is likely involved in VHL elongin BC complex dysfunction in PCC and this could impact HIF1-target expression (Figures 2(b) and 2(c)).

3.3. Gene Dosage Affects Expression of VHL Elongin BC Complex Components in PCC. We next evaluated whether gene copy number alterations to the VHL elongin BC complex components were correlated with expression of these genes. RNA sequencing data for the 171 PCC tumors was downloaded from TCGA, and samples were grouped according to their copy number status. Intriguingly, gene dosage in four of the five VHL complex components that were altered at the copy number level—*RBX1*, *CUL5*, *VHL*, and *TCEB1*—was significantly positively correlated with expression ($P < 0.0001$) (Figure 3). *TCEB2*, which had a gene CNL frequency of 2.9%, was not significantly correlated with expression; *CUL2* was not altered at the copy number level. These findings suggest that underexpression of *RBX1*, *CUL5*, *VHL*, and *TCEB1* is a selected event in PCC.

3.4. DNA-Level Alterations Affecting VHL Elongin BC Complex Components Differ between VHL-Related Cancers. We evaluated if gene CNL affects components of the VHL elongin BC protein complex in another cancer type characterized by inactivation of *VHL*: RCC. We queried gene CNL and mutation frequencies of the six VHL elongin BC complex genes in RCC using resources available at the cBioPortal for Cancer Genomics. In RCC, negligible gene CNL and mutation frequencies were observed for all of the VHL complex genes except for *VHL* itself (Figure 4). Across 522 RCC tumors, *VHL* was mutated in 179 (34.3%) samples and lost at the copy number level in 419 (71.3%) samples, while 39.7% of samples exhibited concurrent mutation and CNL, *RBX1*, *CUL2*, *CUL5*, *TCEB2*, and *TCEB2* did not appear to be significantly altered in RCC at DNA level, with no complex components exceeding a CNL frequency of 4% and no mutation frequency reaching 1%.

By contrast, *VHL* mutation frequency in PCC was only 2% according to TCGA data and, of the 171 PCC tumors from TCGA, only 26.3% of samples exhibited CNL of *VHL*, with *RBX1* and *CUL5* also undergoing frequent CNL. From these data, we observe markedly different genetic profiles when comparing genes coding for the VHL elongin BC complex between RCC and PCC. In RCC, it appears that *VHL* is the sole contributor to disruption of VHL elongin BC complex, whereas, in PCC, VHL elongin BC complex loss of function may occur frequently through CNL of *VHL*, *RBX1*, and *CUL5* and through mutation of *VHL*.

3.5. Correlation of VHL Elongin BC Protein Complex Status with Other Frequent Somatic Mutations in PCC. We evaluated the mutation status of three other genes known to be frequently mutated in PCC (*HRAS*, *RET*, and *NF1*) to assess their relationship with alterations in *VHL* complex

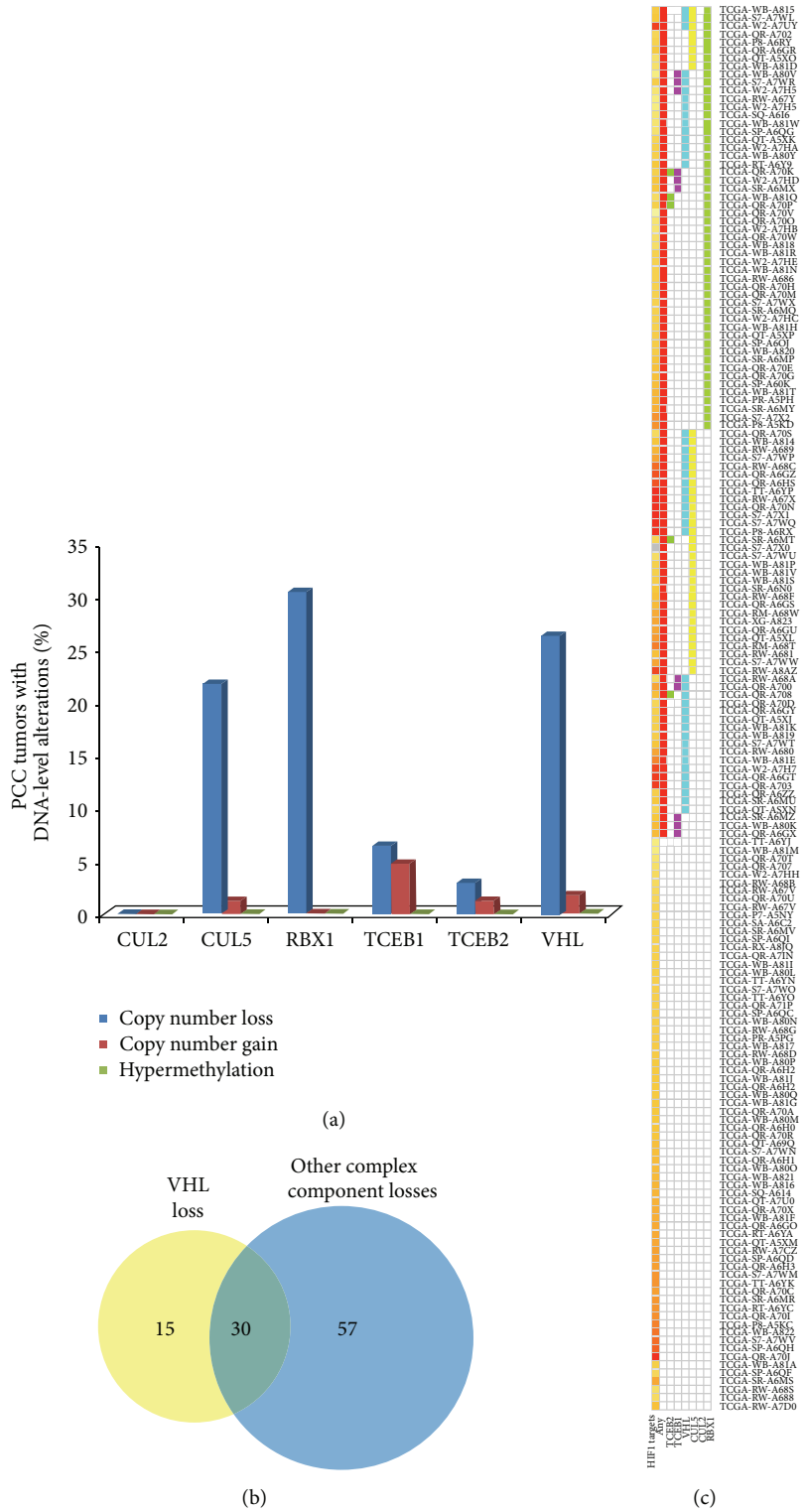

Figure 2: Disruption of VHL complex component genes. (a) Frequency of DNA copy number alterations and promoter hypermethylation for each component of the VHL elongin BC complex. (b) Venn diagram detailing PCC samples that have copy number loss of VHL complex components either alone or in combination. (c) Incidence of copy number loss of individual components of the VHL complex (*RBX1, CUL2, CUL5, TCEB1, TCEB2,* and *VHL*) across a panel of 171 pheochromocytomas. Each row represents an individual complex component affected by copy number loss: *RBX1* (light green), *CUL5* (yellow), *TCEB1* (pink), *TCEB2* (dark green), or *VHL* (aqua). Presence of any alteration in any individual component is shown in red. The bottom row (yellow to red gradient) indicates the gene enrichment score HIF1 gene targets with red bands representing greater enrichment. All samples appear to be enriched for HIF1 targets though not all samples have loss of function events affecting *VHL*.

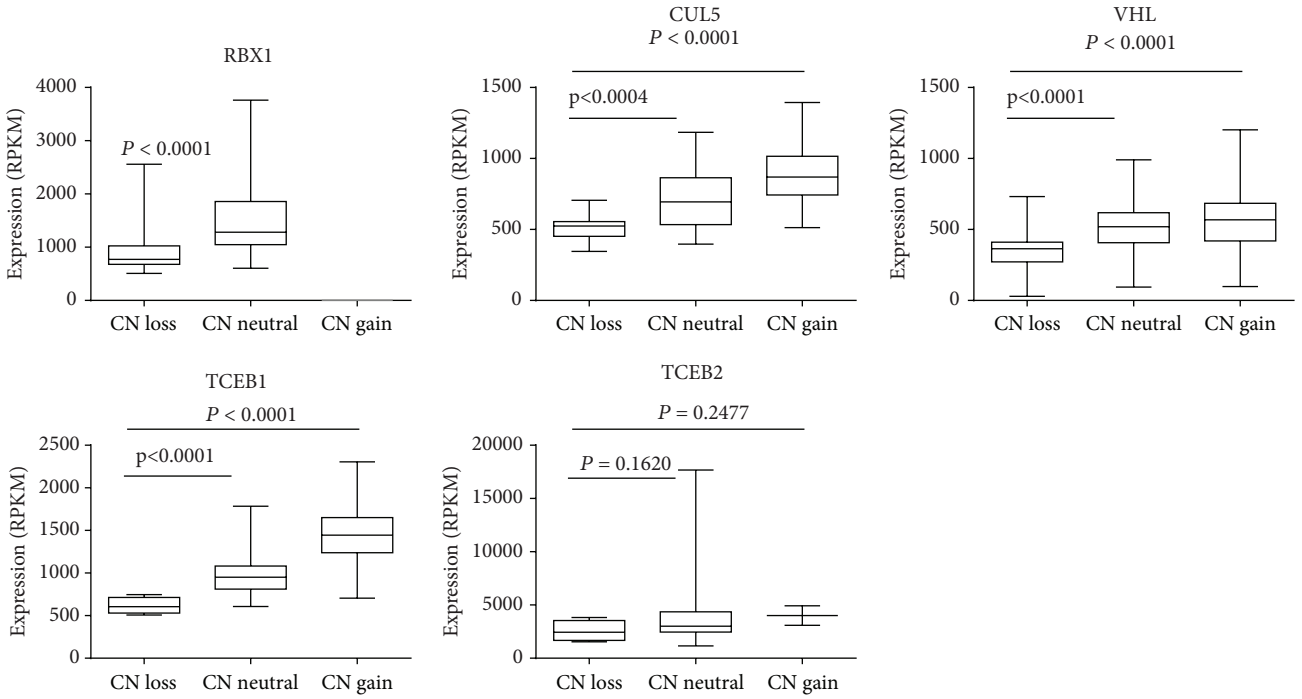

Figure 3: Gene dosage of VHL elongin BC complex components affects expression in PCC. *RBX1* expression is positively correlated with copy number loss (Mann-Whitney P value < 0.0001) and no copy number gain was seen for *RBX1* in PCC samples. Copy number was significantly positively correlated with expression for *CUL5*, *VHL*, and *TCEB1* complex components (Kruskal-Wallis P value < 0.0001).

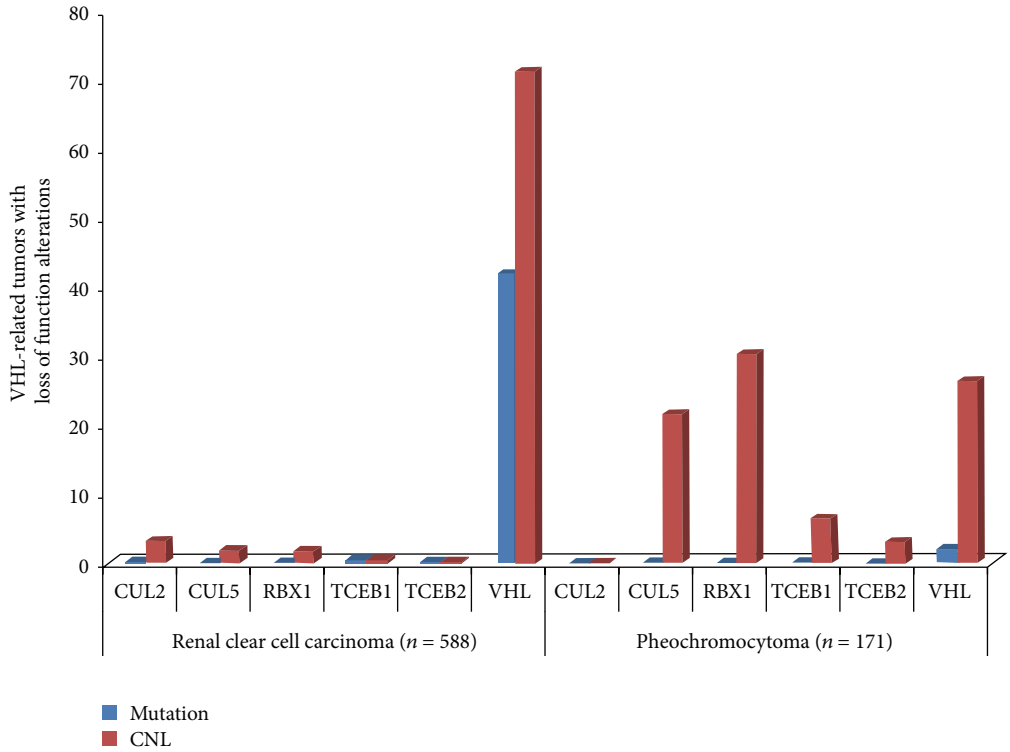

Figure 4: Frequency plot displaying two VHL-related tumors, renal clear cell carcinoma and pheochromocytoma, and corresponding types of DNA-level alterations that affect each individual complex component. The figure shows patterns and frequency of DNA copy number loss (red) and mutation (blue) affecting each component of the VHL complex in the two tumor types.

component genes [13, 50]. Of the 171 PCC tumors analyzed, 39 had mutations in either *HRAS*, *RET,* or *NF1* (Supplementary Figure 2). Twelve of the 39 cases had no disruption in the *VHL* complex, while 27 displayed VHL elongin BC protein complex disruption. The frequency of cases harboring mutations in *RET*, *HRAS,* or *NF1* did not significantly differ between cases with or without VHL complex disruption ($P = 0.1942$, chi-square test). In RCC, *NF1*, *RET,* and *HRAS* were not frequently mutated (2%, 0%, and <1%, resp.), and these mutations were not mutually exclusive with *VHL* mutations.

4. Discussion

Oxygen-sensing pathways are paramount for cell survival and normal cellular function, while they also play a key role in tumor progression and aggressiveness. HIF1 pathways allow cells to survive in conditions of temporary oxygen deprivation (e.g., HIF is essential in embryonic development). Since abnormal accumulation of HIF1-α subunits can induce HIF1 pathways to promote tumor progression and aggressiveness, its levels need to be tightly regulated. This function is mainly achieved by the VHL elongin BC complex [28]. In VHL-related tumors, such as PCC and RCC [20, 34], HIF1 activity is aberrantly and constitutively high, mimicking a hypoxic environment, irrespective of oxygen levels [31, 51, 52]. Genetic lesions affecting the *VHL* gene are usually considered the cause of HIF1-α accumulation [25, 26].

Since genetic mechanisms disrupting the *VHL* gene are only present in a fraction of PCC, we have tested the hypothesis that deregulation of other protein components of the VHL elongin BC complex might also result in activation of HIF1 pathways. Indeed, gene CNL affecting *RBX1* and *CUL5* significantly impacted gene expression. We also noticed a negative correlation between hypermethylation and expression for *RBX1*; however, the low methylation levels of probes across all samples, including nonmalignant samples, imply that hypermethylation is not a major mechanism of *RBX1* downregulation. It has been shown that underexpression of *RBX1*, due to gene *CNL*, might interfere with the *KEAP1*/*CUL3*/*RBX1* complex, which also displays a E3 ubiquitin-ligase activity in thyroid and ovarian cancer [53]. Similarly, underexpression of *CUL5* linked to genetic loss events has been documented in breast tumors [54]. Together, these results suggest that gene CNL has an impact on decreased gene expression of *RBX1* and *CUL5*, in addition to *VHL*, and may subsequently contribute to dysfunction of the VHL elongin BC complex in PCC. Interestingly, other frequent somatic mutations in PCC, such as those affecting *HRAS*, *RET,* and *NF1*, seem to occur independently of VHL elongin BC protein complex disruption.

It has been well documented that loss of function alterations to *VHL*, components of the succinate dehydrogenase (SDH) complex, and HIF2A, as well as pseudohypoxia characterize cluster 1 PCC tumors and correlate with increased HIF1 signaling [25, 26, 55, 56]. We propose the fact that tumors involving DNA-level alterations of the VHL complex component genes should be considered as part of this cluster. An enrichment analysis (GSEA) of the panel of 171 PCC tumors revealed that HIF1 gene targets were positively enriched in all cases (Figure 2(c)). The high frequency of samples showing alterations in at least one of the complex components (59.6%) at least partially explains the positive enrichment of HIF1 targets. In cases that did not have clear DNA-level alterations to members of the VHL elongin BC complex, upregulation of HIF1-target genes may simply be due to the presence of hypoxic cells in the biopsy of samples used to generate the data. From these observations, we suggest that gene CNL of other VHL complex components, namely, *RBX1* and *CUL5*, along with *VHL,* CNL, and mutation, facilitates dysfunction of this complex and the consequent accumulation of HIF1-α.

VHL is also frequently disrupted in RCC. Previous studies have shown that the *VHL* gene is affected by somatic mutations in 50% of cases, while hypermethylation is observed in 10–20% of sporadic RCC [20]. We analyzed disruption of *VHL* and other complex components in RCC and compared these results to their disruption in PCC. Interestingly, *VHL* seems to be the only gene significantly disrupted in RCC, with 71.3% of cases undergoing CNL and other 34.3% with *VHL* mutation. These results indicate that RCC tumors are likely dependent on elimination of *VHL* rather than other complex components in order to generate conditions of pseudohypoxia. The genetic landscape of the VHL elongin BC complex genes in PCC, however, showed *VHL* gene to be less frequently inactivated at the DNA level, with the burden of genetic inactivation of the VHL elongin BC complex seeming to fall somewhat equally on *RBX1*, *CUL5,* and *VHL*.

The data presented here provide a rationale for a more comprehensive interrogation of the role of other *VHL* complex components (namely, *RBX1* and *CUL5*) in the HIF1-mediated oxygen-sensing pathway in PCC. In summary, we present compelling evidence that HIF1-mediated pseudohypoxic conditions are genetically selected in PCC via the disruption of multiple VHL complex components and we provide further rationale for exploring this pathway as a therapeutic target in PCC with potential application to RCC and other VHL-related diseases.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

David Rowbotham, Katey S. S. Enfield, and Victor D. Martinez contributed equally to this paper.

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Research Article

Endoscopic Retroperitoneal Adrenalectomy for Adrenal Metastases

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Objectives. To evaluate whether retroperitoneal approach for adrenalectomy is a safe and effective treatment for adrenal metastases (AM). *Methods*. From June 2004 to January 2014, nine consecutive patients with AM were treated with endoscopic retroperitoneal adrenalectomy (ERA). A retrospective study was conducted, and clinical data, tumor characteristics, and oncologic outcomes were acquired and analyzed. *Results*. Renal cancer was the primary site of malignancy in 44.4% of cases. The mean operative time was 132 ± 10.4 min. There were 5 synchronous and 4 metachronous AM. One patient required conversion to transperitoneal laparoscopic procedure. No mortality or perioperative complications were observed. The median overall survival was 11 months (range: 2–42 months). Survival rates of 50% and 25% were identified at 1 and 3 years, respectively. At the end of the study, 4 patients were alive with a mean observed follow-up of 20 months. No patients presented with local tumor relapse or port-site metastases. *Conclusions*. This study shows that ERA is a safe and effective procedure for resection of AM and advances the surgical treatment of adrenal disease. The use of the retroperitoneal approach for adrenal tumors less than 6 cm can provide very favorable surgical outcomes.

1. Introduction

Metastases to the adrenal glands represent the second most common type of adrenal mass after adrenal adenomas [1]. Lung, breast, stomach, and kidney cancers and melanomas and lymphomas most commonly metastasize to the adrenal glands [2]. Management strategy of adrenal metastases (AM) varies depending on the different clinical situation and can include close observation, chemotherapy, local ablative therapy, radiotherapy, or surgical resection [3–6]. Several studies have confirmed prolonged survival after an adrenal metastasectomy in selected patients who presented with isolated AM when that is the only site of disease spread [7–10].

Over the last few decades, laparoscopic technique radically changed adrenal surgery, making access to the adrenal glands easier and less traumatic. Laparoscopic adrenalectomy (LA) has become the gold standard for removing benign tumors of the adrenal glands because it offers lower morbidity rates, reduced postoperative pain, shorter hospital stay, perfect cosmetic results, and other benefits compared to open surgery [11, 12]. LA could be performed transperitoneally or retroperitoneally [13, 14]. The advantages of the transperitoneal approach include the wider working space and readily identifiable anatomic landmarks [14]. The retroperitoneal approach was considered to be associated with more direct access to the gland, avoidance of intraperitoneal organs, avoidance of adhesions in previously operated patients, and the ability to perform bilateral adrenalectomy without repositioning [15]. Different surgical methods could be selected according to the characteristics of patients, such as tumor diameter, location, histologic type, extent of deterioration, and metastasis [16].

Advantages of laparoscopic surgery have prompted interest in expanding this method to the treatment of adrenal malignancies [17–19]. Long-term survival after LA for isolated AM was demonstrated in several reports [3, 7]; however,

the utility of laparoscopic methods for malignancies is less certain because of concerns regarding the risk of tumor cell spillage [20].

Although controversial, LA was recommended as an appropriate initial approach for isolated AM in some studies, because it achieved the same level of results of tumor control and less traumas compared with open surgery [21–23]. Furthermore, there was no quantitative assessment concerning the association between retroperitoneal adrenalectomy and patients with AM. In response, we conducted the study to evaluate the efficacy and effectiveness of endoscopic retroperitoneal adrenalectomy (ERA) for AM.

2. Materials and Methods

From June 2004 to January 2014, 145 ERA were performed at Vilnius University Hospital Santariskiu Klinikos. Nine patients (6,25%) were found to have a histologically confirmed AM. These 9 patients were included in the present retrospective study.

The diagnosis of AM from primary tumor was suspected in any case of newly diagnosed adrenal mass, characterized by growing size on sequential imaging studies (abdominal ultrasonosgraphy and computed tomography (CT)). Percutaneous adrenal biopsy was ruled out for prevention of tumor seeding. All patients were evaluated with the assistance of an oncologist, with tumor-specific markers and global imaging (CT of the chest, abdomen, and pelvis) to search for other sites of metastatic involvement. The hormonal examination for adrenal metabolic dysfunction was carried out in conjunction with endocrinologist.

ERA was decided for patients with solitary adrenal mass inferior to 6 cm without evidence of periadrenal malignant infiltration or regional lymphadenopathy on imaging examinations, negative serologic tests for adrenal metabolic dysfunction, and patient history of malignant disease. Surgery was performed with curative intent in all patients.

Patient demographic characteristics (age, gender, tumor size and side, diagnosis of primary malignancy, and operative history) are summarized in Table 1.

Operation reports were reviewed to obtain operative time, estimated blood loss, need for conversion to transperitoneal LA or open adrenalectomy, status of resection margin, and complications. In addition, information regarding postoperative course (postoperative hospital stay, postoperative complications, the use of adjuvant therapy within 1 year of the procedure, and survival rates) was recorded. Pathology reports were reviewed to obtain removed tumor weight and final diagnosis.

Metastases were considered as synchronous (<6 months) or metachronous (≥6 months) depending on the interval after primary surgery. The completeness of adrenal surgery was defined in terms of R0 (complete resection with no microscopic residual tumor), R1 (complete resection with no grossly visible tumor as defined by the surgeon, but margins are microscopically positive according to the pathologist), R2 (partial resection, with grossly visible tumor left behind), and RX (presence of residual tumor cannot be assessed). Local recurrence was defined as radiological or biopsied confirmation of a recurrent disease in the adrenal bed. After surgery, the patients were followed up by endocrinologist and an oncologist every 6 months by physical examination and systemic CT, or sooner if they become symptomatic. Overall survival was calculated from the time of adrenalectomy up to death or end of the follow-up.

2.1. Surgical Technique. The posterior retroperitoneal approach was used for all ERA as described by Walz et al. [24]. After induction of general anesthesia, the patient is placed prone, in the jackknife position. A 2 cm sized transverse skin incision is made just below the tip of the 12th rib. The abdominal wall of the back is then opened and the retroperitoneal space exposed. A small cavity in the retroperitoneum is then prepared using a finger to accommodate the other trocars. A 10 mm trocar is then inserted through as second incision (4 cm removed medially from the first incision) guided by an index finger inserted through the first incision. A third skin incision for another 10 mm trocar is made along with the lowest margin of the 11th rib 4 cm laterally from the first incision. A 10 mm blunt trocar is inserted through the first incision, and carbon dioxide is insufflated to 20 mm Hg for creation of capnoretroperitoneum. The skin sutures were secured around the gas port preventing a gas leak. After creating the retroperitoneal working space, Gerota's fascia is opened, perirenal fat is dissected, and the kidney upper pole is mobilized to expose the adrenal gland. Dissection of gland starts with lower margin detachment from the upper kidney pole in a lateral to medial direction using 5 mm ultrasonic dissector. After exposing adrenal gland from surrounding tissue and medial isolation of the main suprarenal vein, the vessel is clipped and divided with scissor. Adrenal gland with surrounding fat was resected with the greatest of care to prevent tumor disruption. The surgical specimens were always extracted in a bag and retrieved via the first trocar site. No drain was inserted and the incision was closed subcutaneously.

In a case when ERA cannot safely be performed we convert it to transperitoneal LA. It is necessary to change the patient's position on the operating table. Patient is placed in the lateral decubitus position. The surgical technique for transperitoneal LA has previously been described by our team in detail [25, 26].

2.2. Statistical Analysis. Categorical variables are expressed as frequencies and percentages and continuous variables as mean and standard deviation (±SD). Follow-up time variable and survival time are expressed as median values. Survival probabilities were estimated by using the Kaplan-Meier method. The level of statistical significance was set at $P < 0.05$. We conducted all statistical analyses using SPSS version 13.0 for Windows (SPSS Inc.).

Table 1: Patient characteristics and perioperative data.

Patient	Age (years)	Gender	Location	Primary malignancy	Metastases type	Treatment for primary malignancy
1	69	M	Right	Kidney cancer	S	Surgery, chemotherapy
2	65	F	Left	Lung cancer	M	Surgery, chemotherapy
3	46	M	Right	Melanoma	M	Surgery, chemotherapy
4	70	F	Left	Kidney cancer	M	Surgery
5	80	M	Right	Colon cancer	S	Surgery, chemotherapy
6	81	F	Right	Colon cancer	M	Surgery, chemotherapy
7	60	M	Left	Kidney cancer	S	Surgery
8	68	F	Left	Kidney cancer	S	Surgery
9	59	M	Left	Stomach cancer	S	Surgery, chemotherapy

F: female; M: male; S: synchronous; and M: metachronous.

Table 2: Perioperative and postoperative data.

Patient	Tumor size (mm)	Operative time (min)	Blood loss (mL)	Resection status	Specimen weight (g)	Hospital stay (days)	Survival (months)
1	30	110	50	R0	13	1	38
2	25	120	10	R0	15	5	10
3	40	135	10	RX	40	5	6
4	30	90	10	R0	46	2	42*
5	60	195	50	RX	64	5	6
6	40	120	20	RX	88	8	26*
7	15	120	20	R0	9	3	24*
8	43	140	60	R0	32	3	11
9	50	165	50	RX	106	2	2*

*Still alive.

3. Results

Patients' characteristics are summarized in Table 1. The mean age of all patients undergoing ERA for AM was 66.4 ± 3.6 years. The female/male ratio was 1/1.25. All AM were unilateral (left: five patients; right: four patients). The primary sources of the metastasis are shown in Table 1. The most common primary tumor site was kidney (4 patients, 44%), followed by colon (2 patients, 22%). All patients have been operated on for primary malignancy previously. The surgical procedure consisted of contralateral ERA in all patients with previous kidney cancer. The mean tumor size in the preoperative radiologic imaging studies was 37 ± 4.5 mm. Five (56%) patients presented with synchronous metastases, while four (44%) patients presented with metachronous metastases. No percutaneous adrenal biopsy was performed for the diagnosis of malignancy.

Perioperative and postoperative data are presented in Table 2. The mean operative time and estimated blood loss during ERA were 132 ± 10.4 min and 31 ± 7 mL, respectively. One conversion to transperitoneal LA occurred because of dense adhesions and difficult interpretation of anatomical structures of retroperitoneal space caused by previous left colon resection. After open insertion of the first optical trocar in to the abdominal cavity we found few intra-abdominal adhesions that do not interfere successfully to perform LA. The operation time was 120 min in this case. There were no perioperative complications and mortality. The mean postoperative hospital stay was 3.7 ± 0.7 days.

Five patients (55,5%) had complete macroscopic resection and negative margins (R0 resection). Four patients (44,5%) had complete macroscopic resection too but margins on pathology examination were not assessed because the surgical specimen was morcellated with forceps in a bag to make it easier to remove via trocar incision (RX resection).

In all cases, pathological examinations revealed the diagnosis of metastasis related to primary malignancies. The mean specimen weight in the pathologic studies was 45.9 ± 11.4 g.

The mean observed follow-up (FU) was 20 (range: 2–42) months. The median overall survival was 11 months (range: 2–42 months, Figure 1). Survival at 1 year was 50% (95% CI, 15–77; Figure 1) and at 3 years, 25% (95% CI, 1–64).

The difference between the median survival for patients with standard duration and long duration of surgery was significant ($P = 0,024$) (Figure 2). In patients with standard duration of surgery (≤120 min) median survival was 38 months (95% CI, 23, 25–44, 75) compared with long duration of surgery (>120 min) where median survival was 6 months (95% CI, 4, 4–10, 93).

The differences between the median survival for patients with small and big tumors ($P = 0,180$; Figure 3), with different origin of metastases ($P = 0,103$; Figure 4) and with

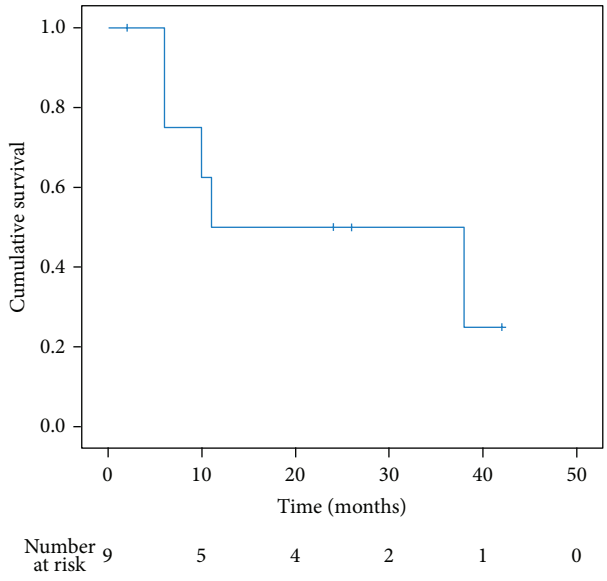

Figure 1: Overall survival curve according to the Kaplan-Meyer method.

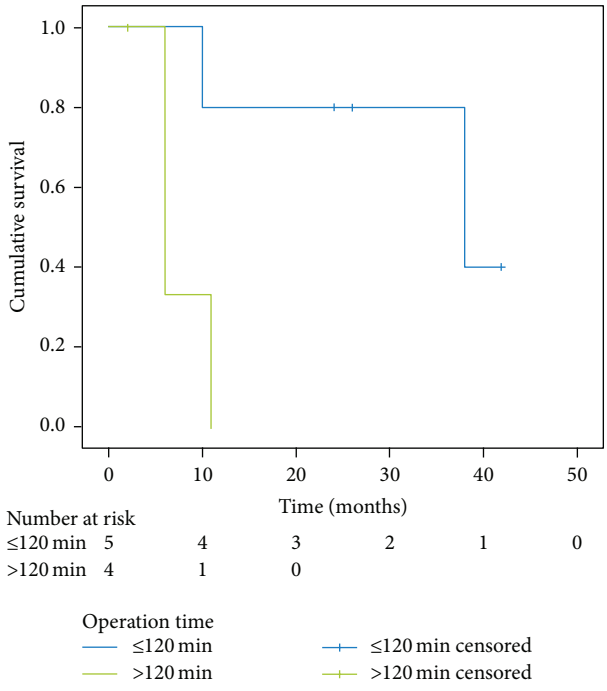

Figure 2: Kaplan-Meier survival curve of subgroups of patients with standard duration (≤120 min) and long duration (>120 min) of surgery; $P = 0,024$.

synchronous and metachronous metastases ($P = 0,711$; Figure 5), were not significant.

At the end of the study, 4 (45%) patients were alive with a mean FU of 20 months. Three patients were alive without evidence of disease 42, 26, and 24 months after ERA. The longest survival (42 months) was observed in a female patient with metachronous AM and primary malignancy

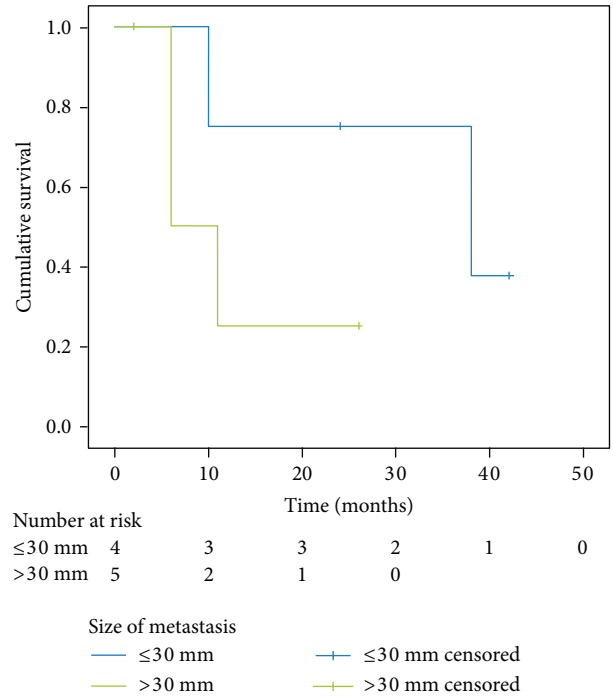

Figure 3: Kaplan-Meier survival curve of subgroups of patients with small (≤3 cm) and big (>3 cm) tumors; $P = 0,180$.

of kidney. Causes of death in the 4 patients who did not survive 1 year were progression of disease in 3 (75%) and causes unrelated to the malignant disease in 1 (25%). No local tumor recurrence or port-site metastases were observed during follow-up period.

4. Discussion

The role of minimally invasive surgery for adrenal malignancies remains controversial. The number of patients successfully undergoing LA reported by most authors is small, and many studies have failed to stratify patients according to whether they had primary adrenal cancer or metastatic disease [9, 18, 19, 27]. These two conditions must be assessed separately [28]. Metastasis to the adrenal gland should be suspected in patients with adrenal incidentaloma and a history of cancers most frequently metastasizing to the lung, breast, kidney, or colon [28]. Many reports in the literature have demonstrated success of LA in cases with solitary metastases, achieving a very low incidence of local recurrences or peritoneal dissemination [17, 29, 30]. The main aspect to consider for the success of laparoscopy is a small size of the adrenal tumor. In present study, the mean diameter of such metastases was 3.7 cm and we performed ERA in all cases.

The main concern of the surgical procedure is to make the patient tumor-free and it is therefore of a great importance to follow oncological principles [30]. We recommend excision of any adrenal metastases without touching the tumor or the gland; the surgeon should start the procedure from the perirenal fat tissue, to avoid the risk of tumor spillage or

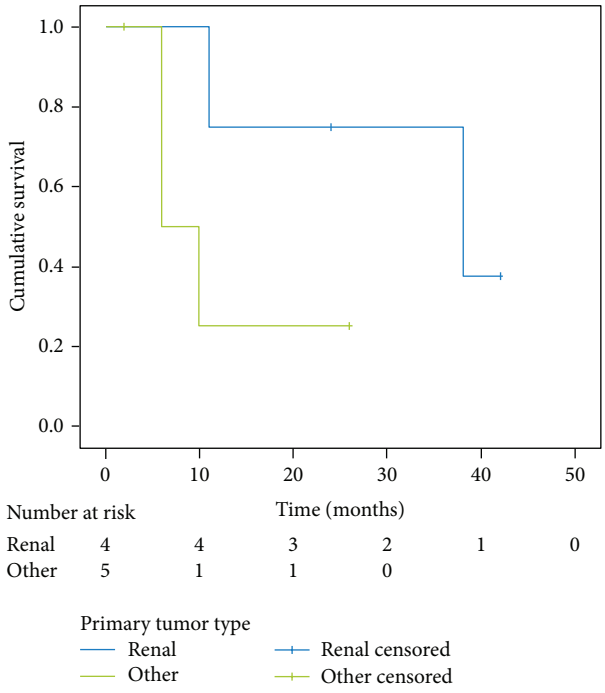

Figure 4: Kaplan-Meier survival curve of subgroups of patients with different origin of metastases (kidney versus other); $P = 0,103$.

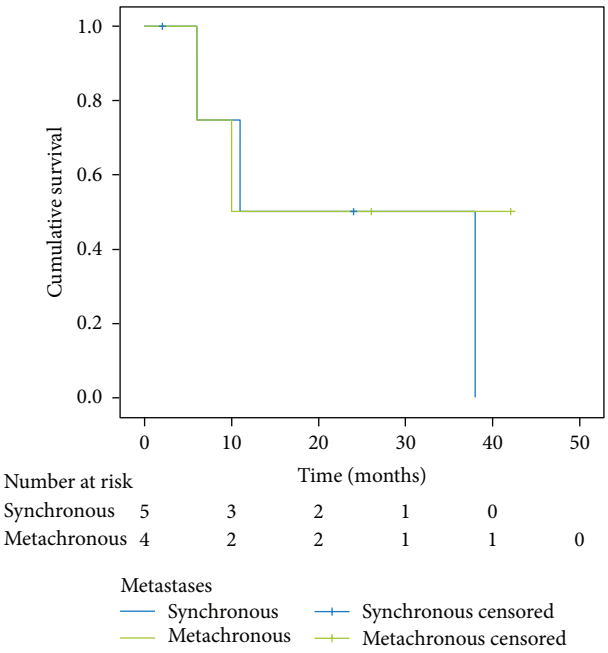

Figure 5: Kaplan-Meier survival curve of subgroups of synchronous and metachronous metastases; $P = 0,711$.

incomplete resection. Retroperitoneal approach is the ideal way in order to fulfill these objectives.

We evaluated the safety and efficiency of ERA for AM and found a trend toward decreased operation time (132 min versus 144 min), blood loss (31 mL versus 130 mL), and complication rate (0% versus 8,35%) in our study group compared to results of LA for metastasis in other published series summarized in Table 3 [7, 20, 23, 28, 30–38].

Walz et al. [24] reported that despite the narrower working space and unfamiliar retroperitoneal landmarks, the ERA was associated with decreased operative time and rapid patient recovery. The local recurrence rate was satisfied, which may be attributed to the en bloc resection of metastases in ERA. The mean postoperative hospital stay 3,7 days was similar like that in other studies [9, 22, 23]. Approving that ERA for AM is superior to LA based on our data is still too early due to lack of a control group and low number of cases in our series.

Despite potential improved surgical outcomes, the oncological outcomes of laparoscopic approach have remained in question with concerns about inadequate oncological resection. In the largest series to date of patients undergoing adrenal metastasectomy, Strong et al. [23] reported a 5-year survival of 31% in 92 patients. When they compared the survival rate between the open and laparoscopic groups, they found no differences, suggesting equal oncological outcomes between the two approaches. Moreno et al. [39] also reported that the median overall survival was 29 months and 5-year survival rate was 35% after adrenalectomy for solid tumor. In our study, the median overall survival was 11 months and 1 and 3 years survival rates were 50% and 25%. The longest survival was found in patients with metastases for renal carcinoma and colorectal cancer. Even in situations of single metastasis, the survival rate was less than two years and only three patients were in complete remission of their disease. One reason for these differences may come from the populations studied.

In the literature, the prognostic factors for survival after resection for adrenal metastasis were variable, including tumor type, tumor size, operation occasion (synchronous or metachronous), margin status, and previous surgery for metastases. Ma et al. [40] found that body mass index (BMI), tumor type, tumor size, and margin status were four independent prognostic factors of survival. Renehan et al. [41] concluded that increased BMI is associated with increased risk of common and less common malignancies.

Tumor type was previously suggested to be an important prognostic factor for survival. Lo et al. [8] suggested that patients with metastases from adenocarcinoma had the best chances of survival. However, in our material, kidney cancer had the best survival period, whereas patients with melanoma and colon cancer had the worst survival. The difference in survival period may be attributed to the intrinsic biological behavior of different tumor types or presence of occult concurrent metastases in other organs. Therefore all patients have to carefully undergo preoperative staging including chest, abdominal, and cerebral CT scans before the operation.

Tumor size also was a predictive factor in previous studies. Strong et al. [23] reported that the <4.5 cm group had a better survival period than the ≥4.5 cm group. In our study, the median survival period of the ≤3 cm group was 38 versus 6 months for the >3 cm group. The difference may be attributed to the increased size of adrenal lesions correlated with the operative complexity and possible subsequent disease behavior. We also found that standard duration of surgery

Table 3: Surgical results of LA for metastasis in previously published series.

Author	Year	Number of patients	OT (min)	EBL (mL)	Conversion rate (%)	Complication rate (%)
Heniford et al. [7]	1999	8	181	138	10	9
Valeri et al. [31]	2001	8	160	260	0	0
Sarela et al. [34]	2003	11	NR	NR	0	NR
Sebag et al. [32]	2006	16	NR	NR	31	18,7
Castillo et al. [33]	2007	32	87	89	0	6
Adler et al. [20]	2007	9	165	63	11	0
Strong et al. [23]	2007	31	175	106	NR	NR
Marangos et al. [30]	2009	31	104	100	3,2	7,4
Crenn et al. [35]	2011	13	174	351	23	NR
Zakoji et al. [36]	2012	5	142	38	NR	NR
Toniato [28]	2013	15	80	NR	6,7	6,7
Chen et al. [37]	2014	21	159	NR	14	19
Hirayama et al. [38]	2014	8	156	30	NR	NR
Total		195	144	130	9,9	8,35

OT means operation time, EBL means estimated blood loss, and NR means not reported.

is statistically significant associated with better survival rate ($P = 0,024$), but these findings are limited by the low number of cases in our series.

Metachronous metastases had better survival rate in several studies [34, 42]. This result can be attributed to the different intrinsic biology of the tumors in the two groups (metachronous versus synchronous). Synchronous lesions were more aggressive and grew faster than metachronous ones. In the current study, the median survival period of the metachronous group was 10 versus 11 months for the synchronous group. The difference between the median survival for the patients with synchronous and metachronous metastases was not significant in our material; thus we cannot consider the time between findings of the primary tumor and the metastasis in adrenal gland as a strong survival predictor.

In conclusion, many studies have documented that surgery for AM contributes to a more favorable prognosis than when these tumors are not resected [3, 30]. In agreement with Zografos et al. [43] our study shows that the retroperitoneal approach can be justified and is feasible for adrenal metastases less than 6 cm. Minimally invasive surgery gives us an opportunity to minimize surgical trauma which also may be more tolerable to a patient with several previous surgical procedures.

In summary, we found that ERA for AM offers the same advantages as those amply reported for benign adrenal disease, with no morbidity and mortality, and the acceptable oncological results.

Conflict of Interests

The authors declare that there are no conflicting financial interests.

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Research Article

Study of Microvessel Density and the Expression of Vascular Endothelial Growth Factors in Adrenal Gland Pheochromocytomas

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Angiogenesis (neoangiogenesis), a process of neovascularization, is an essential step for local tumor growth and distant metastasis formation. We have analysed angiogenesis status: vascular architecture, microvessel density, and vascular endothelial growth factors expression in 62 adrenal pheochromocytomas: 57 benign and 5 malignant. Immunohistochemical evaluation revealed that vascular architecture and vessel density are different in the central and subcapsular areas of the tumor. Furthermore, we have observed a strong correlation between number of macrophages and microvessel density in the central and subcapsular areas of the tumor and between the expression of VEGF-A in tumor cells and microvessel density in central and subcapsular areas of the tumor. Secondary changes in these tumors influence the results and both vascular architecture and microvessel density are markedly disturbed by hemorrhagic and cystic changes in pheochromocytomas. These changes are partially caused by laparoscopic operation technique. However, no differences in vascular parameters were found between pheochromocytomas with benign and malignant clinical behavior. Our observation showed that analysis of angiogenesis, as a single feature, does not help in differentiating malignant and benign pheochromocytomas and has no independent prognostic significance. On the other hand, high microvessel density in pheochromocytoma is a promising factor for antiangiogenic therapy in malignant cases.

1. Introduction

Adrenal pheochromocytoma (PCC) is an uncommon, neuroendocrine, catecholamine-secreting tumour arising from chromaffin cells of the adrenal medulla [1, 2]. Its clinical behavior is uncertain [3–5]. The histological separation between benign and malignant cases is usually difficult, and a definitive diagnosis of malignant PCC should be restricted to lesions displaying distant metastases [1, 2]. Metastases are defined by the finding of tumour cells in sites where chromaffin cells are normally absent [6]. According to most authors, recurrent disease occurring months or even years after the initial operation allows for classification of the tumour to a malignant group [1, 6, 7]. The most common metastatic sites are lymph nodes, liver, lungs, and bones [1, 2, 8]. PASS criteria (Pheochromocytoma of the Adrenal Gland Scaled Score) were proposed in 2002 as a tool for differentiating between benign and malignant cases [9] but this scoring system is not perfect and has some limitations. Using this scale, a significant proportion of PCC receives boundary PASS values (PASS = 4 or 5) which do not allow for an unequivocal assignment of the tumor to a benign or

malignant group. It is necessary to find additional features that allow better prediction of the clinical malignant behavior of the tumor (future recurrence or metastasis). Microvessel density (MVD) may be such parameter.

Neoangiogenesis, a process of neovascularization, is a complex phenomenon which plays a vital role in many physiological processes like organ development, wound healing, and tissue regeneration as well as in the pathology of many diseases, especially inflammatory and neoplastic diseases [10]. Angiogenesis is also essential for tumor growth and metastasis formation. Cancers, after a so-called angiogenic switch, acquire the ability to induce new vessel formation. The process of neovascularization depends on the ability to release specific factors stimulating and inhibiting new blood vessel formation. Both blood vessels formation stimulating and inhibiting factors can be released by neoplastic cells, stromal components, and immune cells like macrophages. Many strategies are used to evaluate the role of neoangiogenesis in tumor progression, and one of them is assessing microvessel density (MVD) [11, 12].

The aim of the study was to compare MVD, expression of vascular endothelial growth factors (VEGF-A, VEGF-C, and VEGF-D), and the number of macrophages in different areas of 57 benign and 5 malignant tumors and to determine if angiogenesis evaluation can be useful in routine pathomorphological practice for predicting the clinical outcome of a particular PCC tumor.

2. Material and Methods

The PCC samples were obtained from the Pathomorphology Department, Medical College Jagiellonian University (MCJU) in Krakow, Poland. The study was approved by the Jagiellonian University Bioethical Committee (KBET/82/B/2010).

The material under study consisted of 62 PCCs diagnosed in 58 patients (30 males and 28 females) in the Pathomorphology Department of MCJU during a period of 15 years from 1996 to 2010. Four patients, three women and one man, had bilateral tumours. Seven patients were known to have one of the syndromes associated with increased incidence of adrenal pheochromocytoma (two patients with MEN 2A syndrome, four with NF, and one with VHL syndrome). Three of these patients (two with MEN 2A and one with NF syndrome) had bilateral tumours. Ten patients were 30 or younger—only one tumor in this group presented malignant clinical behaviour. The mean tumour diameter was 4.98 cm (median: 4.2 cm, range: 1.5–13 cm, SD = 2.38) with no significant difference between male and female group (5.1 and 4.9 cm, resp.)—Table 1.

Five tumours were malignant: three PCCs gave distant metastases (to liver, lungs, and bones) and two had locally recurred. All PCCs with a malignant clinical course were unilateral. Clinical data were derived from patients' records and were available in 49 of the 58 cases (mean time of the follow-up: 46.3 months, median: 39 months). Nine cases were lost to follow-up, but we know that these patients were not treated for any recurrence and/or metastasis in our department.

Table 1: Characteristics of patients with the diagnosis of pheochromocytoma.

	Male	Female	Total
Age (y)	48.4	46.9	47.6
Site			
Left	11	17	24
Right	17	13	34
Unknown	3	1	4
Diameter (cm)	5.1	4.9	5.0
PASS	4.09	4.25	4.17

Haematoxylin and eosin-stained slides and paraffin blocks from tumors and adjacent adrenal glands were available in all 62 cases. Each diagnosis of PCC was reevaluated and confirmed by immunohistochemical staining with four antibodies against chromogranin A (CrA), synaptophysin (Syn), S-100 protein (S-100), and melan A [13]. Tumour cell immunoreactivity for chromogranin and synaptophysin with simultaneous lack of immunoreactivity for melan A and the presence of S-100 positive elongated cells, at least focally, was taken as confirmation of the diagnosis of PCC. Severity of haemorrhagic changes in the tumor was estimated in each case. The hemorrhagic changes within the tumor were scored from 0 (none or minimal) to 3 (extensive hemorrhagic changes disrupting at least half of the tumor surface visible in the histological slides).

In each case, a single H-E section and corresponding paraffin block including well-preserved tumor tissue as well as capsule were chosen, and seven 3 μm sections were prepared from the paraffin block. MVD was evaluated after immunostaining endothelial cells with antibodies against CD31 and CD105 for blood vessels and D2-40 for lymphatic vessels. Additionally, the expression of vascular endothelial growth factors (VEGF-A, VEGF-C, and VEGF-D) was evaluated. Immunohistochemistry was performed by standard method: the slides were dewaxed, rehydrated, and incubated in 3% peroxide solution for 10 minutes to block the endogenous peroxidase activity. Antigen retrieval was carried out by microwaving in citrate buffer (pH 6.0) or EDTA for 5 minutes at 700 W and then for 5 minutes at 600 W. The Lab-Vision (Thermo Fisher Scientific, Waltham, USA) detection system was used. 3-Amino-9-ethylcarbazole served as the chromogen. The slides were counterstained with Mayer's haematoxylin (DAKO, Denmark). The primary antibodies and the respective technical details are summarised in Table 2.

Positive structures (Figures 3 and 4), morphologically identifiable vessels and collections of immunopositive cells as well as single endothelial cells, were counted independently by two of the authors (MB and GD) who were blinded to the clinical and pathological data in two different areas of the tumor: the subcapsular and intratumoral spaces of each tumor. The subcapsular space was defined as the area within one high power field (0.5 mm) beneath the outer border of the tumor. The remainder of the tumor was defined as the intratumoral (central) area.

Table 2: Primary antibodies used in the study.

Specificity	Clone	Manufacturer	Dilution	Antigen retrieval
CD31	JC70A	DAKO, Denmark	1 : 20	EDTA
CD105	4G11	Novocastra	1 : 50	Citrate buffer
D2-40	D2-40	Covance	Ready to use	Citrate buffer
VEGF-A	Polyclonal	Santa Cruz	1 : 100	EDTA
VEGF-C	Polyclonal	Santa Cruz	1 : 100	—
VEGF-D	78923	R&D Systems	1 : 200	EDTA
CD68	PG-M1	DAKO, Denmark	1 : 50	EDTA

Table 3: The vessel counts in the whole study group of PCCs.

Marker	Location	Method	Mean	Min.	Max.	SD
CD31	Subcapsular	Hot spot	56.88	15.00	120.00	23.72
	Intratumoral	Hot spot	60.07	19.00	142.00	27.91
	Subcapsular	Chalkey	40.31	0.00	75.68	13.54
	Intratumoral	Chalkey	46.08	0.00	79.08	14.37
CD105	Subcapsular	Hot spot	30.15	6.00	120.00	20.84
	Intratumoral	Hot spot	37.91	9.00	124.00	24.48
	Subcapsular	Chalkey	23.51	0.00	70.58	12.75
	Intratumoral	Chalkey	33.15	0.00	64.63	15.22
D2-40	Capsular	Hot spot	1.92	0.00	7.00	1.34
	Subcapsular	Hot spot	0.12	0.00	4.00	0.56
	Intratumoral	Hot spot	0.00	0.00	0.00	—

Two different methods of counting were used. In the first method the number of all vessels in 10 high power fields, HPF (×10 ocular, ×40 objective), was added up after prescanning on low magnification (×10 ocular, ×10 objective) to choose the area with the impression of the highest vessel profiles number ("hot spot") in the intratumoral space. In the subcapsular area all vessels in 10 consecutive HPF were counted. The result calculated was the mean count of vessels for one HPF. In the second method, the Chalkley eyepiece graticule (Chalkley grid area 0.196 mm^2) with 25 randomly positioned dots was applied to the ocular of the Olympus microscope. On higher magnification (×10 ocular, ×40 objective) a Chalkley eyepiece graticule was applied to each "hot spot" area and then orientated and rotated so that the maximum number of points would hit on or within the vessel structure in the "hot spot" area. In the Chalkley method dots are counted, not the individual vessels. The Chalkley count was expressed as the total number of dots per square millimeter.

Lymphatic vessels (after D2-40 immunostaining (Figure 5)) were counted subcapsularly in 10 consecutive HPF and in 10 HPF in the intratumoral space.

Macrophages were counted in 10 HPF after prescanning on low magnification (×10 ocular, ×10 objective) to choose the area with higher cell density. The result calculated was the mean count of CD68 positive cells for one HPF.

The extent of immunoreactivity for VEGFs was expressed as the sum of grade and intensity of staining. Staining was graded according to the percentage of positive tumour cells (0: no staining; 1: <10%; 2: 10–50%; 3: 51–100% of positive cells). Intensity of staining was described as none (0), weak (1), moderate (2), or strong (3) (Figures 6–9). As a result, combined VEGF immunoreactivity could range from 0 to 6. All evaluations were done using an Olympus BX51 microscope equipped with a 40x UPlanFLN eyepiece (field of view diameter: 0.55 mm).

Statistical analysis was performed using Statistica 10 (StatSoft Inc., Tulsa, USA). Comparison between groups was done with Student's t-test, Mann-Whitney U test, and Kruskall-Wallis ANOVA test; the relationship between variables was assessed using Spearman's correlation coefficient. The significance level was set to 0.05.

3. Results

The material consisted of 62 cases of PCC from 58 patients: 30 males and 28 females. Three females and one man had bilateral PCC. The average age of the patients was 47.66 years (range: 19 to 75, SD: 15.41). The age in males and females (48.42 versus 46.90) did not differ significantly. 29 tumors (46.8%) were located at the right adrenal gland, 21 tumors (33.9%) were located at the left adrenal gland, and in 4 patients tumors were bilateral. In 4 cases (6.4%) laterality was not stated.

Angiogenesis was evaluated by MVD by two different methods. The overall results showing the number of blood vessels in the subcapsular and central areas of tumors are summarized in Table 3.

In both counting methods, MVD in the central areas of the tumors was higher than in the subcapsular areas. Strong correlation was found between both the numbers of CD31

Table 4: Correlation between the number of macrophages and the number of CD31 and CD105 positive blood vessels in subcapsular and central areas of the tumors.

CD68	CD31		CD105	
	Subcapsular	Intratumoral	Subcapsular	Intratumoral
Subcapsular	$r = 0.4534$	$r = 0.4803$	$r = 0.6013$	$r = 0.5701$
Intratumoral	$r = 0.4972$	$r = 0.5391$	$r = 0.5806$	$r = 0.6124$

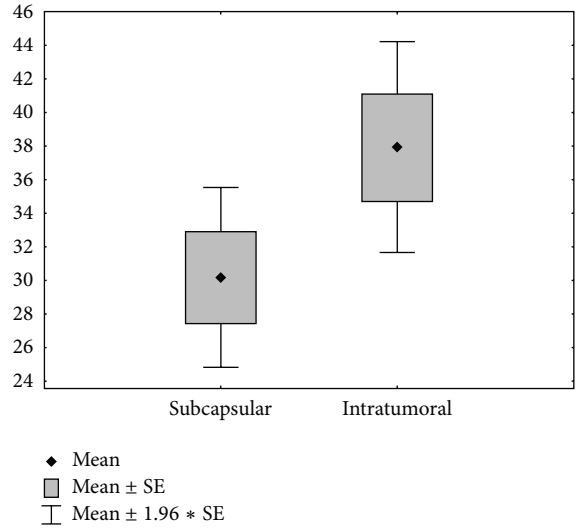

Figure 1: Number of CD105 positive blood vessels in subcapsular and central areas of the tumors.

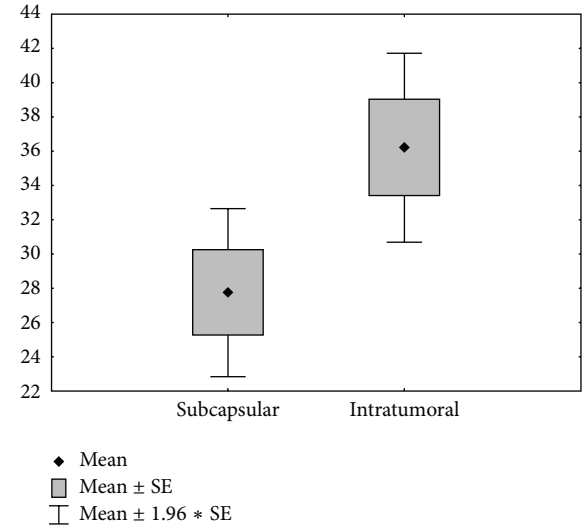

Figure 2: Number of macrophages in subcapsular and central areas of the tumors.

positive blood vessels in subcapsular and central areas of the tumors ($r = 0.8653$, $P < 0.001$) and between the numbers of CD105 positive blood vessels in subcapsular and central areas of the tumors ($r = 0.8837$, $P < 0.01$)—Figure 1. The difference between the variables for CD105 positive vessels was statistically significant (30.155 versus 37.91, Student's t-test $P < 0.001$).

Lymphatic vessels were absent in central parts of all investigated PCCs. In 4 cases (6,4%) single lymphatic D2-40 positive vessels were present in subcapsular areas. In 55 PCCs few lymphatic vessels were present within the capsule.

Mean subcapsular CD68 positive cell count was 27.68 (range: 4 to 87, SD: 18.97); mean intratumoral CD68 positive cell count was 36.14 (range: 11 to 97, SD: 21.24)—Figure 2. Strong correlation was found between the numbers of macrophages in subcapsular and central parts of the tumors ($r = 0.9166$, $P < 0.01$) and the difference between the values (27,68 versus 36,14) was statistically significant (Wilcoxon and Student's t-test $P < 0.001$).

Strong correlation was found between the number of macrophages and the number of CD31 positive blood vessels and between the number of macrophages and CD105 positive blood vessels in subcapsular and central parts of the tumors—all correlation coefficients were statistically significant ($P < 0.0001$)—Table 4.

The detailed individual values and statistical analysis for VEGFs expression are presented in Table 5. VEGF-A showed the strongest expression and was correlated with the number

Table 5: The expression of VEGF-A, VEGF-C, and VEGF-D.

	Mean	Min.	Max.	SD
VEGF-A	4.57	2	6	1.05
VEGF-C	3.58	0	6	1.09
VEGF-D	1.93	0	4	1.42

of both intratumoral and subcapsular CD31 positive and CD105 positive vessels in both counting methods—Table 6. No correlation was found between the expression of VEGF-C and MVD and between VEGF-D expression and MVD.

An inverse correlation between haemorrhagic changes and the number of CD105 positive vessels in subcapsular parts of the tumor was found ($P = 0.018$).

The differences in vascular parameters between PCCs with benign and malignant clinical behavior were slight and not statistically significant—Table 7.

4. Discussion

Angiogenesis (neoangiogenesis, NA) is the formation of new capillaries from already existing vessels. NA is regulated by a variety of proteins, inter alia, vascular growth factors and their receptors, angiogenesis modulating proteins, integrins, and angiogenesis inhibitors [14–16]. NA is a complex phenomenon and many strategies are used to evaluate its role in physiological and pathological processes. Most commonly used methods consist of assessing microvessel

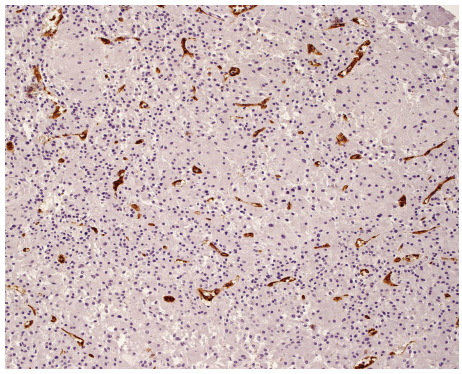

Figure 3: Numerous blood vessels with small round lumens (immunostaining for CD31).

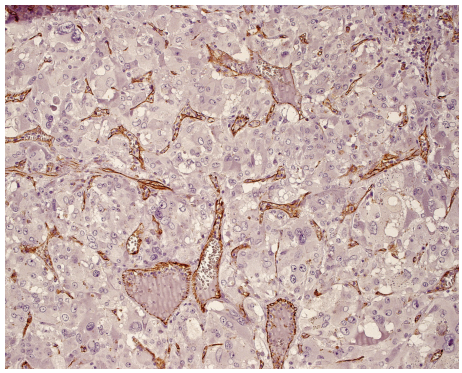

Figure 4: Blood vessels with irregular, expanded lumens (immunostaining for CD31).

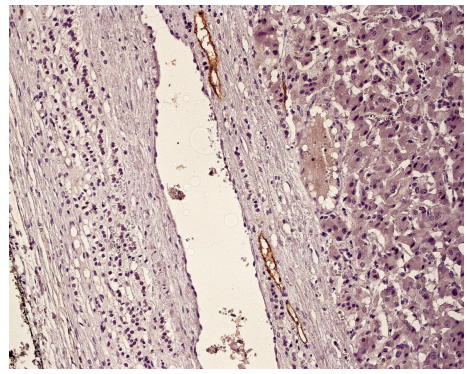

Figure 5: Lymphatic blood vessels present only in the tumor capsule (immunostaining for D2-40).

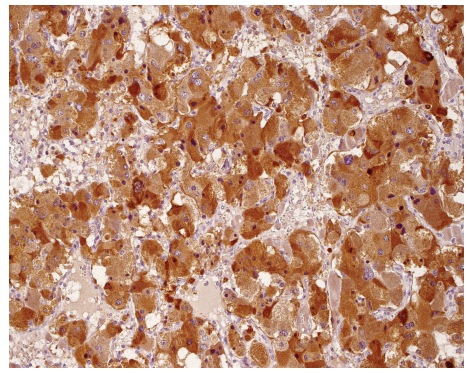

Figure 6: Positive, strong (3+/2+), granular immunostaining for VEGF-A in 100% of tumor cells.

density (MVD) and expression of angiogenic factors, among which the most important are vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) [11, 12, 17]. NA is essential both in nonpathological processes like embryogenesis or wound healing and in tumorigenesis [10, 18] where it is an essential step for tumor growth, progression, and metastasis formation [11]. Formation of new blood capillaries is also dependent on the extracellular matrix which serves as structural support for existing and developing vessels and on the ability of different cells to release specific factors stimulating new blood vessel formation and factors which downregulate vessel formation inhibitors [19]. The sources of those factors are both neoplastic cells and various stromal and immune cells, inter alia, macrophages. Microvascular density can be a prognostic factor in some human cancers [17, 20, 21], as metastasis formation is dependent on the possibility of tumor cells to enter the lumen of small vessels and to flow with blood to distant places and organs. Importantly, this means that neovascularisation is necessary not only for local tumor growth but also for allowing distant spread of the neoplasm.

The currently accepted standard method for quantifying tumor angiogenesis is to assess MVD based on immunohistochemistry (IHC). Groups of scientists had chosen different antibodies to evaluate MVD in various tumors [17, 22–26]. Our group had found in previous studies that the choice of IHC marker used for endothelial cells detection may influence the results, and the CD31 antibody as an endothelial marker provides the most unequivocal and conspicuous results [27]. CD31-highlighted endothelial cells are clearly visible and easy to count. On the other hand, CD34 antibody highlighted not only blood vessels but also other structures in the vicinity, such as connective tissue fibers, and usually CD34 gives much higher counts than CD31 [26]. Another endothelial marker commonly used in assessing MVD is endoglin (CD105). CD105 is a proliferation-associated and hypoxia-inducible protein abundantly expressed in angiogenic endothelial cells. Endoglin is a receptor for transforming growth factor- (TGF-) beta1 and TGF-beta3, and it modulates TGF-beta signalling. CD105 is required for endothelial cell proliferation [28], and CD105-based MVD is an independent prognostic factor for survival in patients with some tumor types [29–31]. CD105 is strongly expressed in the blood vessels of tumor tissues.

We have investigated the angiogenic status by comparing vascular architecture, microvessel count (based on both CD31 and CD105 IHC), and the expression of VEGFs in different areas of benign and malignant PCC tumors.

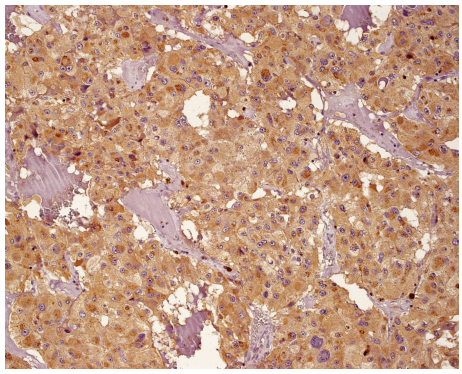

Figure 7: Positive (2+) immunostaining for VEGF-A in about 10% of tumor cells.

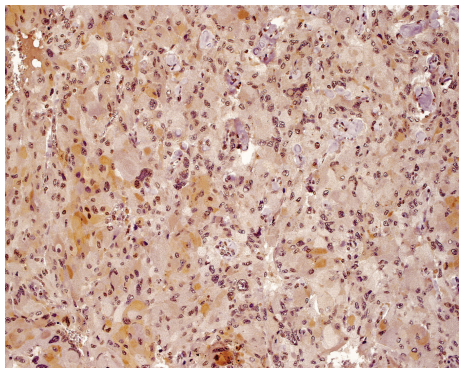

Figure 8: Immunostaining for VEGF-C weakly positive (1+) in 20% of tumor cells.

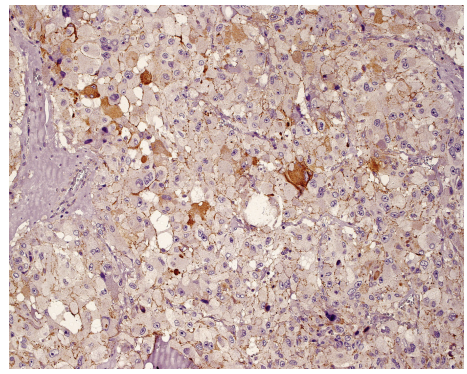

Figure 9: Immunostaining for VEGF-D only focally positive (2+) in single cells.

In the analyzed group of PPC, both benign and malignant neoplasms were highly vascularized tumors. Vascular architecture pattern was not equal, and vascular channels had different shapes and sizes in different parts of the tumor. Favier et al. had reported the differences in vascular architecture between benign and malignant PPC: benign tumors exhibited a regular pattern of small vessels while malignant PCC exhibited a more irregular pattern of vessels along with the presence of larger vascular channels between tumor cell nodules [24]. We have found highly heterogeneous vascular architecture patterns within particular PCC tumors, both benign and malignant. In areas with hemorrhagic and/or cystic changes, relevant quantification of vascular pattern was much more difficult and results were not always reliable. Changing operating techniques (prevailing laparoscopic procedures) increases the incidence and extent of hemorrhages in adrenal tumors (data prepared for publication) and therefore assessment of vascular architecture seems not to be a reliable procedure in PCC.

Angiogenesis (NA) was evaluated by assessing MVD using immunohistochemistry with CD31 and CD105 and assessing the expression of VEGFs. We have found that the MVD was higher in central areas of the tumor compared with subcapsular areas for both vessel counting strategies and with the use of both antibodies (CD31 and CD105). A strong correlation was found between the numbers of CD31 and CD105 positive blood vessels in both subcapsular and central areas of the tumors. The difference between the variables for CD105 positive vessels was statistically significant (30.155 versus 37,91, Student's t-test $P < 0.001$). This could be an indication that NA is more efficient in oxygen-reduced central parts of the tumor. Low oxygen conditions activate the hypoxia signaling pathway in neoplastic cells. Hypoxia-inducible target genes mediate multiple biological functions involved in the development of new blood vessels. Oxygen deprivation shifts the balance between factors stimulating and inhibiting angiogenesis toward the former.

We have also observed a strong correlation between the number of macrophages (in both subcapsular and central areas of the tumors) and MVD assessed by IHC with both CD31 and CD105 and between expression of VEGF-A in the tumor cells and MVD. A more than twofold excess in VEGF-A expression level was observed compared to VEGF-D levels. Expression of VEGF-A was also higher than expression of VEGF-C. The overexpression of VEGF-A and correlation between the number of macrophages and MVD indicate that neoangiogenesis in PCC is VEGF-A dependent and macrophages are highly involved in the process. VEGF-C and VEGF-D seem to be less involved in the vascularization of PCC. As we have stated in a previous study, mast cells also participate in vessel formation in PCC [32].

There are reports that MVD could influence the prognosis of various solid tumors. The literature concerning angiogenic status in PCC is still scanty and the results are ambiguous; some authors had found an increase in vascular density (MVD) in malignant versus benign PCC but some did not confirm these results [23, 24, 33–39]. Our investigation showed that there was no correlation between angiogenic status of PCCs and their malignant (recurrent or metastatic) behavior. We did not observe overexpression of any VEGFs or higher MVD in malignant versus benign PCCs, but the lack of significant differences in MVD and VEGF expression between groups of PCC in our study may be due to a small number of cases in the second investigated group. Increase in MVD in malignant PCCs was previously described by Favier in a group of PCCs, 50% of which harbour the SDHB-mutation (so-called cluster 1 tumors, C1) and were mostly extraadrenal PCCs (paragangliomas). The group of

Table 6: Correlations between the expressions of VEGF-A and MVD ("hot spot" method).

	CD31		CD105	
	Subcapsular	Intratumoral	Subcapsular	Intratumoral
VEGF-C	$r = 0.1757$	$r = 0.0637$	$r = 0.0999$	$r = -0.0227$
VEGF-A	$r = 0.3330$	$r = 0.4028$	$r = 0.4702$	$r = 0.4282$
VEGF-D	$r = -0.1068$	$r = -0.1526$	$r = -0.0193$	$r = -0.1338$

Table 7: Vascular parameters in benign and malignant pheochromocytomas.

Marker	Location	Method	Benign		Malignant	
			Mean	SD	Mean	SD
CD31	Subcapsular	Hot spot	57.47	23.69	50.40	25.78
	Intratumoral	Hot spot	61.19	27.71	48.00	30.32
	Subcapsular	Chalkey	39.71	13.49	47.11	13.69
	Intratumoral	Chalkey	45.61	14.22	51.53	16.70
CD105	Subcapsular	Hot spot	29.94	21.07	28.60	20.26
	Intratumoral	Hot spot	37.75	23.61	39.60	35.91
	Subcapsular	Chalkey	23.09	13.13	28.23	5.69
	Intratumoral	Chalkey	32.34	15.01	42.35	16.22
D2-40	Capsular	Hot spot	1.87	1.32	2.50	1.73
	Subcapsular	Hot spot	0.11	0.57	0.20	0.45
	Intratumoral	Hot spot	0.00	0.00	0.00	0.00
VEGF-A			4.64	1.01	3.80	1.30
VEGF-C			3.60	1.12	3.40	0.89
VEGF-D			1.98	1.41	1.40	1.67

tumors analysed in our study consisted of 62 adrenal PPCs in which SDH-mutations are very rare—only two of 62 tumors (3,2%) harbour SDHB-gene mutations (data prepared for publication). On the other hand, Ohij et al. reported the absence of statistical association between MVD and malignancy in PCC [38].

Lymphatic vessel density was analyzed in the same 62 PCC tumors after IHC with the lymphatic endothelial marker D2-40. D2-40 labelling revealed a complete absence of lymphatic vessels in the central parts of all PCCs. We have found single lymphatic vessels in 4 PCCs (6,4%) in subcapsular areas and in 55 PCCs (88.7%) within the capsule. With only a few lymphatic vessels that are found only in the subcapsular areas of the tumor, it can be assumed that the spread through lymphatics to lymph nodes will be much rarer than the spread by blood to distant organs.

For the majority of patients with both benign and malignant PCC, the surgical removal of the tumor is the treatment of choice. In malignant cases with distant metastases, chemotherapy (CVD combination: cyclophosphamide, vincristine, and dacarbazine), radiotherapy, and/or radiometabolic therapy using 131J-MIBG can also be used [39, 40]. These therapies may lead to remission and symptom relief in up to 50% of patients [22, 40]. Even so, half of the patients with malignant, metastatic PCC do not benefit from these therapies, and there is a need to find other treatment possibilities. Because all PCCs are highly vascularized neoplasms, malignant tumors may be candidates for molecular targeted therapies, especially antiangiogenic therapies targeting the vascular endothelial growth factor pathway. Monoclonal anti-VEGF antibody (bevacizumab) and tyrosine kinase inhibitors are already used in patients with advanced renal carcinoma and gastrointestinal stromal tumors (GIST).

In summary, PCCs differed in vascular density in central and subcapsular areas of the tumor, but there were no statistically significant differences in vascular density between benign and malignant cases, so MVD is not appropriate to differentiate between benign and malignant PPC. Moreover, secondary changes in these tumors influence the results and both vascular architecture and MVD are markedly disturbed by hemorrhagic and cystic changes in PCCs. These changes are partially caused by laparoscopic operation technique. High MVD in all PCCs is a promising factor for antiangiogenic therapy, especially in the subgroup of PCC belonging to the cluster 1 group (with SDHX or VHL-gene mutation [22].

5. Conclusion

(1) Microvessel density, as a single feature, does not help in differentiating malignant and benign PCC and has no independent prognostic significance in PCC.

(2) The results of assessing vascular architecture and MVD are biased by secondary changes in tumor tissue, especially hemorrhages and cystic changes.

(3) High MVD in all PCCs is a promising factor for antiangiogenic therapy, especially in the subgroup of malignant PCC belonging to the cluster 1 group (with SDHX or VHL-gene mutation).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Adrenal Disorders and the Paediatric Brain: Pathophysiological Considerations and Clinical Implications

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Various neurological and psychiatric manifestations have been recorded in children with adrenal disorders. Based on literature review and on personal case-studies and case-series we focused on the pathophysiological and clinical implications of glucocorticoid-related, mineralcorticoid-related, and catecholamine-related paediatric nervous system involvement. Childhood *Cushing syndrome* can be associated with long-lasting cognitive deficits and abnormal behaviour, even after resolution of the hypercortisolism. Exposure to excessive *replacement of exogenous glucocorticoids* in the paediatric age group (e.g., during treatments for adrenal insufficiency) has been reported with neurological and magnetic resonance imaging (MRI) abnormalities (e.g., delayed myelination and brain atrophy) due to potential corticosteroid-related myelin damage in the developing brain and the possible impairment of limbic system ontogenesis. *Idiopathic intracranial hypertension* (IIH), a disorder of unclear pathophysiology characterised by increased cerebrospinal fluid (CSF) pressure, has been described in children with hypercortisolism, adrenal insufficiency, and hyperaldosteronism, reflecting the potential underlying involvement of the adrenal-brain axis in the regulation of CSF pressure homeostasis. Arterial hypertension caused by *paediatric adenomas* or *tumours* of the *adrenal cortex* or *medulla* has been associated with various hypertension-related neurological manifestations. The development and maturation of the central nervous system (CNS) through childhood is tightly regulated by intrinsic, paracrine, endocrine, and external modulators, and perturbations in any of these factors, including those related to *adrenal hormone imbalance*, could result in consequences that affect the structure and function of the paediatric brain. Animal experiments and clinical studies demonstrated that the developing (i.e., paediatric) CNS seems to be particularly vulnerable to alterations induced by adrenal disorders and/or supraphysiological doses of corticosteroids. Physicians should be aware of potential neurological manifestations in children with adrenal dysfunction to achieve better prevention and timely diagnosis and treatment of these disorders. Further studies are needed to explore the potential neurological, cognitive, and psychiatric long-term consequences of high doses of prolonged corticosteroid administration in childhood.

1. Background

The assumption that children respond similarly to adults with respect to disease pathophysiology, medication efficacy, and adverse reactions is often erroneous [1].

It is largely known that the developing central nervous system (CNS) is qualitatively different from the adult nervous system, as the latter represents the final result of a complex ontogenetic process that requires various steps of cellular proliferation, angiogenesis, migration, synaptogenesis, differentiation, and myelination [2]. Evidence from numerous sources has demonstrated that neural development processes extends from the embryonic period through adolescence [2, 3].

The development of the nervous system is sensitive to potential insults during vulnerable periods because the process is dependent on the temporal and regional emergence of critical developmental processes (i.e., proliferation, migration, differentiation, synaptogenesis, myelination, and apoptosis) [3].

The ontogeny of the CNS in childhood is under tight regulation by intrinsic, paracrine, endocrine, and external modulators, and perturbations in any of these factors could result in long-term consequences that possibly lead to long-term impairment of the structure and function of the developing brain [3, 4].

In this context, among the modulator substances, adrenal hormones exert very important regulatory activities and trophic effects on cell survival, differentiation, maturation, and synaptogenesis of the CNS [5].

Adrenal hormones are secreted by the adrenal glands, which contain three zones within the cortex (i.e., glomerulosa, fasciculate, and reticularis), whereas the adrenal medulla is located in the central portion of the gland. The adrenal cortex is derived from the mesoderm, while the adrenal medulla is derived from the neuroectoderm, and its chromaffin cells secrete catecholamines in a process regulated by the preganglionic sympathetic neurons. The zona reticularis adjacent to the medulla secretes sex hormones, and the middle zone (i.e., fasciculate) secretes glucocorticoids (e.g., cortisol); the secretion from both of these zones is under the control of the hypothalamus-pituitary axis. The outer zone (i.e., glomerulosa) secretes mineralocorticoids (e.g., aldosterone) and is under the control of the renin-angiotensin system (RAS) [6].

A wide array of conditions can cause adrenal disorders in the paediatric age group, with a higher percentage of underlying causative genetic diseases compared to the adult age group. Despite extensive evidence from animal experiments regarding the higher risk of corticosteroid-related neurological sequelae in the developing brain, a limited number of human studies have functionally investigated the impact of adrenal disorders on the paediatric brain and have fully longitudinally explored the potential neurological and psychiatric long-term consequences of both endogenous and exogenous adrenal hormone imbalances.

In this paper, we discuss the most frequent paediatric adrenal disorders, with an emphasis on their neurologic manifestations, their pathophysiology, and their diagnosis.

2. Hypercortisolism and the Paediatric Brain

Cushing syndrome is a metabolic disorder caused by chronically high levels of endogenous cortisol or by exogenous exposure to corticosteroids that impairs carbohydrate, protein, and lipid metabolism and includes all causes of hypercortisolism; on the other hand, the term Cushing disease is reserved for cases of pituitary-dependent Cushing syndrome. The overall incidence of Cushing syndrome is approximately 2 to 5 new cases per million people per year, and only approximately 10% of the new cases each year occur in children [7]. A common cause of Cushing syndrome in children is chronic glucocorticoid administration. Cushing disease (i.e., pituitary dependent) accounts for approximately 75% of all cases of Cushing syndrome in children older than 7 years. In children under 7 years, Cushing disease is less frequent, and the adrenal causes of Cushing syndrome (i.e., adenoma, carcinoma, or bilateral hyperplasia) represent the most common causes of the condition in infants [8]. Adrenal cortical neoplasms in paediatric patients are more difficult to diagnose, and it is also more difficult to separate benign from malignant tumours in this age group [9, 10].

Ectopic corticotropin (ACTH) production accounts for less than 1% of the cases of Cushing syndrome in adolescents, and it is very rare in younger children [8].

Primary pigmented adrenocortical nodular disease (PPNAD; MIM 610489) is a dominantly inherited genetic disorder with the majority of cases associated with Carney Complex type 1 (CNC1; MIM 160980). CNC1 complex is a multiple endocrine neoplasia (MEN) syndrome that affects endocrine glands such as the adrenal cortex (causing Cushing's syndrome), the pituitary, and the thyroid. PPNAD is associated with germ line inactivating mutations of the PRKAR1A gene, which is located at 17q22-24 and encodes protein kinase A [10].

Bilateral adrenocortical hyperplasia has also been reported in McCune-Albright syndrome (MAS; MIM 174800), but in these cases, it is not always associated with hypercortisolism. In MAS, there is a somatic mutation of the GNAS1 gene leading to constitutive activation of the Gsα protein and non-ACTH-dependent adrenal cortex steroidogenesis [7, 9].

The onset of Cushing syndrome in children is often subclinical and insidious, with the most common presenting symptom being weight gain in the absence of a concomitant height gain [7, 8]. This abnormal growth can be associated with various other clinical signs and symptoms including facial plethora (see Figure 1), hypertension, amenorrhea, and skin manifestations such as acne, easy bruising, striae rubrae, and acanthosis nigricans [7–9]. Hyperandrogenism is another frequent feature of Cushing syndrome, which is due to the increase of adrenal androgens related to ACTH or to their autonomous production by adrenal tumors. Signs and symptoms include *acne*, scalp hair loss (*androgenic alopecia*), and excessive facial and body hair (*hirsutism*) [8, 9].

Of note, adults with Cushing syndrome frequently have cognitive impairment and psychiatric disturbances (mainly depression and anxiety) with a ratio between 57% and 79% [11, 12], while children with this disorder show rates of

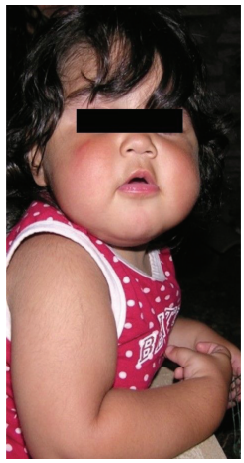


Figure 1: A 5-year-old girl with Cushing Syndrome due to adrenal adenoma that we followed up at our Institution (University of Messina) for sleep disturbances and mood disorders. Note the characteristic facial plethora.

psychiatric symptoms of 44%, with compulsive behaviours predominating [13].

Also exogenous corticosteroid administration has been often reported to be associated with psychiatric manifestations, including psychotic symptoms, hyperactivity, and mild changes in mood and cognition; these manifestations can appear during corticosteroid treatment or during withdrawal [14]. Although the pathophysiology of these side effects is still not fully understood, some authors have proposed potential glucocorticoid-related hippocampus damage as the cause [15]. Concerns have been raised regarding the potential for hippocampus damage that is not completely reversible in some children treated with corticosteroids (especially those on the highest doses and long-term therapies), with possible long-lasting negative effects on cognition, but no longitudinal studies have been performed to clarify the cognitive outcomes in these patients [16].

Of note, several studies have reported a significant global loss of brain volume and CNS atrophic changes based on radiological investigations of both adult and paediatric patients with Cushing Syndrome [17–19]. In addition, supraphysiological doses of exogenous glucocorticoids were found to possibly cause cerebral atrophy and volume loss in different paediatric series [20, 21], and other studies demonstrated a possible link between the increased activity of the pituitary-adrenal axis and cerebral atrophy in the context of depression and stress [22].

In most patients, the MRI atrophic changes related to hypercortisolism are detected in the amygdala, temporal lobe, and hippocampus [17–19, 21], and this could explain the behavioural abnormalities and the impairment of cognition and memory, given the high biological importance of these brain regions in the processing of emotions and in cognition.

Of note, significant recovery of depressive symptoms and improvement in cognition and reversal of cerebral atrophy have been observed in adult patients with Cushing syndrome after clinical remission [17].

Data on the evolution of the cerebral atrophy following correction of hypercortisolism are limited in the paediatric age group, but in a study of 11 children with Cushing syndrome followed up after surgery, a long-lasting significant decline in cognitive function was observed after a return to eucortisolism, despite almost complete reversal of the cerebral atrophy [19]. This observation is in contrast to the adult experience, and the possible decline in cognitive function after curing Cushing syndrome seems to be unique to the paediatric population.

2.1. Pathophysiology of Glucocorticoid-Related Paediatric Brain Damage. The pathogenesis of the loss of brain volume induced by chronic glucocorticoid excess appears to be multifactorial and is not yet fully understood.

Glucocorticoids can act both on mineralcorticoid receptors (MRs) and on glucocorticoid receptors (GRs). MRs are usually protected by glucocorticoids exposure by the effects of 11β-HSD2 enzyme, which converts cortisol into the inactive cortisone; however, 11β-HSD2 is not expressed in the hippocampus or other limbic structures, allowing MRs activation by glucocorticoids in these latter brain regions [23]. Therefore, glucocorticoids excess increases the occupation of MRs/GRs, with predominant involvement of some (but not other) brain regions (e.g., limbic system).

Notably, studies on hippocampal cell cultures showed that supraphysiological doses of glucocorticoids lead to a reversible phase of atrophy of the apical dendrites of pyramidal neurons [24]. In addition, glucocorticoids have been shown to increase the synaptic accumulation of glutamate and to stimulate the N-methyl-D-aspartate (NDMA) receptors with a subsequent increase in intracellular cytosolic Ca^{+} in postsynaptic neurons, which activate several processes leading to neuron cell death [19, 22].

Of note, the partial reversibility of brain atrophy after a return to eucortisolism would indicate that the above-discussed MRI abnormalities are not exclusively related to neuronal death. Interestingly, among the exogenous corticosteroids, dexamethasone has been classically regarded as very potent in treating cerebral oedema [25]; therefore, the loss of brain volume in Cushing syndrome could be putatively secondary also to a decrease in water content of the brain due the hypercortisolism [26].

Interestingly, the complications related to exogenous and endogenous corticosteroid excess on the paediatric brain might depend on several variables, including the stage of CNS development at the time of exposure and the duration of exposure, and in the case of exogenous compounds, the pharmacological characteristics of the corticosteroids used and their dosage [5].

A very important variable might be represented by the stage of CNS development at the time of exposure. In this regard, the neurological manifestations could to be directly proportional to the immaturity of the brain, and the foetal age appears as the most "at risk" period of life [5, 27]. In fact, there is considerable evidence from animal models (e.g., baboon and sheep) that antenatal administration of steroids can have detrimental effects on the developing CNS, causing

a dose-dependent brain atrophy (e.g., predominantly in the hippocampus) and delay in myelination [27, 28].

Notably, the prenatal administration of corticosteroids in humans is common clinical practice in women with possible preterm delivery and is used to stimulate lung maturation and prevent respiratory distress syndrome. The neurological outcomes of those treated infants are still a matter of debate. In fact, several long-term clinical studies have shown no impact of antenatal corticosteroid therapy on neurodevelopmental outcomes during the follow-up of these children (e.g., at 2 years of age) [29–31]. Conversely, other studies suggest a higher rate of cerebral palsy in infants exposed to repeat courses of corticosteroids [32], as well as neurodevelopmental abnormalities [33] and behavioural effects [34].

These observations suggest that prolonged exposure to excess glucocorticoids from an endogenous or exogenous source, not only significantly impacts the weight, height, and development of children, but may also have permanent effects on the paediatric developing brain, behavior, and cognition.

3. Adrenal Insufficiency and the Paediatric Brain

Primary adrenal insufficiency, or Addison's disease, is the destruction or dysfunction of the adrenal cortex gland causing impaired secretion of glucocorticoids and mineralocorticoids [35, 36]. Adrenal insufficiency is a rare disorder with a prevalence in developed countries of up to 93–144 cases per million, with an estimated incidence of 4.44–6 new cases per million population per year [36, 37]. In developed countries, has been estimated that 80–90% of cases of primary adrenal insufficiency are caused by the destruction of the adrenal cortex by cell-mediated immune mechanisms, which can be isolated (40%) or part of an autoimmune polyendocrinopathy syndrome (60%) [36].

Of note, in the paediatric age group, adrenal insufficiency is in most cases related to genetic disorders including impaired steroidogenesis due to defects in enzymes involved in hormone production (e.g., congenital adrenal hyperplasia) or developmental defects of the glands, often associated with mental retardation and neurological features (e.g., Smith-Lemli-Opitz syndrome, triple A syndrome) [11, 36].

In a large series of 103 children with Addison's disease, most reported causes were genetic conditions, which accounted for 78% of cases, whereas autoimmune disease was diagnosed in only 13% of cases [38]. Secondary adrenal insufficiency results from pituitary disease that hampers the release of ACTH exclusively impairing glucocorticoid secretion; tertiary adrenal insufficiency is in most cases related to the long-term administration of exogenous glucocorticoids, which leads to prolonged suppression of hypothalamic secretion of CRH [35, 36].

Adrenal insufficiency in children typically presents with insidious nonspecific symptoms such as fatigue, malaise abdominal pain, weight loss, nausea, and vomiting. Physical signs appear as later manifestations of the disease and include hypotension and hyperpigmentation (e.g., in the primary forms). Classic biochemical signs include hyponatremia, hyperkalaemia, hypoglycaemia, and ketonaemia [36, 37]. A life-threatening adrenal crisis can be the first presentation of adrenal insufficiency, with sudden onset of vomiting, abdominal pain, myalgia, severe hypotension, and hypovolaemic shock. This acute presentation is usually precipitated by a physiological stress, such as surgery, trauma, or a concurrent infection.

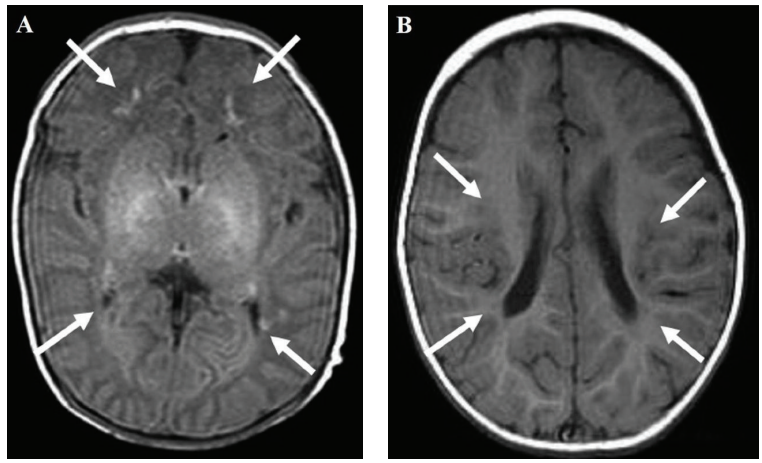
In some paediatric reports, adrenal insufficiency has been reported in association also with idiopathic intracranial hypertension (IIH; see Section 4.2), with neurological symptoms (e.g., headache, diplopia) that appeared as the first clinical manifestations, before those related to the adrenal insufficiency [39, 40].

There have been a number of studies or single case descriptions reporting on isolated episodes of hypoglycaemic coma and on multiple cases of extrapontine and central pontine myelinolysis with encephalopathy and coma in children with Addison disease [41–43]. Adrenal insufficiency is often associated with chronic severe hyponatraemia. Rapid correction of chronic hyponatremia leads to a significant brain dehydration as potassium and organic substances cannot be introduced into the cells as fast as required [44]. As a result of that, the myelin sheath is stripped from the axon and the oligodendrocytes are damaged, especially in some brain areas, which are more vulnerable to the osmotic damage (e.g., pons, basal ganglia), with consequent regional-limited demyelination [45].

Therefore, both extra pontine and central pontine myelinolysis can occur as a consequence of a rapid correction of hyponatremia in individuals with chronic, severe hyponatraemia [45]. The initial symptoms of pontine myelinolysis, which manifest shortly after the rapid correction of hyponatremia, include a depressed level of awareness, difficulty in speaking (dysarthria or mutism), and difficulty in swallowing (dysphagia). Seizures, global tremor and a wide array of movement disorders can be also present at onset, especially when are involved the extra pontine regions (e.g., basal ganglia). Additional symptoms often arise over the following 1-2 weeks including loss of consciousness, global weakness, paralysis in the arms and/or the legs, stiffness, and difficulty with coordination [46]. Myelinolysis can be reversible or, in the most severe cases, can also lead to coma and death. In patients with chronic hyponatremia (e.g., in adrenal insufficiency), the rate of correction should be less than 0.5 mmol/L/h to prevent central and extra pontine myelinolysis [47, 48].

3.1. Congenital Adrenal Hyperplasia. In the paediatric age group, the most common cause of primary adrenal insufficiency is *congenital adrenal hyperplasia* (CAH), a complex and heterogeneous condition resulting from a genetic defect in the biosynthetic pathway of cortisol and/or aldosterone in the adrenal cortex.

In a large series of 103 children with Addison's disease, CAH was revealed as the most frequent cause of adrenal insufficiency, accounting for 71.8% of the cases [38]. Classic CAH is an autosomal recessive disorder with a prevalence rate estimated at one in 15,000 live births [53].



(a) (b)

Figure 2: Brain MRI. Newborn with classic adrenal congenital hyperplasia, on day 12 after birth, white matter abnormalities that consisted of bilateral small diffuse hyperintensities (white arrow) are depicted on T1-weighted images (a). At 1 yr. of age, bilateral small diffuse hyperintensities are documented (white arrow) on T1-weighted images (b). (Reprinted with permission from [49], Copyright Japanese Society for Pediatric Endocrinology).

The most common form is classic CAH due to *21-hydroxylase* deficiency (MIM 202010), a condition characterised by low synthesis of glucocorticoids and, in many cases, mineralocorticoids (i.e., in the salt loosing variant) and adrenal hyperandrogenism. More rare forms are caused by deficiency of *11β-hydroxylase*, *17α-hydroxylase*, *3β-hydroxysteroid dehydrogenase*, or *P450 oxidoreductase* [53].

Replacement of glucocorticoids is necessary for patients with CAH; treatment with mineralocorticoids is necessary in these patients only when aldosterone production is deficient (e.g., in the salt loosing variant). Since intrauterine life, prenatal glucocorticoid deficiency has a potential impact on the foetal brain, and as evidence of this, a significant decrease in the amygdala volume has been observed in CAH infants, suggesting a prenatal critical effect of hormone deficiency on the developing CNS [54].

Of note, several studies based on MRI have described brain white matter abnormalities in children with CAH, with the radiological changes that can be sometimes detected from the first days of life in children with classic CAH [49, 55]. In addition to the white matter changes, moderate atrophy in the right temporal cortex, small volume hippocampus, and agenesis or thinning of the corpus callosum were observed in the long term follow-up of children with classic CAH [56–58].

The pathophysiology of white matter abnormalities is still not fully understood, but many authors have proposed that exposure to excess exogenous glucocorticoids during CAH treatment is the most feasible explanation for these MRI findings due the potential inhibitory role of cortisol in the process of neuronal maturation and myelination by inhibiting the differentiation of oligodendrocyte precursors [5, 28, 56, 57]. In addition, hyponatremia, potentially leading to myelinolysis, has been suggested as a potential contributing factor to the impaired myelin formation in these patients [55, 56]. Some authors have also suggested that hormonal imbalance related to a deficiency in cortisol and aldosterone and an overproduction of 17-OH-progesterone and androgen may further cause a destabilisation of the myelin molecule leading to its degeneration [56].

Clinically, these children may present with seizures, lethargy, postural and intentional tremor, tendon reflex asymmetry, or cerebellar symptoms [49, 55–58]. Standard MRI sequences performed in the first days of life in some newborns with classical CAH typically showed diffuse hyperintensity on T1-weighted sequences (Figure 2(a)), with reduced diffusion in the periventricular white matter on the diffusion-weighted imaging (DWI) sequences. Follow-up radiological studies often showed complete reduction in the previously detected imaging abnormalities (Figure 2(b)), although long-lasting abnormalities and irreversible atrophic changes have also been documented in some patients [55].

Neurological outcomes have not been systematically reported in these children, but a mild reduction in cognitive capacities and memory has been described in some, likely due to the effects of supraphysiological doses of corticosteroid replacement on the amygdala and hippocampus development [59, 60].

3.2. X-Linked Adrenoleukodystrophy. X-linked adrenoleukodystrophy (X-ALD; MIM 300100) is the most common peroxisomal disorder, with an estimated birth incidence of 1 in 17,000 newborns (male and female) [61]. It is caused by mutations in the *ABCD1* gene located on the X-chromosome that encodes the peroxisomal membrane protein ALDP, which is involved in the transmembrane transport of very long-chain fatty acids (VLCFA); therefore, a defect in ALDP results in elevated levels of VLCFA in the plasma and tissues [62].

Adrenoleukodystrophy has a variable age of onset in childhood and exhibits different phenotypes. The clinical

spectrum in males with X-ALD ranges from isolated adrenocortical insufficiency and slowly progressive myelopathy to devastating diffuse cerebral demyelination.

X-ALD is a frequent cause of Addison's disease in boys and adult males. Adrenal insufficiency (or even Addisonian crisis) can sometimes be the presenting symptom of X-ALD in boys and men, years or even decades before the onset of neurological symptoms [10, 36, 62]. The most aggressive and devastating phenotype is cerebral ALD, consisting of a rapidly progressive demyelinating condition that affects the cerebral white matter. It is by definition confined to boys who develop cerebral involvement before the age of 10 years. The boys are normal at birth and have unremarkable development. The mean age of onset is approximately 7 years [62].

The disease usually manifests early with behavioural manifestations including inattention and hyperactivity. It often becomes apparent through school difficulties. It progresses to visual symptoms, auditory processing difficulties, and motor incoordination. Later neurological manifestations can include moderate dystonia, pyramidal signs, gait disturbances with features of cerebellar and pyramidal tract involvement, hemiparesis, or spastic tetraparesis [10, 61, 62]. Typical MRI white matter changes include signal hyper intensities on T2-weighed and FLAIR sequences in the parietooccipital region and the splenium of the corpus callosum [61, 62].

Although it is still impossible to predict which patients will develop the neurological manifestations of the disease, it has been estimated that approximately 35–40% of children with mutations in the ABCD1 gene will develop cerebral ALD before adulthood [61, 62].

However, the prognosis is variable with a great variability between the patients and depends on the neuroinflammatory stage of the disease, which correlates with the cerebral demyelination and the neurological manifestations. It is likely that if they survive into adulthood, all patients with X-ALD eventually develop adrenomyeloneuropathy (AMN), a condition characterised by paraparesis, spasticity, and signs of neuropathy, with onset usually after 20 years of age [10, 63].

The only treatment that has shown some clear benefits in early symptomatic X-ALD is based on haematopoietic stem cell transplantation (HST), with stabilisation or improvement in the clinical and/or MRI findings in 50–75% of treated boys [64]. In the future, HST with autologous cells that have been genetically corrected with lentiviral vectors and reinfused might become an effective treatment because of the promising preliminary results [65].

Paediatric endocrinologists and neurologists may face X-ALD and should potentially consider this disease in any boy presenting with Addison's disease. Early recognition of X-ALD is very important because in some cases, treatment is available, such as HCT in the early stages of cerebral ALD and endocrine replacement therapy for adrenal insufficiency.

3.3. Other Genetic Forms of Adrenal Insufficiency. Autoimmune polyglandular syndrome type 1 (APS-1; MIM 240300) is a rare autosomal recessive disorder caused by mutations in the autoimmune regulator (AIRE) gene, that is characterised by autoimmune adrenal insufficiency, hypoparathyroidism, chronic mucocutaneous candidiasis, ectodermal dystrophy, and many other potential autoimmune disorders. Children with APS-1 may have autoimmune activation against CNS antigens such as autoantibodies directed against aromatic L-amino acid decarboxylase (AADC), tyrosine hydroxylase (TH), tryptophan hydroxylase (TPH), and glutamic acid decarboxylase (GAD). Neurological symptoms previously described in association with APECED include the stiff-man syndrome, cerebellar ataxia, inflammatory demyelinating polyneuropathy, and acute reversible CNS demyelination [66, 67].

Congenital adrenal hypoplasia (MIM 300200), due to *complex glycerol kinase* deficiency, is an X-linked condition in which the adrenal cortex development is prevented. In this disorder, the onset of symptoms varies from birth to childhood, and the clinical presentation includes adrenal insufficiency, psychomotor retardation, muscular dystrophy, hypertelorism, strabismus, short stature, and osteoporosis [68].

Triple A syndrome (MIM 231550) is an autosomal recessive disorder that consists of the triad of ACTH-resistance adrenal insufficiency, alacrima, and achalasia. Aldosterone deficiency may also be observed in patients with gradual neurologic dysfunction with polyneuropathy, mental retardation, hyperreflexia, muscle weakness, dysarthria, ataxia, and abnormal autonomic function [69, 70].

Smith-Lemli-Opitz syndrome (SLOS; MIM 270400) is a neurodevelopmental disorder caused by inborn errors of cholesterol metabolism resulting from mutations in 7-dehydrocholesterol reductase (DHCR7) characterised by intellectual disability, CNS malformations (e.g., abnormalities in septum pellucidum and in corpus callosum, colpocephaly, arachnoid cysts, type I Chiari malformation), and multiple congenital anomalies and is possibly associated with adrenal insufficiency due to the impaired steroidogenesis [36, 37].

Kearns-Sayre syndrome (KSS; MIM 530000) is a mitochondrial disease characterised by myopathy, ptosis, ophthalmoplegia, and deafness and can also manifest with endocrine anomalies, including adrenal insufficiency [10, 36].

4. Hyperaldosteronism and the Paediatric Brain

Hyperaldosteronism is a condition characterised by an increase in mineralocorticoid secretion due a disorder of the zona glomerulosa of the adrenal glands [71].

Primary hyperaldosteronism (PAL) is an exceptional entity in the paediatric age group and is caused by aldosterone secreting adenomas (or carcinomas), bilateral adrenal hyperplasia (e.g., idiopathic hyperaldosteronism), or primary unilateral adrenal hyperplasia [71, 72]. The renin activity is typically low in all forms of PAL because of the negative feedback effect.

Familial hyperaldosteronism (FH) is a rare autosomal dominant disorder with two main typical presentations (i.e., types 1 and 2). The FH type 1 (MIM 103900) is characterised by hypertension, weakness, failure to thrive, and increased

incidence of intracranial aneurysms [73]. Typical laboratory features are hyperaldosteronism and high levels of hybrid steroids (e.g., 18-hydroxycortisol and 18-oxocortisol); excellent clinical response to exogenous glucocorticoid administration has been achieved. The FH type 2 (MIM 605635) is characterised by aldosterone-producing adenomas or bilateral idiopathic hyperaldosteronism (or both), and it usually does not respond to glucocorticoids [73].

Secondary hyperaldosteronism (SAL) results from overactivation of the renin-angiotensin system (RAS) and is therefore characterised by elevated aldosterone and renin levels and usually occurs in adults with renovascular hypertension or renin-secreting tumours. The most frequent causes of SAL in the paediatric age group are the salt-losing tubulopathies (e.g., Bartter syndrome, Gitelman syndrome), autosomal recessive conditions characterised by the loss of Na^+ from the distal nephron with consequent RAS activation and persistent SAL. These disorders variably manifest with growth failure, hypokalaemia, metabolic alkalosis, hyperreninaemia due the hyperplasia of the juxtaglomerular apparatus, and hyperaldosteronism; the arterial pressure is usually within normal limits [72, 74].

Other causes of hyperaldosteronism include potassium sodium-wasting nephropathy, renal tubular acidosis, diuretic or laxative abuse, nephrotic syndrome, nephropathic cystinosis, oestrogen administration, and recombinant growth hormone (r-GH) treatment [75].

Hyperaldosteronism (and the consequent activation of MRs pathways that are present in different organs and tissues including kidney and brain) is one of the most important causes of endocrine-related arterial hypertension, potentially leading to neurological complications as stroke and hypertensive encephalopathy (see Sections 4.1 and 5) [76]. Additionally, hyperaldosteronism has been recently regarded as a cause of IIH, due to a potential impairment of cerebrospinal fluid homeostasis in the presence of an underlying MRs overactivation in the choroid plexus epithelium (see Section 4.2) [77].

4.1. Pseudohyperaldosteronism and Pseudohypoaldosteronism and the Paediatric Brain. Congenital absence of adrenal enzymes such as *11β-hydroxylase* and *17α-hydroxylase* lead to CAH with elevated serum deoxycorticosterone (DOC). Supraphysiological levels of DOC promote salt retention, volume expansion, and arterial hypertension, similarly to what occur in hyperaldosteronism [75].

Apparent mineralcorticoid excess (AME; MIM 218030) is caused by deficiency of *11β-hydroxysteroid dehydrogenase type 2* (11βHSD2). This condition is characterised by decreased conversion of biologically active cortisol into inactive cortisone, with consequent constitutional MRs activation, resulting in pseudohyperaldosteronism and suppressed levels of both renin and aldosterone [78].

AME has been frequently reported in association with neurological manifestations in the pediatric population. In a series of 14 children with AME, 8 had neurologic symptoms at the time of diagnosis including developmental delay, abnormal electroencephalographic evaluation with a pattern of generalized seizure disorder, history of lapses of awareness and signs of cerebral infarcts on MRI [79]. Notably, stroke represents one of the most dangerous risk factors for children with AME, possibly due the detrimental effects on the cerebral vessels of constitutive activation of the MRs, largely expressed in many CNS sites [80]. In a report, a female adolescent with AME developed stroke despite adequate treatment of her hypertension and the authors suggested a pathophysiological role of 11βHSD2 deficiency as causative factor of endothelial dysfunction involving the cerebral circulation, due to the excessive exposure to high levels of circulating active cortisol [81].

Pseudohypoaldosteronism (PHA) is a condition characterised by mineralocorticoid resistance, with a great genetic and clinical heterogeneity [82]. PHA type 1 can result from autosomal dominant mutations in the mineralocorticoid receptor coding gene NR3C2 (PHA1A; MIM 177735) or from autosomal recessive mutations in the epithelial sodium channel (PHA1B; MIM 264350). Both autosomal dominant and recessive forms of PHA1 are characterised by salt wasting and hyperkalaemia with increased plasma renin and aldosterone levels, reflecting a resistance of the kidney and other tissues to mineralocorticoids [83]. The onset of clinical manifestations in PHA1 is mainly confined to early childhood, with hyperkalaemia, hyponatremia, and dehydration. Salt supplements are required (especially in the recessive form that is more severe) coupled with attainment of control of hyperkalaemia [83]. PHA1 has been described in association with encephalopathy and convulsions, due to rapid (erroneous) salt supplementation and possible underlying pontine and/or extrapontine myelinolysis [84].

PHA type 2 (PHA2) represents a group of conditions commonly characterised by hyperkalaemia despite normal renal glomerular filtration, metabolic acidosis, and a low plasma renin level, with high incidence of hypertension. Plasma aldosterone levels are low to mildly (but inadequately) increased. PHA2 is characterised by a great genetic variability with several potentially causative genes (including *WNK1*, *WNK4*, *KLHL3*, and *CUL3*), most of them crucially involved in the (negative) regulation of the NaCl cotransporter (NCC) [85, 86].

In PHA2, recurrent myalgia and periodic paralysis had been recorded in the original description by Gordon [87]. Mutations in the *WNK1* gene have been shown to be causative of the hereditary sensory neuropathy in a French family with PHA2 and defects in peripheral sensory perception; the authors demonstrated that WNK1 *is highly* expressed in the sensory components of peripheral nervous system and is associated with relaying sensory and nociceptive signals in sensory neurons [86, 88].

4.2. Hyperaldosteronism and IIH. IIH, also known as *pseudotumor cerebri*, is a neurological disorder of uncertain aetiology, characterised by increased intracranial pressure (ICP) in the absence of a tumour, hydrocephalus and no apparent cause based on neuroimaging or other routine evaluations [89]. Its main symptoms and signs are headache, nausea, and vomiting, as well as visual field defects and papilledema. MRI imaging can sometimes reveal significant

changes, including flattening of the posterior sclera, distension of the perioptic subarachnoid space, and partial empty sella, often correlated to a long lasting elevation of ICP [89]. Clinically, patients typically present with varying combinations of headache, tinnitus, and diplopia [77]. Children with IIH sometimes present with signs that mimic a posterior fossa lesion, including ataxia, nuchal rigidity, facial palsy, or torticollis [89]. Typical symptoms of papilledema include loss of visual acuity and/or transient visual blurring. Papilledema is frequently identified at the time of presentation but could be also observed on routine fundoscopic examination in asymptomatic patients. Ophthalmoplegia can also sometimes occur in patients with IIH, as a result of sixth cranial nerve palsy [89].

While the exact pathophysiology of IIH remains unknown, there have been many proposed theories. Notably, various exogenous and endogenous disorders of the hypothalamic-pituitary-adrenal axis including Addison's disease and Cushing's disease, chronic use of corticosteroids as well as withdrawal from chronic use, adrenal androgen excess and more recently PAL and SAL have been all shown to be potentially associated with the development of IIH [39, 40, 50, 90, 91].

A recent study proposed a theory unifying various neuroendocrine effects on the mineralocorticoid receptor (MR) pathway to explain a possible mechanism for the increased cerebrospinal fluid (CSF) production and ICP in IIH [50, 92]. The MR(s) are abundantly expressed in the choroid plexus epithelial cells (CPEC), which are putatively crucial in the regulation of CSF production [93]. Activation of the MR or its downstream pathways can enhance and stimulate the generation of Na/K ATPase pumps, which can lead to the movement of sodium ions at the CPEC apical membrane into the cerebral ventricle and actively create an osmotic gradient to drive CSF secretion and increase CSF pressure [94, 95]. Based on this perspective, the MR signalling at the CPEC level could therefore be a key pathway in IIH pathophysiology and could explain several reported endocrine-metabolic causes of IIH (see Figure 3), including PAL and SAL, obesity, metabolic syndrome, Cushing syndrome, chronic steroid administration, hypervitaminosis A, recombinant growth hormone (r-GH) therapy, and estro-progestin supplementation [50, 89–91].

Aldosterone, a mineralocorticoid responsible for Na^+ reabsorption and K^+, Ca^{2+}, and Mg^{2+} excretion in target tissues, including the CPEC, can exert its biological effects on these cells via the MR pathway [50]. An increase in its activity in PAL and SAL disorders may potentially directly affect the ICP in IIH. Support for this perspective includes numerous recent studies demonstrating that in children and adults with PAL or SAL, those treated with spironolactone, an aldosterone receptor antagonist, had resolution of the neurological symptoms and of the ophthalmological manifestations [95, 96]. Additional paediatric cases further strengthened this association of IIH and hyperaldosteronism in which children with various conditions (e.g., metabolic syndrome, SAL due tubular dysfunction) have been successfully treated with spironolactone after a lack of clinical response to the other diuretic treatments (e.g., acetazolamide) [95–97].

This emergent perspective has been proposed as potentially unifying because of the many reported endocrine metabolic associations that may have a common action on the MR pathway, reflecting the possible important involvement of the adrenocortical hormones in the homeostasis of CSF production and pressure [50, 92].

CSF cortisol levels are regulated by 11βHSD1, that is abundant the CPEC and converts inactive cortisone to cortisol and acts with high affinity at the MR(s) (similarly to aldosterone) in the CPEC; a pathophysiological link between the enzyme activity and the predisposition to IIH has therefore been suggested [98].

Thus, derangements of this putative adrenal-brain axis in children, through exogenous or endogenous mechanisms, may lead to the development of IIH via this mechanism. Vitamin A has been shown to induce expression of neurosteroids in glial cell lines, which could also theoretically interact with MR(s) [99]. Additionally, human fat, an active endocrine tissue, secretes various cytokines and aldosterone-releasing factors, providing another possible link to elevated ICP in obese patients with IIH (in addition to the link of aldosterone-related predisposition to arterial hypertension, insulin resistance, and metabolic syndrome in the obese population) [50, 100, 101].

Finally, a relationship between IIH and r-GH therapy has been reported numerous times and r-GH is known to be associated with RAS activation and elevated aldosterone levels, especially in the first phases of treatment [102, 103]. Thus, this emerging perspective suggests that the above mentioned associations with IIH may all have a unified action on the MR pathway in the CP, through the adrenal-brain axis, resulting in altered CSF fluid dynamics and elevated ICP (Figure 3) [103, 104].

However, further experimental work should to be performed to confirm these potential perspectives for a complete understanding of the pathophysiology of IIH when related to other reported risk factors (e.g., adrenal insufficiency, steroid withdrawal) and to assess the best etiologically targeted treatment for this increasingly recognised neurological disorder.

5. Disorders of the Adrenal Medulla and the Paediatric Brain

The adrenal medulla is located at the centre of the adrenal gland, is surrounded by the adrenal cortex, and consists of secreting cells called "*chromaffin cells*" (or pheochromocytes because they stain brown with chromium salts) that secrete *epinephrine* (adrenaline), *norepinephrine* (noradrenaline), and a small amount of *dopamine* in response to stimulation by *sympathetic pre-ganglionic neurons* [72].

Catecholamine-secreting tumours of the adrenal medulla are called pheochromocytomas, which are very rare tumours in children; they can be solitary or multiple, benign or malignant and are frequently associated with an underlying genetic disease (Figure 4). Those tumours arising in the sympathetic ganglia are called extra-adrenal paragangliomas [72].

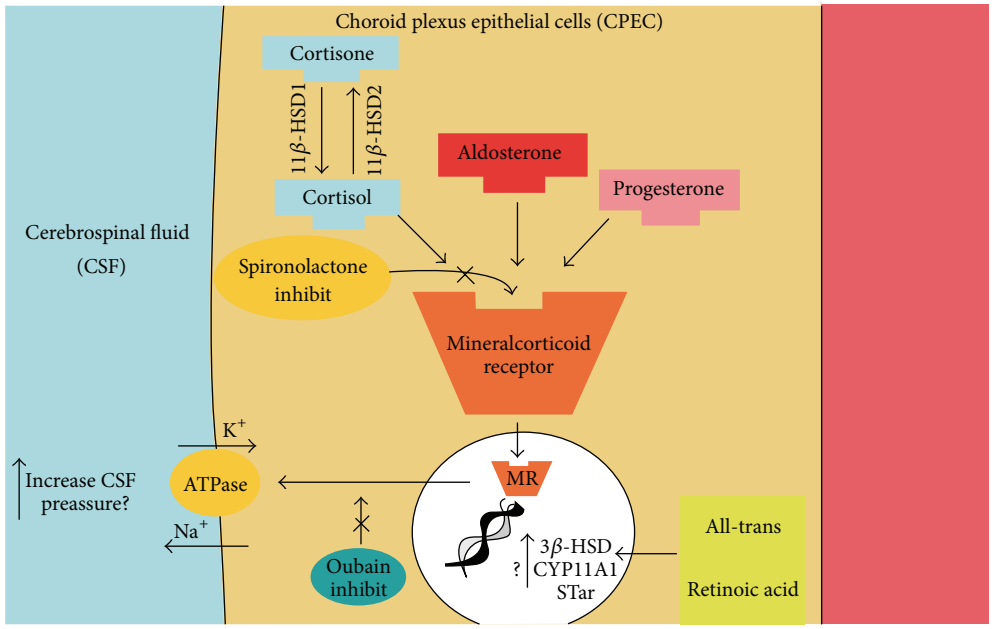
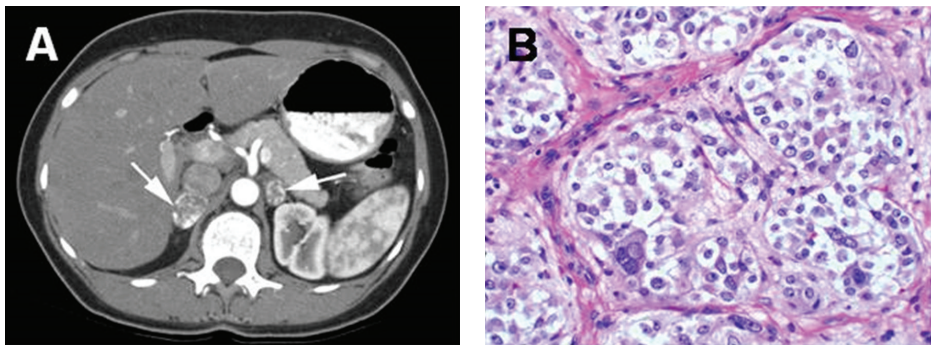


FIGURE 3: The putative effects of a wide array of neuroendocrine interactions on CSF secretion in the choroid plexus epithelium. Detailed figure of CP epithelium illustrating the proposed regulation of CSF secretion by several neuroendocrine interactions. Aldosterone can stimulate MR. In nucleus MR can activate mineralocorticoid responsive elements that stimulate synthesis from DNA of Na^+/K^+ ATPase pumps. Active sodium secretion by the Na/K ATPase at the apical CP membrane lead to movement of sodium ions into the cerebral ventricle and this creates an osmotic gradient to drive CSF secretion. The enzyme 11βHSD1 is highly abundant in the CP, in which its oxidoreductase activity converts inactive cortisone to cortisol, which can activate the MR with similar affinity to aldosterone. All-trans retinoic acid, interacting with DNA, can activate neurosteroidogenesis and *de novo* synthesis of steroids. CPEC = choroid plexus epithelial cells; CSF = cerebrospinal fluid; MR = mineralcorticoid receptor; CSF = cerebrospinal fluid; see [50] (http://www.ncbi.nlm.nih.gov/pubmed/23160227).



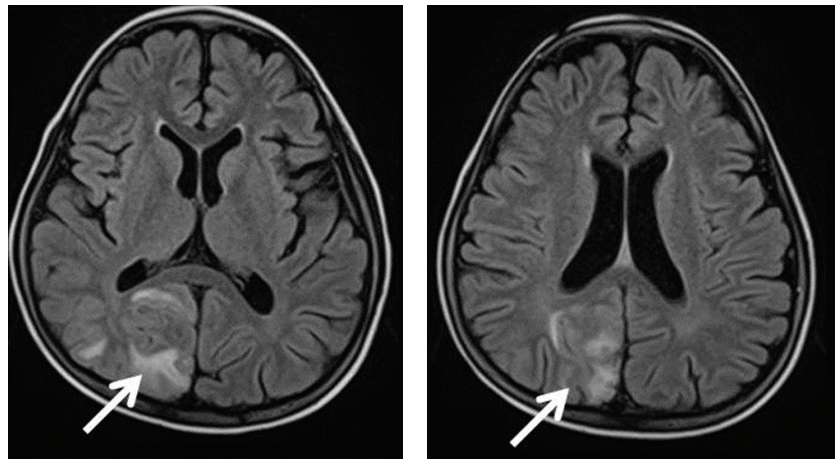
(a) (b)

FIGURE 4: (a) Bilateral pheochromocytomas (arrows) with rim enhancement in the adrenal glands of a 26-year-old woman with VHL disease; (b) Pheocromocytomas tumor cells are arranged in rounded clusters, separated by endothelial-lined spaces, with have vesicles containing norepinephrine and epinephrine (Reprinted with permission from [51], Copyright Springer Verlag). VHL = Von Hippel – Lindau.

Some triggering factors, such as intercurrent illness, hypoglycaemia, and surgical procedures, increase the production of catecholamines from the adrenal medulla with a subsequent impact mainly on the cardiovascular system and onset of manifestations such as increasing heart frequency, elevation of blood pressure, increased myocardial contractibility, and development of cardiac conduction anomalies [72].

Clinical manifestations of pheochromocytomas and paragangliomas are related to catecholamine action on the cardiovascular system, with some other symptoms that include increased (cold) sweating, tremors, weakness, and psychological agitation associated with palpitations [10]. Tumours that affect the adrenal medulla cause increased secretion of norepinephrine and epinephrine. Tumours that secret norepinephrine usually produce severe sustained hypertension, whereas those that secrete primarily epinephrine produce episodic hypertensive crises [72].

Neurologic complications are usually caused by changes in blood pressure and include episodic headaches, and



(a) (b)

Figure 5: FLAIR brain MR images of a 12-year-old girl with sporadic paraganglioma admitted to our Institution with headache and seizures. Unilateral right occipitoparietal lobe vasogenic edema (arrows), typical of PRES, is well depicted after a severe hypertensive crisis. PRES = Posterior reversible encephalopathy syndrome.

ischemic or haemorrhagic cerebrovascular events have been reported in the paediatric literature [105–107]. Furthermore, it is important to be cognisant of the possibility of posterior reversible encephalopathy syndrome (PRES) in children with pheochromocytoma or paraganglioma presenting with hypertension and cerebrovascular manifestations [108]. PRES is a recently recognised clinical-radiological entity, previously called hypertensive encephalopathy. In fact, arterial hypertension could be regarded as the most well-known risk factor for this condition, and therefore, several children with PRES caused by pheochromocytoma or paraganglioma have been reported in the literature [109–111].

Clinically, children with PRES typically manifest headache, encephalopathy, confusion, visual symptoms, and seizures [108, 110]. Typical MRI changes predominantly involve the white matter of territories supplied by the posterior cerebral circulation (Figure 5), and the radiological abnormalities as well as the clinical manifestations are usually reversible, once the causal factor (e.g., hypertensive crisis) returns to within normal physiological limits.

Correct and timely diagnosis of this condition in children has important therapeutic and prognostic implications because the reversibility of the clinical and radiologic abnormalities is contingent on the prompt control of the blood pressure. Although the pathophysiology of PRES is still unclear, the current most accepted theory is that severe hypertension exceeds the limits of autoregulation, leading to breakthrough brain oedema and the onset of neurological manifestations coupled with correlated radiological findings [110].

Interestingly, some authors postulated that the risk of hypertension-related PRES is higher in the paediatric population compared to the adults due the immaturity of the regulatory mechanisms that protect the brain from damage related to blood pressure elevations [112].

5.1. Genetic Disorders with Pheochromocytoma. At least 24% of pheochromocytomas and sympathetic paragangliomas have been estimated to be correlated with familial cancer syndromes and various genetic disorders [51, 52, 113]. Identification of these syndromes and disorders is therefore of prime importance for these children and their relatives.

Multiple endocrine neoplasia type 2 (MEN 2) is a distinct hereditary syndrome that has an autosomal dominant pattern of inheritance and different subtypes (e.g., MEN 2A; MEN 2B), associated with specific mutations in the proto-oncogene *RET* [52, 114, 115].

Multiple endocrine neoplasia type 2A (MEN 2A; MIM 171400) is an autosomal dominant disease characterised by parathyroid hyperplasia or adenoma, medullary carcinoma of the thyroid, and bilateral pheochromocytomas [114, 115].

Multiple endocrine neoplasia type 2B (MEN 2B; MIM 162300) is also an autosomal dominant disorder and is phenotypically characterised by the combination of pheochromocytomas, mucosal neuromas, and thickening of the optic nerves. Patients with MEN 2 B also develop medullary thyroid carcinoma (100%) and may also have intestinal ganglioneuromatosis. Although medullary thyroid cancer and pheochromocytoma account for most of the morbidity and mortality associated with MEN 2B, the nonendocrine physical findings are important in identifying at-risk individuals early in life [52]. Patients with MEN 2B often have skeletal abnormalities such as talipes equinovarus, pes cavus, dorsal scoliosis, kyphoscoliosis, and lordosis; small joint hyper laxity and chest deformities (e.g., pectus excavatum) are also common clinical findings (Figures 6(a) and 6(b)). Almost 100% of patients with MEN 2B have mucosal neuromas and abnormal dentition (Figures 6(c) and 6(d)) [52].

Von Hippel-Lindau disease (VHL; MIM 193300) is an autosomal dominant familial neoplasia syndrome that results from a germline mutation of the *VHL* gene. It is characterised

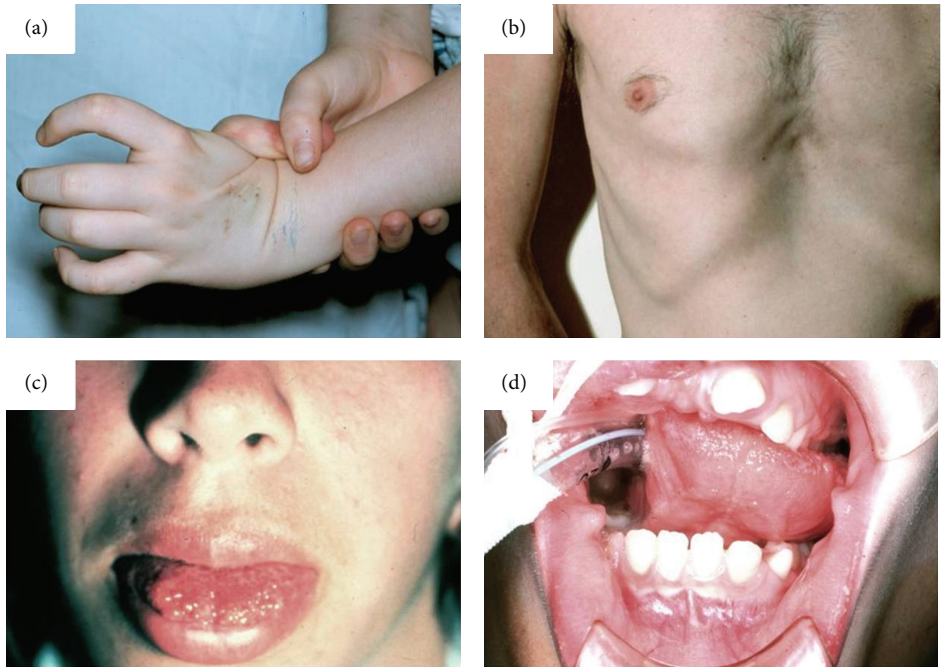


Figure 6: Clinical Features of MEN2B syndrome. Note (a) the laxity in the first metacarpal joint with complete hyperextension causing no pain; (b) typical chest deformity with pectus excavatum; (c) mucosal neuroma in the anterior tongue; (d) abnormal dentition (Reprinted with permission from [52], Copyright Springer Verlag). MEN2B = Multiple endocrine neoplasia type 2b.

by the presence of paragangliomas, pheochromocytomas, retinal angiomas, cerebellar hemangioblastomas, renal and pancreatic cysts, and renal cell carcinomas [51].

Neurofibromatosis type 1 (NF1; MIM 162200) is an autosomal dominant disease characterised by café-au-lait spots, axillary/inguinal freckling, iris hamartomas, neurofibromas, optic nerve gliomas, and skeletal abnormalities including sphenoid dysplasia, caused by mutations in the *NF1* gene [116, 117].

Pheochromocytomas are a very rare feature of NF, affecting approximately 0.1% to 6% of all patients, although a study suggested that they may be missed in some NF1 patients [118]. However, NF-1 associated pheochromocytomas occur in adults and very rarely in paediatric patients [119] and almost never in other NF-related phenotypes [120, 121].

Familial paragangliomas are a group of autosomal dominant disorders caused by mutations in the genes encoding the succinate dehydrogenase (SDH) mitochondrial complex and are characterised by paragangliomas, usually located in the head and neck [52]. These tumours are usually benign, although approximately 10% of them may undergo malignant transformation, depending on their local invasion and degree of vascularisation. The prognosis depends on the extension of the disease at the time of the diagnosis [114].

6. Conclusions

The paediatric nervous system is vulnerable to potential insults during a wide critical period, which extends from the first (embryonic) developmental phases through the entire adolescence and involves tight ontogenic regulatory and feedback activities. These activities are mediated by a number of (intrinsic and external) modulators: any perturbation of these activities/modulators could result in long-term consequences leading to (reversible or nonreversible) impairment of the neurological structure(s) and/or function.

The *adrenal hormones* are among the most sophisticated and important regulatory modulators of the cellular and surrounding environment in the developing (i.e., paediatric) nervous system. Thus, physicians should be aware not only to (the well-known) systemic manifestations but also to the frequent potential neurological and/or psychiatric abnormalities related to a wide array of adrenal diseases and to (acute or chronic) exposure to supra- (or infra-) physiological levels of corticosteroids in children so as to achieve better prevention and timely diagnosis and treatment of these disorders. In the present review we focused on the pathophysiological and clinical implications of glucocorticoid-related, mineralcorticoid-related, and catecholamine-related neurological manifestations and/or paediatric brain damage secondary to *endogenous* (i.e., Cushing syndrome, Addison's disease, congenital adrenal hyperplasia, X-linked adrenoleukodystrophy, autoimmune polyglandular syndrome type 1, congenital adrenal hypoplasia, triple A syndrome, Smith-Lemli-Opitz syndrome, Kearns-Sayre syndrome, hyperaldosteronism, pseudohyperaldosteronism and pseudohypoaldosteronism, genetic and non-genetic tumours of the adrenal cortex or medulla) or *exogenous* (e.g., replacement or acute/chronic corticosteroid therapies) exposure to low or high levels of adrenal hormones.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Diagnosis and Treatment of Adrenal Medullary Hyperplasia: Experience from 12 Cases

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Objective. To dissect the characteristics of adrenal medullary hyperplasia (AMH) and share our experience of diagnosis and treatment of AMH. *Methods*. From 1999 to 2013, 12 cases of AMH have been pathologically diagnosed after operation in our hospital. The clinical characteristics, process of diagnosis, treatment, and prognosis during follow-up of all patients are summarized retrospectively. *Results*. Four cases were trended to be AMH and 6 cases were trended to be pheochromocytoma before operation; moreover, the other two patients were diagnosed accidentally. All patients, except for the patient with mucinous tubular and spindle cell carcinoma of left kidney by open surgery, experienced a smooth laparoscopic adrenalectomy, including 2 with radical nephrectomy, 10 of which experienced unilateral adrenalectomy, 1 was bilaterally partial adrenalectomy, and the remaining one was unilaterally complete removal and then 2/3 partially contralateral excision. After a medium follow-up of 6.5 years, it demonstrated a satisfactory outcome of 8 cured patients and 4 symptomatic improved patients. *Conclusions*. AMH presents a mimicking morphology and clinical manifestation with pheochromocytoma. Surgery could be the only effective choice for the treatment of AMH and showed a preferable prognosis after a quite long follow-up.

1. Introduction

Catecholamine syndrome is a frequent factor leading to hypertension, including adrenal medullary hyperplasia (AMH) and pheochromocytoma. However, AMH is much rarer than pheochromocytoma, which is characterized by paroxysmal hypertension clinically and adrenal medullary cell mass hyperplasia pathologically [1]. Patients with AMH are frequently misdiagnosed to have pheochromocytoma as similar manifestations, laboratorial results, and radiological presentations.

We aim to analyze the detailed characteristics, share the experience of surgical treatment and prognosis of AMH, and provide some possible advices of distinguishing AMH from pheochromocytoma through a systematic review of literatures and cases in hand.

2. Materials and Methods

Twelve patients were pathologically diagnosed as AMH from January 1999 to December 2013 in our hospital. All data were searched and collected retrospectively.

Before operation, imaging examinations, including urinary color Doppler ultrasonography (US), enhanced computed tomography (CT), magnetic resonance imaging (MRI), and ^{131}I-metaiodobenzylguanidine (^{131}I-MIBG) scintigraphy, were used to confirm and identify the position and details of adrenal lesions. When specific or suspicious lesions were ascertained, adrenal hormones were routinely detected, including serous electrolytes, cortisol, aldosterone, and 24-hour catecholamine in blood and 17-hydroxy (17-OH), 17-ketones (17-KS), vanillylmandelic acid (VMA), and 24-hour catecholamine in urine. In addition, Regitine suppression test was conducted.

Table 1: Clinical characteristics of patients.

	Male number of patients	Female number of patients	Total
Total	8	4	12
Medium age (range)	47 (39–71)	33.5 (27–45)	41.5 (27–71)
Symptoms			
Hypertension	7	3	10
Headache	3	2	5
Dizziness	4	2	6
Nausea and vomiting	3	1	4
Heart anomalies	2	1	3
Operation			
Laparoscopic	7	4	11
Open	1	—	1
Lesions			
Hyperplasia only	1	3	4
Mass only	4	1	5
Hyperplasia and mass	3	0	3
Size of mass (cm)			
Medium	0.5	0.5	0.5
Range	0.2–1.5	—	0.2–1.5
Follow-up (yr)			
Medium	6.5	4.5	6.5
Range	2–14	1–9	1–14

Perioperative prescriptions, including one to four weeks of routinely oral α-adrenergic receptor blockers, phenoxybenzamine (usually from 5 mg b.i.d to maximal 30 mg t.i.d) or prazosin (usually from 1 mg q.d. to maximal 3 mg t.i.d), and 3 days of alternately transvenous use of a dose of 1000 mL crystalloid and colloidal solutions, respectively, were applied to reduce the incidence of turbulence of haemodynamics during operation. For patients with tachycardia, different doses of β2-receptor blockers, such as Betaloc, would be used till heart rate was less than 90 per minute.

After all preparations were completed, laparoscopic surgery through retroperitoneal approach would be carried out on all patients. According to results of imageology, unilateral adrenal gland would be abscised in patients with significantly one-side hyperplasia or masses, while bilaterally subtotal adrenalectomy could be taken into consideration when bilateral masses were found. Severer side could be removed in patients with bilateral hyperplasia, and if clinical manifestations could not be controlled after surgery, contralateral partial adrenalectomy would be considered. Blood pressure, laboratory studies (mainly catecholamine), and imageology examinations were routinely conducted during follow-up.

3. Results

In summary, 10 patients complained of hypertension, 1 of paroxysmal blood urine, and 1 of renal mass merging elevating blood pressure during health examination. In patients with only hypertension, 6 were persistent and 4 were paroxysmal. The patients complaining of blood urine and renal mass were diagnosed to have right nephrectomy combined with nephrolith and mucinous tubular and spindle cell carcinoma (MTSCC) of left kidney, respectively. Unilateral nephrectomy was carried out in these two patients, 1 by laparoscopic surgery and the other one by open surgery, and then AMH was accidentally diagnosed, which made all examinations focus on adrenals neglected. Therefore, all examinations mentioned were excluded for these two cases. All patients had a degree of slight to occasionally severe headache when ascending of blood pressure seizure, with a possible inducement of tension, overworked or position altering, and so forth. Detailed characteristics of these patients were summarized in Table 1.

All patients had no evident abnormalities in serous electrolytes (mainly potassium), cortisol, and aldosterone, whereas different degrees of elevating could be demonstrated in uric 17-OH, 17-KS, vanillylmandelic acid (VMA), and uric and serous catecholamine, respectively. Regitine suppressing tests were all positive.

Enlargement of unilateral adrenal and low-echoes of adrenal masses were shown in 4 cases and 3 cases, respectively, based on urinary US, and no bilateral lesions were found. According to CT scans, 4 cases of bilateral abnormalities and 6 cases of unilateral abnormalities could be found. In patients with bilateral lesions, 1 patient was diagnosed to have bilateral masses, 2 to have unilateral mass and contralateral hyperplasia, and 1 to have bilateral hyperplasia. However, in patients with unilateral lesion, both hyperplasia and mass were diagnosed in 3 cases, respectively. The sizes of masses

ranged from 0.3 to 1.2 cm, except that one adrenal mass was sized of about 3.0 cm but diagnosed as adrenocortical adenoma. For the patient with MTSCC, 1.5 cm of mass in his left adrenal was reported.

Further, 6 patients with adrenal hyperplasia had experienced a MRI scan, which showed nearly similar results with CT scan, except for 1 case of no anomaly in MRI. ^{131}I-MIBG was applied in 6 patients with hyperplasia and showed enhanced signal gathering. No ectopic gatherings were found in ^{131}I-MIBG.

In total, 4 cases were suspected to be AMH while 6 were suspected to be pheochromocytoma before operation. All patients underwent a smoothly, unilaterally, and retroperitoneally laparoscopic adrenalectomy, except for 1 with bilateral partial adrenalectomy and 2 with radical nephrectomy, without any evident hemodynamic instability during operation. Left AMH and right adrenocortical adenoma were then diagnosed in patient suffering from bilaterally partial surgery. After a routinely postoperative follow-up of 1 month, 1 patient with unilateral mass and contralateral hyperplasia complained of unsatisfactorily blood pressure (BP) controlling and received a reoperation of 2/3 left partially excision and 12 months of phasedown of hormone replacement therapy with nearly normal BP.

Pathology showed that (1) 14 adrenals from 12 patients were macroscopic hyperplasia and bodiness, of which mass could be found in 9 adrenals from 8 patients with a diameter ranging from 0.2 to 3.0 cm, (2) increased medulla could be found in all samples with a corticomedullary ratio ranging from 1 to 10, from which medulla could be found in all of adrenals, (3) consequently, 13 specimens excised were diagnosed to be AMH and four patients were diagnosed to have diffuse hyperplasia while other 8 were diagnosed to have nodal, with a diameter ranging from 0.2 to 1.5 cm, and multiple nodes were found in 1 patient, (4) cortical lesions had been merged in 3 patients, with 2 adrenocortical adenomas and 1 nonfunctional adenoma, and (5) MTSCC of kidney and ganglioneuroma were diagnosed in 1 patient, respectively.

After a medium follow-up of 6.5 (range: 1–14) years, 8 patients were cured with almost normal BP, symptoms, examinations, and studies and 4 patients obtained a significant improving of discomforts and decreasing of BP with only 2 patients who need a low dose of single antihypertensive drug. The patient receiving unilateral total and contralateral subtotal surgery acquired a compensatory hyperplasia of the remaining adrenal tissue and recovered to orthobiosis without of hypotensor taken.

4. Discussion

AMH, usually and bilaterally, is an infrequently clinicomorphologic entity which is characterized by hypertension clinically and medullary hyperplasia pathologically. It has been reported to be associated with familial or type 2 multiple endocrine neoplasia (MEN 2) syndrome and is regarded as a precursor of pheochromocytoma [2].

The etiology of AMH remains unknown, which may be caused by a synthetic of heredity, nerve system, and endocrine system. Quantities of susceptibility genes mutation, including RET, SDHB, and NF-1, have been recognized as being possible to the development of AMH to pheochromocytoma [3–6]. Multiple limitations, such as deficient cases, insufficient basic researches, and difficulties of establishing models, have made the pathology, diagnosis, and therapy of AMH indefinite and controversial for a long time [7].

Although the primary diagnosis of AMH in our study mainly has relied on imageology, including US, CT, and MRI, the effectiveness of these technologies is still under debate. Yung et al. have presented the superior diagnostic sensitivity of MIBG scans through a case without abnormality in CT and MRI [8]. Similarly, the advantages of ^{131}I-MIBG in the diagnosis of MEN 2 have been verified [9]. Combination application of MRI and MIBG could be more valuable for diagnosis of pheochromocytoma [10]. These conclusions might be similarly applied to AMH. Further, MIBG might provide a guidance of excluding ectopic lesions, which would be significant for protocols of treatment and prognosis. However, routine preoperative I-MIBG seems unnecessary for patients with pheochromocytoma [11, 12].

Additionally, after adrenal lesions were explicit or suspected, laboratory inspections of increasing of blood and urinary catecholamines could be helpful for distinguishing AMH from aldosteronism, cortical adenoma, and paragangliomas, but difficult from pheochromocytoma.

To prevent severe complications caused by AMH, surgery is still the most effective choice. Laparoscopic surgery has been prevalent for over a decade in our hospital, as quantities of advantages of no significant differences in security, smaller trauma, less blood loss and shorter hospital stay, and so forth, compared with the traditional open surgery. We would like to have an extraperitoneal approach which could exert less interference in gastrointestinal system. Similar outcomes and shorter hospital stay have been proven in retroperitoneoscopic adrenalectomy compared with traditional laparoscopic surgery [13–15]. In addition, the safeties and efficiencies of robot-assisted and laparoscopic single-site adrenalectomy compared to conventional laparoscopic surgery have demonstrated insignificant differences by systematic review and meta-analysis, respectively [16–18].

What is more, surgical techniques for adrenal neoplasms have experienced a variation from total adrenalectomy to unilateral adrenalectomy and to bilateral subtotal adrenalectomy with preservation of normal tissue [19]. Moreover, prophylactic bilateral adrenalectomy has not been advocated, as Addisonian crisis could occur in one-quarter of the patients over 10 years despite adequate corticosteroid replacement [20].

From our experience, patients with AMH should undergo a similar preoperative preparation with pheochromocytoma; then an adrenalectomy (partial or total) could be carried out at the evident side. If the symptoms could not be relieved, contralateral partial adrenalectomy should be taken into consideration. Importantly, bilaterally partial adrenalectomy should not to be neglected for patients with bilateral nodes. Bilateral adrenalectomy should be the final choice to avoid lifelong hormone replacements and complications of cortisol taking in. In our study, one patient received bilaterally

subtotal adrenalectomy simultaneously, while another received 2/3 of left adrenalectomy at his one-month follow-up after right adrenalectomy. Both gained a quite satisfactory prognosis except one with 12 months of phasedown of replacement therapy.

Though macroscopic differences between pheochromocytoma and AMH seem inconspicuous, slight distinction could be detected. Valdés et al. reviewed literatures and tried to summarize the main differences of clinical and pathological features between pheochromocytoma and AMH [21]. However, small lesions could be hard to be distinguished macroscopically according to this principle. Additionally, some believed that pheochromocytoma, mostly unilateral, is a hypostatic neoplasm which has integral capsule, while adrenal medulla out of neoplasm would be normal or at least not be atrophic though been extruded; however, AMH usually to be bilateral with diffuse or small nodular alterations in medulla as well as contrast presentations in capsule and invasion of entity compared with pheochromocytoma.

Above all, pathology is still the only method to confirm AMH. Pitifully, uniform standards concentrating on pathological diagnosis are lacking. In our opinion, the main criteria about pathological diagnosis of AMH are decreasing corticomedullary ratio with a quotient less than 10 combined with elevating weight of adrenal medullary; medullary hyperplasia is a proliferation of cells containing normal cellular architecture as opposed to the nests of cytologically atypical polygonal cells which characterize pheochromocytoma [22]; medullary could be found in alar part or tail of adrenal with or without nodular hyperplasia and polymorphic cells could exist; furthermore, medullary hyperplasia with small nodes without capsule or only medullary hyperplasia without neoplasm could be also one reference. However, pheochromocytoma but AMH should be diagnosed, no matter the size of entity when capsule or tumor cells larger than normal pheochromocytes microscopically appear. Meanwhile, medullary that appears in tail of adrenal could not be the evidence of AMH. Equally, AMH could not be excluded without medullary in tail.

Our study trended that AMH would be a benign lesion with mild biological behavior. Similar conclusion was presented by Lenders et al. [23]. However, there is still 30%–50% risk to develop contralateral tumors after unilateral adrenalectomy in MEN 2 within 10 years [20]. According to our study, all patients gained preferable efficacy in a mean follow-up of 6.5 years, with no recurrence or malignant transformation.

5. Conclusion

AMH is a rare disease which presents a mimicking morphology and clinical manifestation with pheochromocytoma. Combination of medical history, imageology, and laboratory tests could only provide a tendency in its diagnosis, though MIBG might have a superior advantage. Though surgery could be the only effective choice, surgical modes should be evaluated thoroughly. Preferable prognosis after a quite long follow-up was present.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Lu Yang and Liang Gao contributed equally to this work and share the first authorship.

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Clinical Study

The Optimal Approach for Laparoscopic Adrenalectomy through Mono Port regarding Left or Right Sides: A Comparative Study

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Introduction. Several studies have shown the feasibility and safety of both transperitoneal and posterior retroperitoneal approaches for single incision laparoscopic adrenalectomy, but none have compared the outcomes according to the left- or right-sided location of the adrenal glands. *Materials and Methods.* From 2009 to 2013, 89 patients who received LAMP (laparoscopic adrenalectomy through mono port) were analyzed. The surgical outcomes attained using the transperitoneal approach (TPA) and posterior retroperitoneal approach (PRA) were analyzed and compared. *Results and Discussion.* On the right side, no significant differences were found between the LAMP-TPA and LAMP-PRA groups in terms of patient characteristics and clinicopathological data. However, outcomes differed in which LAMP-PRA group had a statistically significant shorter mean operative time (84.13 ± 41.47 min versus 116.84 ± 33.17 min; $P = 0.038$), time of first oral intake (1.00 ± 0.00 days versus 1.21 ± 0.42 days; $P = 0.042$), and length of hospitalization (2.17 ± 0.389 days versus 3.68 ± 1.38 days; $P \leq 0.001$), whereas in left-sided adrenalectomies LAMP-TPA had a statistically significant shorter mean operative time (83.85 ± 27.72 min versus 110.95 ± 29.31 min; $P = 0.002$). *Conclusions.* We report that LAMP-PRA is more appropriate for right-sided laparoscopic adrenalectomies due to anatomical characteristics and better surgical outcomes. For left-sided laparoscopic adrenalectomies, however, we propose LAMP-TPA as a more suitable method.

1. Introduction

Since the report of the first pioneering experiences [1], laparoscopic adrenalectomy has become the gold standard for the treatment of benign adrenal tumors. Owing to further development in surgical techniques and instruments, as well as a general increase of interest toward minimally invasive procedures in the past decades, a single incision approach was developed [2] and has been applied to conventional laparoscopic adrenalectomy.

A single incision approach has many advantages compared to conventional procedures, including improved cosmesis, reduced postoperative pain, and quicker convalescence [3]. However, single incision adrenalectomy necessitates more advanced techniques compared with multiport laparoscopic surgery [4, 5]; therefore careful selection of an adequate surgical approach is the key to a successful operation.

The aim of this study was to describe surgical techniques, to analyze the outcomes, and to provide insight on the optimal choice of surgical approach for each individual patient receiving laparoscopic adrenalectomy through mono port (LAMP).

2. Materials and Methods

2.1. Patients. From March 2009 to November 2013, 107 patients underwent laparoscopic adrenalectomy in our institute. Among them, 18 patients received conventional laparoscopic adrenalectomy, respectively. The remaining 89 patients, who received laparoscopic adrenalectomy through mono port (LAMP), were included in our study. All operations were performed by a single surgeon.

All patients received LAMP with either the transperitoneal approach or the posterior retroperitoneal approach, regardless of side. Initially, the surgical approach was chosen according to the tumor size and body habitus of the patient. For patients with tumors larger than 4 cm, BMI over 30 kg/m^2, or with thick subcutaneous back tissue (thickness

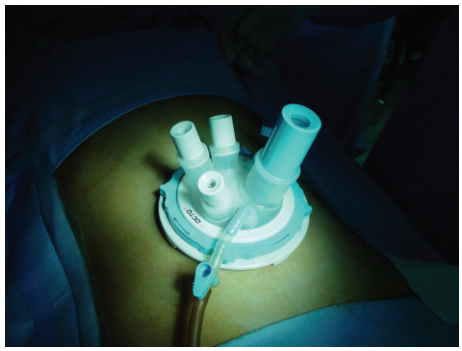

Figure 1: OCTO port.

over 4 cm between the skin and Gerota's fascia), the transperitoneal approach was indicated. We selected patients with tumors less than 4 cm, BMI under 30 kg/m^2, or with previous abdominal operations for the posterior retroperitoneal approach. However, after allocating patients to each approach according to the selection criteria for 33 cases, due to the small number of patients with tumors larger than 4 cm ($N = 7$), patients with BMI over 30 kg/m^2 ($N = 1$), or patients with abundant retroperitoneal fat ($N = 1$), an imbalance occurred between groups, after which patients were randomly selected to receive each surgical approach, except for patients with tumors over 7 cm ($N = 3$), who received LAMP using the transperitoneal approach.

The surgical outcomes attained using the transperitoneal and posterior retroperitoneal approaches according to the right and left sides were analyzed and compared with respect to tumor size, operation time, time to first oral intake, postoperative hospitalization time, estimated blood loss, and postoperative complications. Statistical analysis was performed using SPSS ver. 18.0, and P values of <0.05 were considered statistically significant.

2.2. Operation Methods

2.2.1. Laparoscopic Adrenalectomy through Mono Port Using the Transperitoneal Approach (LAMP-TPA). Under general anesthesia, the patient was positioned in the lateral (right or left) decubitus position. The operation table was flexed just above the level of the iliac crest to maximally widen the space between the iliac crest and the costal margin for the best possible port access. After padding all pressure points (shoulders, elbows, hips, ankles, etc.) to prevent nerve injury and pressure sores, a bean bag placed under the patient before surgery was made firm to securely position the patient on the operation table.

Using preoperative abdominal CT scans, we measured the length from the adrenal tumor to the umbilicus, and with this measurement as a landmark, the selection of the optimal incision location was made. Usually, a single 3 cm incision is made parallel to the lower costal margin, two fingerbreadths below the costal margin in the midclavicular line. After opening the peritoneum, an OCTO port (Dalim Surgnet, Korea) (Figure 1) was inserted into the access site, and pneumoperitoneum to 15 mmHg was established using carbon dioxide insufflation.

For the left adrenal gland, the splenic flexure of the colon and the spleen were mobilized and drawn anteromedially to expose the retroperitoneum. For the right-sided adrenalectomy, after the right lobe of the liver was mobilized, it was retracted superiomedially with a snake retractor to expose the adrenal gland and the inferior vena cava. After exposing the adrenal gland, the feeding vessels were ligated using a Ligasure (Covidien, Mansfield, MA, USA). The central vein of the adrenal gland was usually ligated with conventional laparoscopic clips. After adequate hemostasis, the specimen was retrieved using a retrieval pouch.

2.2.2. Laparoscopic Adrenalectomy through Mono Port Using the Posterior Retroperitoneal Approach (LAMP-PRA). Under general anesthesia, the patient was placed prone in the jackknife position, and the operation table was flexed to maximize exposure of the posterior retroperitoneal space from the subcostal margin to the iliac crest. A 3 cm sized transverse skin incision was made one fingerbreadth below the lowest tip of the 12th rib. The fascia was incised, and the external oblique, internal oblique, and transversalis muscles were bluntly divided to expose the retroperitoneal space. After minimal working space in the retroperitoneum was made for port insertion, the OCTO port was inserted and CO_2 was insufflated to 15 mmHg for pneumoretroperitoneum. After creating adequate working space, Gerota's fascia was opened, and the kidney upper pole was mobilized to expose the adrenal gland. After dissecting the adrenal gland from the surrounding tissue, the adrenal central vein was identified and ligated. Finally, the adrenal gland was placed in a pouch and retrieved.

3. Results

From March 2009 to November 2013, 89 patients underwent LAMP. Forty-seven patients received surgery for left-sided adrenal tumors and 42 patients for right-sided tumors. In cases of left-sided adrenalectomy, the transperitoneal and posterior retroperitoneal approaches were performed in 26 and 21 patients, and on the left, 19 and 23 patients received surgery using each approach, respectively. Table 1 shows the clinical and pathological patient characteristics of all patient groups.

In cases of left-sided adrenalectomy, there were no statistical differences regarding patient factors in both groups. In pathologic diagnosis, one patient in the LAMP-TPA group was diagnosed with metastatic adenocarcinoma and one patient in the LAMP-PRA group with metastatic hepatocellular carcinoma.

With regard to adrenalectomies performed on the right side, no significant differences were found between the two groups except for patient age (TPA 40.79 ± 5.53 years versus PRA 50.22 ± 11.61 years; $P = 0.002$).

The mean tumor sizes of the left-sided groups were similar (3.28 ± 1.72 cm versus 3.45 ± 2.34 cm; $P = 0.779$) (Table 2). However, the LAMP-TPA group had a shorter

Table 1: Patient characteristics.

	Left ($N = 47$)		P value	Right ($N = 42$)		P value
	LAMP-TPA	LAMP-PRA		LAMP-TPA	LAMP-PRA	
Patients (N)	26	21		19	23	
Sex (N)			0.326			0.327
Male	11	9		15	15	
Female	15	12		4	8	
Age (years)	48.35 ± 12.26	53.38 ± 11.55	0.158	40.79 ± 5.53	50.22 ± 11.61	0.002
BMI (kg/m^2)	23.95 ± 3.35	27.28 ± 8.20	0.093	25.93 ± 2.71	25.31 ± 2.99	0.489
Diagnosis			0.936			0.509
Primary aldosteronism	6	3		3	5	
Cushing's syndrome	12	10		4	8	
Pheochromocytoma	3	4		0	0	
Nonfunctioning tumor	4	3		12	10	
Others	1	1		0	0	

LAMP-TPA: laparoscopic adrenalectomy through mono port using the transperitoneal approach, LAMP-PRA: laparoscopic adrenalectomy through mono port using the posterior retroperitoneal approach.

Table 2: Outcomes after surgery.

	Left ($N = 47$)		P value	Right ($N = 42$)		P value
	LAMP-TPA ($N = 26$)	LAMP-PRA ($N = 21$)		LAMP-TPA ($N = 19$)	LAMP-PRA ($N = 23$)	
Tumor size (cm)	3.28 ± 1.72	3.45 ± 2.34	0.779	2.72 ± 1.57	2.61 ± 1.19	0.811
Operative time (min)	83.85 ± 27.72	110.95 ± 29.31	0.002	116.84 ± 33.17	84.13 ± 41.47	0.008
Time to first oral intake (days)	1.81 ± 0.49	1.10 ± 0.30	<0.001	1.21 ± 0.42	1.00 ± 0.00	0.042
Length of hospitalization (days)	3.62 ± 1.02	3.57 ± 0.75	0.870	3.68 ± 1.38	2.17 ± 0.39	<0.001
Estimated blood loss (mL)	26.15 ± 13.21	25.72 ± 12.53	0.743	24.52 ± 7.48	23.28 ± 11.74	0.240
Complications	1	0		0	0	

LAMP-TPA: laparoscopic adrenalectomy through mono port using the transperitoneal approach, LAMP-PRA: laparoscopic adrenalectomy through mono port using the posterior retroperitoneal approach.

mean operative time than the LAMP-PRA group, which was shown to be statistically significant (83.85 ± 27.72 min versus 110.95 ± 29.31 min; $P = 0.002$). In contrast, the average time to first oral intake was shorter in the LAMP-PRA group (1.10 ± 0.30 days versus 1.81 ± 0.49 days; $P \leq 0.001$), but there were no differences in hospitalization time (3.62 ± 1.02 days versus 3.57 ± 0.75 days; $P = 0.870$) and estimated blood loss (26.15 ± 13.21 mL versus 25.72 ± 12.53; $P = 0.743$) between both groups.

In right-sided adrenalectomies, the mean tumor sizes were 2.72 ± 1.57 cm in the LAMP-TPA group and 2.61 ± 1.19 cm in the LAMP-PRA group ($P = 0.811$), respectively (Table 2). The LAMP-PRA group had a significantly shorter mean operative time (84.13 ± 41.47 min versus 116.84 ± 33.17 min; $P = 0.008$). Also, the average time to first oral intake (1.00 ± 0.00 days versus 1.21 ± 0.42 days; $P = 0.042$) and hospitalization time (2.17 ± 0.39 days versus 3.68 ± 1.38 days; $P \leq 0.001$) were shorter in the LAMP-PRA group. Both groups had similar estimated blood losses (23.28 ± 11.74 mL versus 24.52 ± 7.48 mL; $P = 0.240$).

Regarding complications, one patient in the left LAMP-TPA suffered from postoperative ileus and required longer hospitalization, but no additional postoperative complications were reported in the other groups.

4. Discussion

In the search for better patient outcomes, surgery for benign adrenal diseases has gradually changed from the conventional open method to the less invasive methods of laparoscopic adrenalectomy. The laparoscopic approach offers several advantages over open adrenalectomy, such as reduced blood loss, fewer complications, less postoperative pain, and a shorter period of hospital stay [6–8].

To further enhance these advantages, less invasive methods have been introduced, leading to the development of the single incision laparoscopic adrenalectomy [2].

Several studies have shown the feasibility and safety of both transperitoneal and posterior retroperitoneal approaches in single incision laparoscopic adrenalectomy [4, 9–13], but none have compared the outcomes according to the left or right side. Our results show that there may be a difference in surgical results depending on the approach

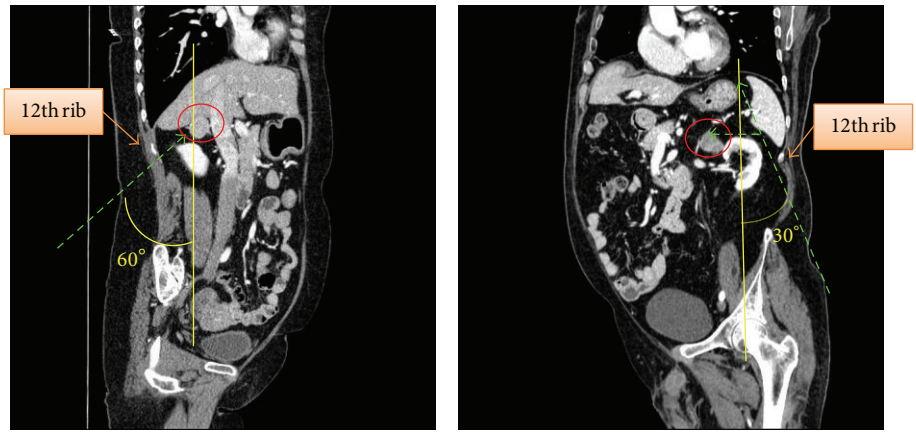

Figure 2: Comparison of right and left-sided LAMP-PRA.

selected according to which side the surgery is performed. We propose that the reason for this disparity is due to the anatomical location and characteristics of the adrenal glands.

The left kidney is located approximately between the vertebral level T12 to L3, and due to the asymmetry within the abdominal cavity caused by the liver, the right kidney is slightly lower than the left [14]. Also, the left kidney is typically slightly larger than the right [15]. Both adrenal glands are located superior to the kidneys. The right adrenal gland is pyramidal in shape and lies at the apex of the right kidney. The left adrenal gland has a semilunar shape and lies on the superomedial or anterosuperomedial aspect of the left kidney. On the right side, the liver is located in front of the right adrenal gland, making the anterior margin, which is just separated by the parietal peritoneum. On the left, the spleen with the pancreatic tail makes up the anterior border to the left adrenal gland.

Due to this anatomical presentation, in the posterior retroperitoneal approach, the right adrenal gland is closer with respect to the subcostal margin, and because of its location just superior to the apex of the right kidney, visualization of the adrenal gland is more feasible on the right compared to the left (Figure 2). Also, by using this approach, the right adrenal gland can be directly accessed without mobilization of any intra-abdominal viscera, typically the liver, which can be time consuming and prone to complications.

According to our data, on the right side, the PRA showed statistical significant improvement in operation time compared to the TPA and also demonstrated better postoperative results.

As for the left adrenal gland, however, the retroperitoneal approach can be somewhat challenging. In single incision laparoscopic adrenalectomy, the incision site is limited to the inferior border of the thoracic cage, and it cannot be made directly above the adrenal glands. Thus, in general, the location of the gland is relatively more further from the incision site on the left side. Also, the field of vision and working space are usually narrow [13], and the range of movement is limited by the incision site itself. Due to this difference in surgical depth, a more acute angle is needed to manipulate the gland on the left (Figure 2). Furthermore, direct visualization of the adrenal gland is usually obscured by the apex of the left kidney, so access can only be achieved by excessive retraction of the left kidney during surgery. On the other hand, the TPA, due to the relative familiarity of surgical anatomy to most surgeons, provides a more feasible access to the adrenal gland, even though it requires dissection of the spleen, pancreas tail and splenic flexure of the colon.

According to our data, the operative time was significantly shorter in the LAMP-TPA group on the left side. Postoperative outcomes were similar, although, the time to first oral intake was shorter in the LAMP-PRA group. This was probably due to CO_2 gas insufflation and manipulation of intra-abdominal organs (colon, spleen, pancreas, etc.) which resulted in delayed peristalsis.

There have been some negative views regarding the need for a single incision approach in laparoscopic adrenalectomy, since the conventional approach is feasible and has good outcomes [6–8]. But, in order to evacuate the specimen after conventional laparoscopic adrenalectomy, an additional incision or elongation of the previous incision is needed, which could result in longer operation times and disfigurement of the postoperative wound. After initial trocar insertion in LAMP, however, no further disruption of the wound is needed, resulting in excellent cosmesis (Figure 3). Furthermore, direct visualization of the abdominal layers during opening of the wound as well as identification of the underlying structures during placement of the trocar is possible, which could reduce trocar-related complications. As seen in this study, no wound related complications were reported in all groups, which supports this finding.

5. Conclusion

In conclusion, we report that the LAMP-PRA is a more adequate approach for right-sided single incision laparoscopic adrenalectomies. Although the anterior approach may offer greater familiarity of the anatomy to the surgeon, visibility of

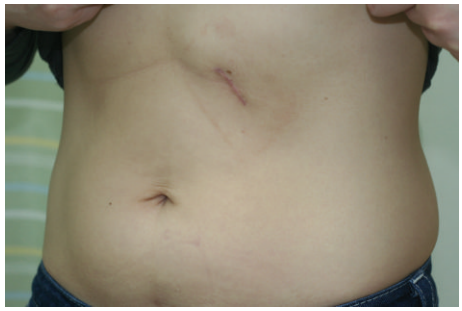

Figure 3: Postoperative scar of left-sided LAMP-TPA.

the right adrenal gland is solely achieved through retraction of the liver, which can be avoided with a posterior approach.

For left-sided adrenalectomies, however, we propose that the LAMP-TPA is more suitable. The anatomical location of the left adrenal gland hinders feasible access through the posterior approach. In contrast, the transperitoneal approach provides easier manipulation of the adrenal gland, which could result in better results for the patient.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Tuberculosis of the Adrenal Gland: A Case Report and Review of the Literature of Infections of the Adrenal Gland

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Infections of the adrenal glands remain an important cause of adrenal insufficiency, especially in the developing world. Indeed, when Thomas Addison first described the condition that now bears his name over 150 years ago, the vast majority of cases were attributable to tuberculosis. Here we describe a classic, but relatively uncommon, presentation in the United States of adrenal insufficiency followed by a review of the current literature pertaining to adrenal infections.

1. Introduction

A 46-year-old man presented to his physician with a 3-month history of generalized weakness and 15-pound unintentional weight loss. He also reported mild dyspnea on exertion and decreased appetite. His past medical history was significant for hypertriglyceridemia, primary hypothyroidism, and vitamin D deficiency. He had emigrated from the Philippines 6 years prior and had been working as a nurse at a skilled nursing facility. He had not left the country since his initial arrival. He denied sick contacts, specifically exposure to tuberculosis, smoking, alcohol consumption, or the use of illicit substances. A tuberculin skin test performed in 2007 resulted in induration (diameter unknown) and it was attributed to prior BCG vaccine. There was no evidence of pulmonary tuberculosis on a chest radiograph. Physical examination revealed abdominal distension and free fluid but was otherwise unremarkable. A diagnostic paracentesis revealed an exudative effusion with a positive Ziehl Neelsen stain for acid fast bacilli. The patient was started on treatment (Isoniazid, rifampicin, pyrazinamide, and ethambutol) for presumed extrapulmonary tuberculosis which was later confirmed by culture.

One month after starting antitubercular therapy he presented to the hospital with worsening fatigue, salt craving, vomiting, loss of libido, and erectile dysfunction. On examination, he had low blood pressure and appeared cachectic. In addition he had bitemporal muscle wasting and hyperpigmentation of skin, oral mucosa, and nails. Laboratory evaluation was significant for hyponatremia, hyperkalemia, and mild hypercalcemia. A random cortisol was 2.5 mcg/dL with an ACTH of 531.2 pcg/mL. The basal and cosyntropin stimulated serum cortisol were, respectively 1.8 mcg/dL and 2.0 mcg/dL, which was consistent with the diagnosis of primary adrenal insufficiency most likely due to tuberculosis. A computed tomography scan of the abdomen with intravenous contrast revealed bilaterally enlarged adrenal glands (4 cm × 3.3 cm on the right, 2.3 cm × 2.1 cm on the left) (Figure 1). On review of his prior CT scan of the abdomen, the patient had bilaterally enlarged adrenal glands at the time of his initial presentation as well.

With the background of tuberculosis and acute adrenal insufficiency diagnosed by laboratory test, bilateral enlargement of adrenal glands was considered most consistent with tuberculosis in our patient. Deterioration of his clinical status following antitubercular treatment could be attributed to accelerated cortisol metabolism by induction of CYP 3A4 by rifampicin. He was initially treated with intravenous hydrocortisone and was subsequently discharged on hydrocortisone and fludrocortisone. His symptoms have improved significantly. However, he is requiring slightly higher dose of hydrocortisone, which could be due to CYP 3A4 induction by rifampicin. He is likely to require lifelong treatment for adrenal insufficiency. A study that looked at tuberculosis

Table 1: Salient features of various adrenal infections.

Organism	Imaging findings	Comments
Mycobacterium tuberculosis	Bilateral adrenal enlargement (active infection). Atrophy and calcification in remote infection	Adrenal enlargement improves with treatment; adrenal insufficiency does not. Steroid dose should be increased if on rifampin
HIV	Depends on the etiology (multiple OIs can involve the adrenals)	Adrenal insufficiency due to viral, fungal, mycobacterial infiltration. "Pseudo-Cushing's" due to antiretroviral drugs and impaired cortisol metabolism
Histoplasma capsulatum	Bilateral adrenal enlargement	Nearly 50% have adrenal involvement
Paracoccidioides	Bilateral adrenal enlargement	Endemic in South America. Adrenal insufficiency does not always improve with treatment of the infection
Blastomyces dermatitidis	Bilateral adrenal enlargement	Similar to paracoccidioidomycosis, overt adrenal insufficiency is less common
Human cytomegalovirus infection	Variable	One of the most common adrenal infections in patients with AIDS. Insufficiency can manifest even when the patient is on glucocorticoid replacement
Bacterial sepsis	Adrenal hemorrhage	A number of bacteria are associated with the Waterhouse-Friderichsen syndrome. Most commonly seen when encapsulated organisms cause overwhelming sepsis
Echinococcus sp.	Adrenal cysts	Causes 6-7% of all adrenal cysts. Treatment is with surgery and albendazole
Trypanosoma sp.	Variable	Adrenals may be the reservoir for *T.cruzi* while *T.brucei* (African sleeping sickness) causes mixed central/peripheral adrenal insufficiency

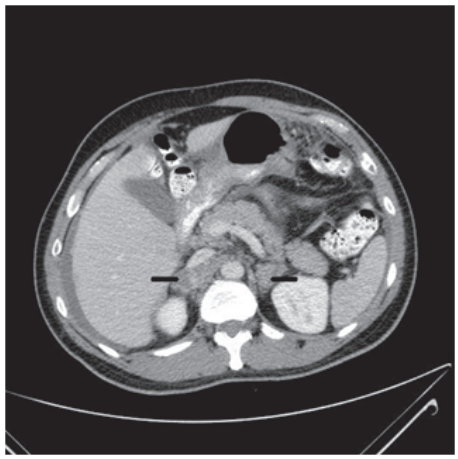

Figure 1: CT scan of the abdomen and pelvis with oral and intravenous contrast showing bilateral adrenal enlargement (black arrows).

patients with bilaterally enlarged adrenal glands found that treatment with antituberculosis drugs does not improve or help recover adrenal functionality [1]. Adrenal biopsy was not performed because the presentation was strongly suggestive of adrenal tuberculosis with active extra-adrenal tuberculosis.

Comment. It is to be noted that BCG vaccine received at birth has no impact on PPD test result 10 years later [2]. The presumption made by the other hospital that this patient's positive TST is secondary to a vaccination at birth was incorrect. Positive PPD in this patient should have prompted further investigations.

2. Background

It is interesting to note that Thomas Addison was in fact seeking an anatomic basis for pernicious anemia rather than the biochemical effects of adrenal insufficiency when he published his seminal paper on the subject. The eleven patients he described in his report all had tuberculosis of the adrenal glands [3]. This consumption has since receded to the background of ailments that afflict the Western world and today is generally considered a disease of immigrants from endemic areas, the immunocompromised or the destitute. In the developing world, however, tuberculosis continues to account for about 20–30% of cases of Addison's disease [4].

The clinical presentation of primary adrenal insufficiency is protean, and an underlying infectious etiology can further obscure the manifestations. The most frequent manifestations are weakness, fatigue, anorexia, weight loss, nausea, vomiting, hypotension, and skin hyperpigmentation (present in 60–100% of patients) [5, 6]. Understandably any of the above symptoms or signs could be easily missed or attributed to the primary infectious process itself.

In the developed world, about 10% of cases of Addison's disease have an infectious etiology; however there are few data available regarding the frequency of organisms that cause clinical adrenal insufficiency. HIV/AIDS and opportunistic infections like cytomegalovirus are the most commonly cited causes following tuberculosis. Various fungi like Cryptococcus, Histoplasma, Coccidioides, Paracoccidioides are also described to involve the adrenal glands in several case reports (Table 1).

3. Tuberculosis

Mycobacterium tuberculosis complex spreads to the adrenal glands hematogenously. Clinical manifestations may take

years to become apparent, and asymptomatic infection is not uncommon. Adrenal involvement was found in 6% of patients with active tuberculosis in an autopsy series [7]. More than 90% of the gland must be destroyed before insufficiency appears [8]. The widespread use of computed tomography has improved our understanding of the patterns of involvement of the adrenal gland in tuberculosis. The majority of patients with active or recently acquired disease (<2 years) have bilateral adrenal enlargement, while calcification and atrophy are the norm with more remote infection or inactive disease [8, 9].

That the adrenals can be enlarged in patients with pulmonary tuberculosis without active involvement of the glands has been demonstrated in various studies. Stress and inflammation could be potential reasons. The activity of the hypothalamic pituitary axis (HPA) has been the subject of numerous studies. The lack of a uniform definition of a "normal cortisol response" to ACTH stimulation has perhaps contributed to some of the heterogeneity in the results. Keleştimur et al. studied the HPA axis in 27 patients with active pulmonary tuberculosis. They also compared responses to 1 mcg and 250 mcg ACTH stimulation. Cortisol responses were consistently higher in the cases when compared to controls [10]. A more recent study by Laway et al. found significantly lower basal and stimulated cortisol levels in active pulmonary tuberculosis when compared to controls, as well as enlarged adrenal glands. None of the patients had clinical adrenal insufficiency. Both of these findings improved after successful antituberculous treatment [4]. However, the absence of serum albumin levels and the lack of adrenal biopsies in the cases limit the interpretation of the results. Other studies have also demonstrated a reduction in adrenal gland size after successful treatment of pulmonary tuberculosis [11].

When tuberculosis results in overt adrenal insufficiency, antituberculous chemotherapy does not appear to restore function. One must also be cognizant of the effect of rifampin, a potent hepatic enzyme inducer on the metabolism of glucocorticoids. Failure to increase the dose of steroid replacement therapy may result in the development of adrenal crisis [1]. Adrenal biopsy is not necessary for primary adrenal insufficiency with bilateral adrenal enlargement in a patient with proven extra-adrenal tuberculosis. However, about 12% of patients with adrenal tuberculosis have no evidence of active extra-adrenal tuberculosis [12]. Adrenal biopsy is generally necessary in these patients to prove adrenal involvement by tuberculosis.

4. HIV Infection

HIV infection affects the adrenal gland in multiple ways. Apart from direct infection, opportunistic infections and antiretroviral medications also have a significant effect on the adrenal glands.

Adrenal insufficiency is prevalent in 17% of patients admitted with AIDS [13]. Due to its high prevalence, recommendations have been made to screen for adrenal insufficiency in HIV patients with symptoms [14]. Most common causes of adrenal insufficiency are infections like CMV, *Mycobacterium tuberculosis* and MAI, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Pneumocystis jirovecii*, and *Toxoplasma gondii*, neoplastic diseases (Kaposi's sarcoma and lymphoma), and bilateral adrenal hemorrhage [15, 16]. Few drugs used for the treatment of HIV infection (protease inhibitors) and drugs used to treat opportunistic infections like rifampicin, ketoconazole, and cotrimoxazole may exacerbate manifestations of primary adrenal insufficiency [17, 18]. Studies have shown decreased level of cortisol, adrenal androgens, and mineralocorticoids in patients infected with HIV [19].

HIV infection can also lead to secondary adrenal insufficiency in advanced stages of the disease by decreasing pituitary and adrenal responses to CRH [20]. Opportunistic infections like CMV, *Mycobacterium tuberculosis*, *Toxoplasma gondii*, *Cryptococcus neoformans*, and *Pneumocystis jirovecii* can also infiltrate the pituitary leading to multiple endocrinopathies.

Treatment of adrenal insufficiency in HIV infection includes hydrocortisone and fludrocortisone (if there is evidence of mineralocorticoid insufficiency).

There are cases of Cushing's like phenotype in patients treated with antiretroviral drugs (protease inhibitors and NNRTIs) often referred to as "pseudo-Cushing's" [21]. A normal cortisol response to the dexamethasone suppression test differentiates pseudo-Cushing's from Cushing's syndrome. Studies have also shown elevated levels of basal plasma cortisol in untreated HIV patients when compared to healthy individuals. The postulated mechanisms include stress due to HIV infection, increased cytokines resulting in stimulation of HPA axis, and reduction in cortisol catabolism [22].

Adrenal tumors found almost exclusively in HIV patients include Kaposi's sarcoma which is secondary to coinfection with the oncogenic human herpes virus type 8 (HHV8) and non-Hodgkin's lymphoma (high-grade malignant B phenotype) which could be secondary to Epstein-Barr virus (EBV) [23, 24].

5. Human Cytomegalovirus Infection

Human cytomegalovirus (HCMV) has been frequently identified as a cause of adrenal insufficiency, especially in patients with HIV/AIDS. The virus has been shown by Trevisan et al. to infect normal human adrenocortical cells and induce cytopathic changes [25]. It also acts as an inducer of steroidogenesis which may explain the discordance between the high rates of CMV adrenalitis in immune suppressed patients in autopsy studies and the relatively rare diagnosis of adrenal insufficiency ante mortem. The virus causes the greatest damage at the cortex-medulla junction [26]. While adrenalitis may be the sole manifestation, the disease is usually disseminated. There have been case reports of exacerbation of CMV infection in patients of adrenal insufficiency after starting glucocorticoids most likely due to the immunosuppressive effect [27, 28].

6. *Histoplasma capsulatum*

Adrenal involvement is typically seen with disseminated chronic histoplasmosis. In one report, primary adrenal

insufficiency occurred in 41.3% of adrenal histoplasmosis cases [29]. Histoplasmosis often coexists with HIV-AIDS and is more commonly seen in the immunocompromised, posttransplant and elderly populations [30]. Histoplasmosis presents with similar constellation of clinical features as tuberculosis and is often missed. Furthermore, it also shares pathological characteristics of necrotizing granulomas and caseous necrosis with tuberculosis. Adrenal glands have bilaterally enlarged radiographic appearance and CT guided biopsy often confirms the diagnosis of adrenal histoplasmosis [31, 32]. In addition to tuberculosis, it can also be often mistaken for a lymphoma, underlining the importance of biopsy in these cases. Management includes treatment with amphotericin B followed by itraconazole (for disseminated disease) and replacement of glucocorticoid and mineralocorticoid if there is evidence of adrenal insufficiency.

7. Paracoccidioidomycosis

Paracoccidioides, a thermal dimorphic fungus, causes infection through the inhalation of infectious conidia. It is endemic in several South American countries. Two species, *P. braziliensis* and *P. lutzi,* are pathogenic in humans. While the acute form usually appears as progressive lymphadenopathy, the chronic form affects the skin, lungs, mucous membranes, and the adrenal glands [33]. Adrenal insufficiency occurs in a large number of patients (2.9%–48.2%) and has even been reported as the initial presenting feature [34, 35]. Autopsy series have demonstrated adrenal involvement in 85–90% of cases [35]. Clinical manifestations range from the asymptomatic to frank Addisonian crisis. This correlates with the extent of granulomatous involvement of the adrenal glands. Similar to tuberculosis, adrenal insufficiency typically persists even after treatment of the infection [35].

8. Other Causes of Adrenal Infections

8.1. Viruses. Many of the herpes viruses infect the adrenal gland including herpes simplex virus types 1 and 2, Epstein-Barr virus, and HCMV. This occurs usually in the setting of disseminated disease and may appear as adrenalitis. Disseminated HSV type 1 and 2 infections in neonates can be fulminant [36]. Murine models suggest the possibility of the adrenals being the initial seat of multiplication of these viruses [37].

EBV, HCMV, and Polyoma BK virus have been identified in resected adrenocortical tumors; the former has also been associated with lymphoma of the adrenal gland [38, 39].

Other commonly occurring viruses that can infect the adrenal glands include echoviruses which can lead to adrenal hemorrhage and necrosis in neonates [40].

The hemorrhagic fever viruses, although rare, can cause devastating damage to the adrenal glands. The Ebola virus, a filovirus, has been shown to cause liquefaction of the adrenals [41]. Other filoviruses and arenaviruses can also damage the adrenals by direct infection [42].

8.2. Fungi. Adrenal cryptococcosis occurs in disseminated cryptococcal infection, usually in the immunocompromised; however, there are case reports of adrenal cryptococcosis in healthy individuals as well. Pneumonia and meningitis are the most common presentations [43]. Like any other fungal infection, the adrenal glands are enlarged on CT scan and the diagnosis is confirmed by a CT guided biopsy [44]. Cryptococcal antigen titers are invariably high and can be used as a biomarker for disease resolution on follow-up [45]. A 6-month course of fluconazole appears to be effective [46]. In contrast to tuberculosis and histoplasmosis, adrenal insufficiency is often improved with resolution of the disease. Adrenal cryptococcal infection resistant to antifungal therapy may respond to adrenalectomy [47].

Pneumocystis jirovecii is an infrequent cause of adrenal insufficiency even in patients with defective cell mediated immunity such as patients with HIV/AIDS. However, it has been known to cause fatal adrenal crisis in the apparently immunocompetent host as well [48].

Blastomyces dermatitidis is a thermal dimorphic fungus that is the North American counterpart of Paracoccidioides in terms of its pathogenesis and propensity for establishing chronic systemic infection. Although pulmonary infection is the most common presentation, it frequently affects the skin, bones, adrenal glands, and the genitourinary system. Almost any organ system may be involved. It appears that subclinical infection of the adrenal glands is more common than overt insufficiency [49]. About 10% of cases in autopsy series revealed adrenal gland infection [50]. Amphotericin B and itraconazole are the drugs of choice in disseminated disease.

8.3. Bacteria. Atypical mycobacteria have been isolated from adrenal glands in patients with HIV/AIDS, however, given the multiple etiologies for adrenal insufficiency and frequent coinfection with other organisms that are known to cause destruction of the adrenal glands; however, it is difficult to establish a causal relationship [15].

The Waterhouse-Friedrichsen syndrome deserves special mention. It is a form of acute adrenal insufficiency that occurs in the setting of bacterial sepsis resulting in adrenal hemorrhage. A number of bacteria are associated with this entity including *N. meningitidis*, *H. influenza*, pneumococcus, *P. multocida*, *K. oxytoca*, *S. aureus*, *Capnocytophaga canimorsus*, Ewingella, and group A streptococcal infections. The organisms are rarely isolated from the adrenal glands at autopsy [51–54].

8.4. Parasites. Of all the organisms mentioned in this review, parasites are perhaps the least commonly reported causes of adrenal infection in the United States. Microsporidia have been reported to cause necrotic lesions in the adrenal glands, particularly in patients with AIDS [55]. Echinococcosis (hydatid disease) is responsible for about 7% of all adrenal cysts. Treatment is surgical excision followed by several months of chemotherapy with oral albendazole to prevent recurrence [56, 57]. Visceral leishmaniasis is another cause of cystic adrenal disease. *Trypanosoma cruzi*, the causative agent of Chagas' disease, has been shown to infect the adrenal gland. Studies have postulated that the adrenals may serve as a reservoir for *T. cruzi* and parasitemia in the central adrenal

vein has been correlated with the development of chronic chagasic cardiomyopathy [58, 59]. African trypanosomiasis has been associated with polyendocrinopathies including hypogonadism, hypothyroidism, and adrenal insufficiency. This can result from a primary glandular or secondary (central) involvement. In one case series of 137 Ugandan patients, treatment of the infection resulted in recovery of adrenal and thyroid function, but hypogonadism tended to persist for years [60].

9. Conclusion

The adrenal glands can be affected by a wide range of organisms through multiple mechanisms including direct infection as well as disturbance of the HPA axis due to physiologic stress and cytokine release being the most common. Many of the diseases described in this review appear as chronic illnesses that have manifestations similar to adrenal insufficiency; therefore, very high index of suspicion is required for making the diagnosis of adrenal insufficiency in these patients. Bilateral adrenal enlargement is a feature of a number of these illnesses, especially the granulomatous infections. It is interesting to note that bilateral adrenal enlargement does not necessarily indicate adrenal infection but may be merely reflective of the response to stress. Successful treatment often results in the reduction of gland size. Once adrenal insufficiency has set in, however, the adrenal hypofunction tends to persist despite appropriate treatment of the underlying infection.

The biochemical diagnosis of adrenal insufficiency is often complicated by acute illness, which ironically is often the setting for it. Lack of uniform definitions and availability of reliability of assays used to measure ACTH and cortisol levels were the limitations encountered in a number of studies. Current guidelines recommend using a 250 mcg postintravenous corticotropin stimulated cortisol level of >18 mcg/dL as a cut-off to rule out primary adrenal insufficiency. In the setting of critical illness a serum cortisol level less than 25 mcg/dL or an increment of less than 9 mcg/dL 30 minutes after a 1 mcg intravenous corticotropin injection may be suggestive of adrenal insufficiency [61].

Steroid replacement strategies are the same irrespective of the etiology, but special attention must be paid to patients on rifampin, isoniazid, azole antifungal agents, and certain antiretroviral agents (ritonavir). These agents significantly impact steroid catabolism by interfering with the cytochrome P 450 system [62].

"The leading and characteristic features which merit attention are anemia, general languor and debility, remarkable feebleness of the heart's action, irritability of the stomach and a peculiar change of the color in the skin, occurring in connection with a diseased condition of the suprarenal capsules." Dr. Addison's classic description can scarcely be improved upon today. It cannot be emphasized enough that the diagnosis of adrenal insufficiency is a clinical challenge and requires a high index of suspicion, especially in the setting of an infectious process [3].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Laparoscopic Adrenalectomy for Adrenal Tumors

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Objective. To evaluate the indication and the clinical value of laparoscopic adrenalectomy of different types of adrenal tumor. *Methods*. From 2009 to 2014, a total of 110 patients were diagnosed with adrenal benign tumor by CT scan and we performed laparoscopic adrenalectomy. The laparoscopic approach has been the procedure of choice for surgery of benign adrenal tumors, and the upper limit of tumor size was thought to be 6 cm. *Results*. 109 of 110 cases were successful; only one was converted to open surgery due to bleeding. The average operating time and intraoperative blood loss of pheochromocytoma were significantly more than the benign tumors ($P < 0.05$). After 3 months of follow-up, the preoperative symptoms were relieved and there was no recurrence. *Conclusions*. Laparoscopic adrenalectomy has the advantages of minimal invasion, less blood loss, fewer complications, quicker recovery, and shorter hospital stay. The full preparation before operation can decrease the average operating time and intraoperative blood loss of pheochromocytomas. Laparoscopic adrenalectomy should be considered as the first choice treatment for the resection of adrenal benign tumor.

1. Introduction

Adrenalectomy is the standard treatment for adrenal gland disorders such as secretory tumors [1]. Laparoscopic procedures have been proposed to reproduce the surgical steps of open surgery and decrease morbidity, postoperative pain, and hospital stay. Laparoscopic surgery for adrenal tumors was firstly reported in 1992 by Gagner et al. who used the transperitoneal approach in three patients [2]. The benefits of laparoscopy on postoperative pain, cosmesis, hospital stay, and convalescence are widely recognized. Current efforts are aimed at further reducing the morbidity associated with minimally invasive surgery [3]. The upper limit of tumor size for laparoscopic surgery was thought to be 6 cm, for two main reasons: (i) the technique to remove a large tumor by laparoscopy is difficult; (ii) large tumors have higher possibility of malignancy [4]. But, there are some series of reports that laparoscopic surgery of the large pheochromocytomas was safe and effective. From 2009 to 2014, we evaluated 110 cases of adrenal diseases including the diameter of >5 cm adrenal tumor who underwent laparoscopic adrenalectomy.

2. Surgical Procedure

All patients were treated by retroperitoneal laparoscopic adrenalectomy. The procedures are usually performed with the patient in a flank position (90°) with the operating table fixed and then we elevated and exposed the surgical area between the costal margin and the iliac crest, and a 2 cm skin incision was made on the middle axillary line above the iliac crest. The fascia was exposed and incised over the same length, the muscle layers were divided bluntly, and the peritoneum was free from the abdominal wall by a finger dissection. Inserting the 10 mm trochar, CO_2 was insufflated to a maximum pressure of 2.0 kPa, and then the laparoscope was introduced. The second trochar was placed on the posterior axillary line under the costal arch. The peritoneal sac was mobilized medially to allow for the introduction of two additional trochars on the anterior axillary line at the level of the two preceding trochars. Gerota's fascia was identified and opened to expose the upper pole of the kidney and the adrenal gland.

3. Statistical Analysis and Patients

Statistical analysis used two-sample Wilcoxon rank-sum (Mann-Whitney) test via Stata7 software. P value less than 0.05 was considered statistically significant.

From 2009 to 2014, 110 patients diagnosed with adrenal benign tumors by CT scan were treated by retroperitoneal laparoscopic adrenalectomy at Huashan Hospital of Fudan University, China. The patients were analyzed retrospectively, including 65 on the left, 39 on the right side, and 6 on both sides, in 43 men and 67 women aged 22–79 (mean 50) years. The laparoscopic approach has been the procedure of choice for surgery of benign adrenal tumors, and the upper limit of tumor size was thought to be 6 cm.

4. Results

109 cases were successfully completed, but one case was converted into open surgery because the bleeding was out of control (pathology diagnosed adrenal cortical carcinoma). The mean operating time and intraoperative blood loss were 102.536 (35–327) mins and 81.454 (5–700) mL, respectively. Postoperative complications developed in 3 cases. One case developed effusion above kidney, and one case developed pneumonia. The last case was infected by MRSA. The postoperative hospitalization times were 6.1636 (2–30) days.

Cortical adenoma was the most common pathology (62.727%), followed by cortical hyperplasia and myelolipoma (9.091%), pheochromocytoma (6.364%), cyst (3.636%), adrenal cortical carcinoma (2.727%), lipoma (1.818%), neurofibroma (0.909%), spindle cell tumor (0.909%), cystic tumor (0.909%), inflammatory mass (0.909%), and lymphangioma (0.909%) (Table 2). Most complaints of the patients were symptomless (Table 1). We divided all patients into 3 groups which were benign tumor, pheochromocytoma, and malignant tumor groups and analyzed the average diameter, operating time, and intraoperative blood loss of each group. Based on pathologic specimens and CT scans, the tumor average diameter was 2.688 cm (0.6–6). The average diameters of benign tumors, pheochromocytomas, and malignant tumors were 2.607 cm (0.6–6), 3.214 cm (2–3.5), and 3.5 cm (3-4), respectively. The malignant tumors were bigger than the other tumors but without statistical significance ($P > 0.05$). The average operating time was 102.536 mins (35–327). One case was converted into open surgery and the operating time was 327 mins because the bleeding was out of control (pathology diagnosed adrenal cortical carcinoma). The average operating times of benign tumors, pheochromocytomas, and malignant tumors were 97.62 mins (35–280), 140.14 mins (90–205), and 178.67 mins (60–327), respectively. The intraoperative blood loss of benign tumors, pheochromocytomas, and malignant tumors was 8 mL (8–400), 85.71 (0–400), and 0 mL, respectively. The intraoperative blood loss and average operating time of pheochromocytomas were significantly more than the benign tumors ($P < 0.05$), but malignant tumors between benign tumors or pheochromocytomas were without statistical significance ($P > 0.05$) (Figure 1).

Table 1: The complaints of all patients.

Complaint	
Hypertension	25
Lumbar/abdominal pain	9
Limb weakness	7
Limb weakness and hypertension	4
Cushing syndrome	6
Limb numbness	4
Hyperglycemia	1
Symptomless	54

Table 2: The number, characteristics, and probability of pathology.

Pathology	Number	Characteristics	Probability
Cortical adenomas	69	Golden yellow	62.727
Pheochromocytoma	7	Grey yellow and red	6.364
Cortical hyperplasia	10	Grey yellow	9.091
Neurofibroma	1	Milky white	0.909
Myelolipoma	10	Grey yellow and red	9.091
Cortical carcinoma	3	Golden yellow or grey red	2.727
The spindle cell tumor	1	Yellow and white	0.909
Cyst	4	Cystic	3.636
Cystic tumor	1	Cystic	0.909
Inflammatory mass	1	Grey white	0.909
Lipoma	2	Grey yellow	1.818
lymphangioma	1	Cystic	0.909

The probability is accurate to 3 digits after the decimal point.

5. Conclusion

Laparoscopic adrenalectomy includes transperitoneal approach and retroperitoneal approach. The choice of the surgical approaches of laparoscopic adrenalectomy depends on the operators' habits and experience. We prefer retroperitoneal approach, which is not disturbed by abdominal organs. The intraoperative blood loss of pheochromocytomas was significantly more than the benign tumors ($P < 0.05$). We recommend the following to prevent intraoperative blood loss and decrease operation time. First, control the blood pressure before operation by Cardura. If it is necessary, use β receptor blocker. The full complement of crystalloid and colloid expands fluid volume. Second, the operators should carefully read the CT and MRI imaging data and ensure the location of the tumor and the relationship with the surrounding organs and tissues. Third, during the operation, clamp the fat around adrenal gland gently and cut off the small blood vessel by ultrasonic knife to decrease bleeding to maintain visual field. The vital management is to dissociate adrenal central vein enough, with 3 titanium clips ligation. If the vessel is thick, using the Ham-o-Lock ligation is necessary. With regard to the diameter >5 cm

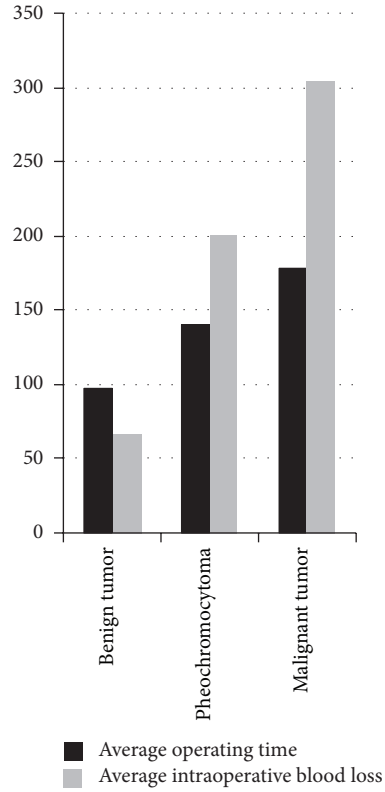

Figure 1: The average operating time and average intraoperative blood loss of different types of tumors. The intraoperative blood loss and average operating time of pheochromocytomas were significantly more than the benign tumors ($P < 0.05$), but malignant tumors between benign tumors or pheochromocytomas were without statistical significance ($P > 0.05$).

of adrenal tumor or large pheochromocytomas, we prefer transabdominal approach.

Compared to retroperitoneal LA, transabdominal approach is risky and can injure intestine, liver, spleen, and other adjacent organs but has the advantages of the obvious anatomical landmarks, wider operation space, and more convenience during the operation.

The large adrenal tumors have higher malignant incident rate, and most of the researchers do not recommend resecting the malignant tumors by laparoscopic adrenalectomy because those are large, rich in blood supply, with thin envelope surrounding tissue invasion. In addition, laparoscopic clamping separation may lead to tumor rupture, local recurrence, or trochar implant. Some researchers held negative attitude of laparoscopic operation for larger adrenal tumors. Nevertheless, confirming the adrenal malignant tumors is difficult by imaging. Under imaging, the malignant tumors present irregular boundary, local invasion, and uneven and high density, and so do benign tumors [5]. Sturgeon et al. discovered more malignant incidence rate of the large adrenal tumor (<4 cm = 5%, ≧4 cm = 10%, and ≧8 cm = 47%) [6]. Our data showed three malignant tumors were 3 cm, 3.5 cm, and 4 cm, respectively (<4 cm = 2.22%, ≧4 cm = 5%). If we only consider the tumor size as the surgical approaching standard, many patients will undergo unnecessary open operation. Kebebew et al. and Lombardi et al showed 3-year disease-free survival rate and local recurrence rate were similar to open operation. As for trocar metastasis, it is a rare complication of laparoscopic operation [7, 8].

Our data showed that the average diameter, operating time, and intraoperative blood loss of malignant tumors were not significantly different to pheochromocytomas and benign tumors ($P > 0.05$) indicating that the laparoscopic operation of adrenal malignant tumor is safe. But for those tumors invading surrounding tissue and blood vessels, they are difficult to control and separate, when patients have serious bleeding circumstances, we should seize the opportunity to convert into open operation. In view of this, implementing laparoscopic operation of adrenal malignant tumors is not the contraindication. But before being widely carried out, multicenter prospective controlled trials on laparoscopic and open radical adrenalectomy of malignant tumors are necessary.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Sun Chuan-yu and Ho Yat-faat contributed equally to this paper.

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Research Article

$CHADS_2$ Scores in the Prediction of Major Adverse Cardiovascular Events in Patients with Cushing's Syndrome

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Vascular events are one of the major causes of death in case of Cushing's syndrome (CS). However, due to the relative low frequency of CS, it is hard to perform a risk assessment for these events. As represented congestive heart failure (C), hypertension (H), age (A), diabetes (D), and stroke (S), the $CHADS_2$ score is now accepted to classify the risk of major adverse cardiovascular events (MACEs) in patients with atrial fibrillation. In this study, participants were enrolled from the National Health Research Institute Database (NHIRD) of Taiwan, and we reviewed 551 patients with their sequential clinically diagnosed CS data between 2002 and 2009 in relation to MACEs risk using $CHADS_2$ score. Good correlation could be identified between the CS and $CHADS_2$ score (AUC = 0.795). Our results show that patients with CS show significantly higher risk of vascular events and the $CHADS_2$ score could be applied for MACEs evaluation. Adequate lifestyle modifications and aggressive cardiovascular risks treatment are suggested for CS patients with higher $CHADS_2$ score.

1. Introduction

Cushing's syndrome (CS) represents the pattern of symptoms and signs caused by prolonged exposure of inappropriately high levels of the hormone cortisol [1–3]. It is relatively rare and most commonly affects adults between the ages of 20 and 50, affecting approximately 2 to 3 per million people in each year [4]. Obesity has been linked with this diagnosis. Other signs and symptoms include thinning of the reddish purple striae, plethora, proximal muscle weakness, weight gain, bruising with no obvious trauma, hypertension, diabetes, and osteoporosis [4–8]. Vascular events, either cardiovascular or cerebrovascular, are the major causes of death [4, 6, 7, 9]. However, due to the relatively low incidents of CS, it is hard to make a risk assessment for these events.

The most frequently used schema designed to stratify risk of thromboembolism in patients with atrial fibrillation is $CHADS_2$ score [10–14], which closely correlate major

adverse cardiovascular events (MACEs), such as myocardial infarction, stroke, and mortality risk. Clinically, $CHADS_2$ score is simple to use, with one point given for congestive heart failure, hypertension, age, and diabetes and two points for stroke or transient ischemic attack. Higher score has been associated with the risk of developing MACEs and for these patients, early anticoagulation therapy should be recommended [13, 15].

Recent studies have indicated that the risks of MACEs other than atrial fibrillation have been reported and the risk assessment was performed by using $CHADS_2$ [16–18]. In this study, participants with clinically diagnosed CS were adopted from the NHIRD of Taiwan. This database enrolls up to 99% of the 23 million residents of Taiwan who receive medical care through the National Health Insurance (NHI) program which consists of ambulatory and inpatient care records and the registration files of the insured. The aim of the study was to explore the relationship between CS and the risk of vascular events. Furthermore, we evaluated the predictive value of $CHADS_2$ on MACEs in patients with CS.

2. Materials and Methods

2.1. Study Population. This observational study was conducted in a retrospective cohort of the Taiwanese population from the year 2002 to 2009, enrolled in NHIRD in Taiwan. The National Health Insurance Bureau of Taiwan randomly reviews the charts of 1 out of every 100 ambulatory cases and one out of every 20 inpatient cases; it also performs patient interviews to verify the accuracy of the diagnosis. In patients ≥18 years of age diagnosed with Cushing's syndrome (ICD-9-CM codes 255.0) between the years 2002 and 2009 were recruited for this study. CS patients with stroke and previous acute myocardial infarction were excluded.

2.2. Risk Score Calculation. The $CHADS_2$ scores were calculated for each patient by assigning 1 point each for the presence of chronic heart failure, hypertension, age below 75 years, and diabetes and by assigning 2 points for history of stroke or transient ischemic attack (TIA). The study patients were divided into 4 groups by their $CHADS_2$ scores: 0, 1, 2, and ≧3, respectively.

2.3. Study End Point and Patient Followup. Mortality data covering the years from 2002 to 2009 were used to calculate the mortality rate in each group. Each patient was tracked for 5 years from the time of their first diagnosis using administrative data to identify all patients who had MACEs such as myocardial infarction, stroke, and mortality or censored during the study period.

2.4. Statistical Analysis. SPSS (version 15, SPSS Inc., Chicago, IL, USA) was used for data analysis. Receiver operating characteristics curve was used to assess the prediction accuracy for MACEs by using $CHADS_2$ score and plots of observed and predicted MACEs were presented. The cumulative rates of MACEs were estimated using the log rank test to examine the differences in the risk of mortality between different groups among the CS patients. The Cox proportional hazards regression model was used to compare the outcomes between different risk groups. We calculated hazard ratios (HR) along with 95% confidence intervals (CI) using a significance level of 0.05. A two-sided P value ($P < 0.05$) was used to determine statistical significance.

Table 1: Baseline characteristics of the patients with Cushing's syndrome from 2002 to 2009 in Taiwan ($n = 551$).

Variables	Study population n (%)
Total	551 (100)
Mean age, years (±SD)	48 ± 20
$CHADS_2$ score	
Mean ± SD	0.96 ± 1.17
0	271 (49.2)
1	121 (22.0)
2	100 (18.1)
≧3	59 (10.7)
Gender	
Male	177 (32.1)
Female	374 (67.9)
Hyperlipidemia	126 (22.9)
Chronic kidney disease	26 (4.7)
Coronary artery disease	42 (7.6)
Atrial fibrillation	5 (0.9)
Socioeconomic status	
Low	227 (50.3)
Moderate	195 (35.4)
High	79 (14.3)

3. Results

There are 551 patients diagnosed with CS from 2002 to 2009 within the NHIRD. The number of patients, age, gender, and distribution of number of patients in different $CHADS_2$ score groups are shown in Table 1. The mean age at diagnosis was 48 ± 20 years. 67.9% of patients were female. The mean $CHADS_2$ score was 0.96 ± 1.17. Severe comorbidity ($CHADS_2$ score ≧3) was noted in 10.7% in all CS patients.

Figure 1 showed that the c-statistics was 0.795 (95% CI, 0.724–0.866). Figure 2(a) was the Kaplan-Meier survival curves. CS patients with higher $CHADS_2$ score were more likely to have MACEs ($P < 0.001$). We further divided the CS patients into four groups based on the $CHADS_2$ scores (Table 2). CS patients with a score of ≧3 are associated with the highest rate in MACEs (Figure 2(b), $P < 0.001$).

In multivariate analysis, each additional $CHADS_2$ score is associated with 1.63-fold (95% CI, 1.29–2.06) increased risk for MACEs when $CHADS_2$ score is a continuous variable (Table 3, model A). In model B of Table 3, where $CHADS_2$ is an ordinal variable and divided into three groups, patients with the score ≧3 remained as an independent prognostic factor for the risk of MACEs with hazard ratios of 7.16 (95% CI, 2.31–22.15), compared with $CHADS_2$ 0-1 after adjusting

Table 2: MACEs among Cushing's syndrome patients with different $CHADS_2$ scores from 2002 to 2009 ($n = 551$).

Variables	Male			Female		
	n	Case (%)	P value	n	Case (%)	P value
$CHADS_2$ score			<0.001			<0.001
0 ($n = 271$)	77	5 (6.5)		194	1 (0.5)	
1 ($n = 121$)	41	6 (14.6)		80	2 (2.5)	
2 ($n = 100$)	38	5 (13.2)		62	8 (12.9)	
≧3 ($n = 59$)	21	9 (42.9)		38	12 (31.6)	

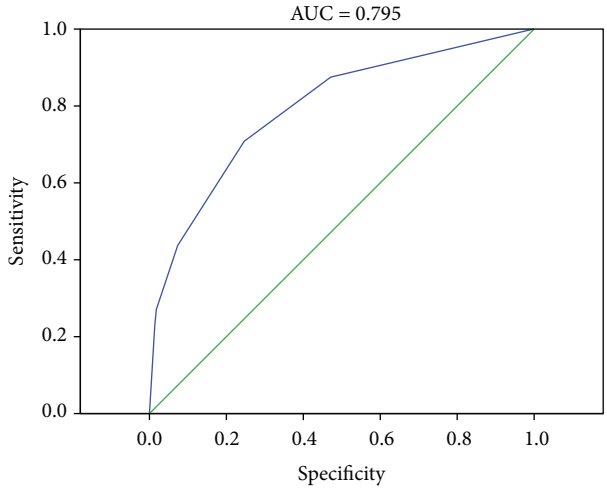

Figure 1: Receiver operating characteristics curve for $CHADS_2$ in prediction of MACEs in Cushing's syndrome patients.

with other factors. As compared to male, female is associated with decreased risk for MACEs in patients with CS (model B, adjusted HR 0.45, 95% CI 0.25–0.81, and $P = 0.009$). Age is associated with increased risk for MACEs (model B, adjusted HR 1.05, 95% CI 1.00–1.05, and $P = 0.026$).

4. Discussion

The main finding of this study is that the $CHADS_2$ score can be useful in the evaluation of the global cardiovascular risk (i.e., MACEs and death) of patients with CS. We provide the general calculation of mortality and morbidity rate as well as a risk assessment for MACEs in cases of CS. We validated the application of $CHADS_2$ score in the MACEs of CS patient for the accuracy of our prediction is 79.5%.

The strengths of these data are based on the fact that it was a nationwide population-based cross-sectional study, with nearly complete follow-up information about access to healthcare institutes, as well as the fact that the dataset was routinely monitored for diagnostic accuracy by the National Health Insurance Bureau of Taiwan. The National Health Insurance (NHI) program was conducted since 1995 which requires mandatory enrollment in the government-run, universal, single-payer insurance system and provides comprehensive benefits coverage. Currently, up to 99% of the 23 million residents of Taiwan receive medical care through this program. Over 97% of the hospitals and clinics in Taiwan are contracted to provide health care services [19], which are reimbursed by the Bureau of NHI, and all data related to these services are collected and stored into the NHIRD by the National Health Research Institutes to provide a comprehensive medical care record. The data consisted of ambulatory care records, inpatient care records, and the registration files of the insured. The dataset included all claims data from Taiwan's NHI program, which was implemented as a means of financing health care for all Taiwanese citizens. The average age and gender distribution of CS patients were also compatible with a previous report [20].

There are many reports about the mortality and morbidity of CS that have been documented [21–24]. However, due to the relative rarity of CS, data collection on the incidence, mortality, and cause of death is scarce. Our results show a 8.7% overall risk of MACEs in CS patients. Poor prognosis was demonstrated in 35.6% of the patients in high risk population ($CHADS_2$ ≧3), 13.0% of the $CHADS_2 = 2$, 6.6% of the $CHADS_2 = 1$, and 2.2% in the cases of the $CHADS_2 = 0$. Current guidelines for atrial fibrillation suggest that the $CHADS_2$ is useful in the selection of antithrombotic therapy; therefore, those with high risk ($CHADS_2$ score ≧2) would benefit from anticoagulation therapy. For those with low (score 0) or moderate (score 1) risk, aspirin may be an alternative given that the risk of bleeding caused by anticoagulation therapy may not justify its use [14]. Antithrombotic therapy is also essential for the CS patients within the high risk population.

Our study had several limitations related to the NHIRD database. The diagnoses of CS, AMI, and stroke were dependent on ICD codes used in the NHIRD database. Another limitation is that the design of the study is retrospective. Validation of scores in retrospective cohort presents always with several limitations that weaken the strength of the results. However, we believe the relatively large number of patients may compensate for these limitations. The National Health Insurance Bureau of Taiwan, however, has made every effort to verify the accuracy of diagnosis based upon random chart reviews and patient interviews. An additional limitation of the NHIRD database was its lack of information on tobacco use, dietary habits, metabolic profiles, or other behavioral factors, which may be risk factors for AMI among CS patients. Nonetheless, given the magnitude and statistical significance of the observed effects in this study, these limitations were unlikely to have compromised our results. In fact, the strength of our study lies in its large sample size. In addition, because

Table 3: Hazard ratios of individual $CHADS_2$ score for MACEs in patients with Cushing's syndrome ($n = 551$).

	Model A*			Model B**		
	Adjusted HR	95% CI	*P* value	Adjusted HR	95% CI	*P* value
$CHADS_2$ score	1.63	1.29–2.06	<0.001			
0				1		
1				1.95	0.63–6.03	0.246
2				2.77	0.90–8.48	0.074
≧3				7.16	2.31–22.15	0.001
Gender						
Male	1			1		
Female	0.50	0.28–0.90	0.022	0.45	0.25–0.81	0.009
Age	1.03	1.00–1.05	0.006	1.02	1.00–1.05	0.026
Hyperlipidemia	0.50	0.23–1.08	0.078	0.55	0.26–1.15	0.114
Chronic kidney disease	1.26	0.52–3.03	0.599	1.35	0.58–3.14	0.484
Coronary artery disease	1.44	0.67–3.09	0.343	1.59	0.76–3.32	0.216
Atrial fibrillation	1.17	0.24–5.53	0.843	1.17	0.25–5.47	0.839
Socioeconomic status						
Low	1			1		
Moderate	1.69	0.90–3.17	0.099	1.57	0.84–2.93	0.156
High	1.22	0.32–4.54	0.764	1.17	0.31–4.32	0.812

Adjusted HR: adjusted hazard ratio; 95% CI: 95% confidence interval.
*Model A: $CHADS_2$ score as continuous variable.
**Model B: $CHADS_2$ score as ordinal variable.

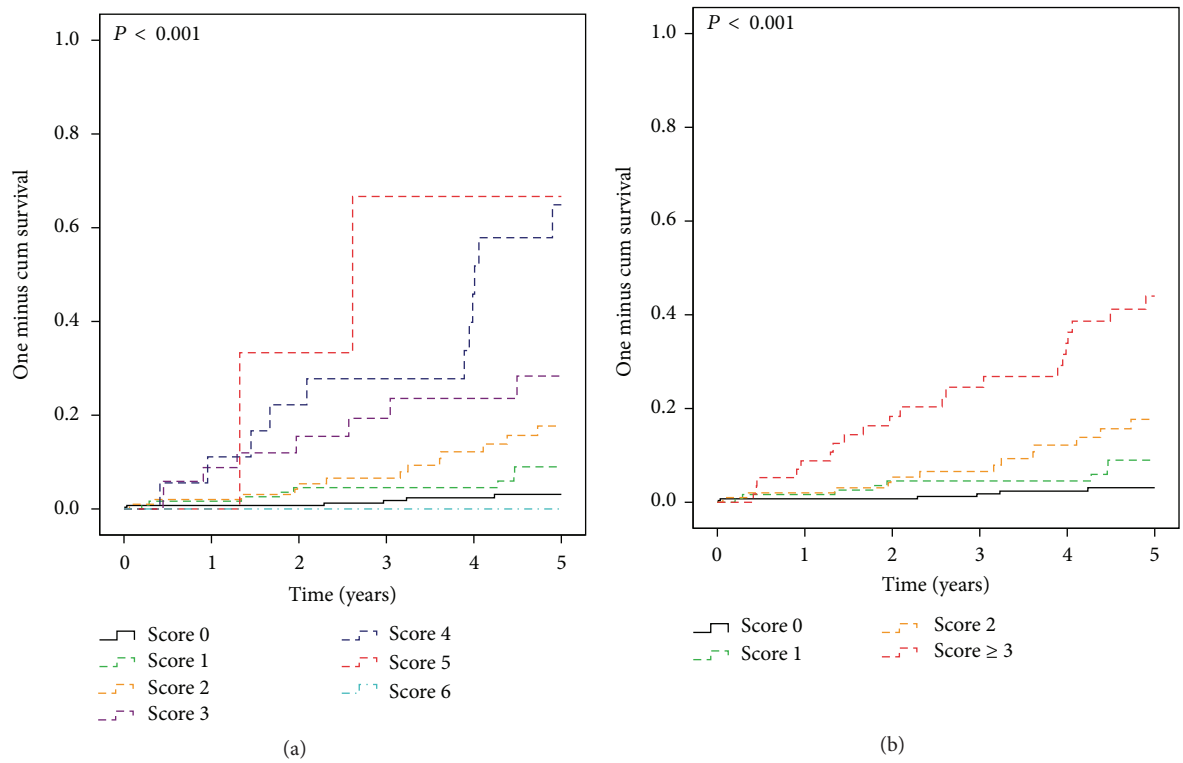

Figure 2: (a) MACEs risk stratified by $CHADS_2$ score. (b) MACEs risk stratified by $CHADS_2$ score categories.

the NHI has 99% coverage in Taiwan, our study had minimal risk of selection bias.

5. Conclusion

In the present study, $CHADS_2$ score could be a good tool to predict the incidence of MACEs in patients with Cushing's syndrome. Cardiovascular risks evaluation and management should be applied more aggressively for CS patients with $CHADS_2$ score $\geqq 3$.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Adipose Tissue and Adrenal Glands: Novel Pathophysiological Mechanisms and Clinical Applications

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Hormones produced by the adrenal glands and adipose tissues have important roles in normal physiology and are altered in many disease states. Obesity is associated with changes in adrenal function, including increase in adrenal medullary catecholamine output, alterations of the hypothalamic-pituitary-adrenal (HPA) axis, elevations in circulating aldosterone together with changes in adipose tissue glucocorticoid metabolism, and enhanced adipocyte mineralocorticoid receptor activity. It is unknown whether these changes in adrenal endocrine function are in part responsible for the pathogenesis of obesity and related comorbidities or represent an adaptive response. In turn, adipose tissue hormones or "adipokines" have direct effects on the adrenal glands and interact with adrenal hormones at several levels. Here we review the emerging evidence supporting the existence of "cross talk" between the adrenal gland and adipose tissue, focusing on the relevance and roles of their respective hormones in health and disease states including obesity, metabolic syndrome, and primary disorders of the adrenals.

1. Introduction

The significance of the adrenal gland as an endocrine organ and the roles of adrenal hormones in physiology and disease have been recognized well over a century [1, 2]. In fact, the history of adrenal endocrinology is almost as old as the study of endocrinology itself. Conversely, adipose tissue was long regarded as a hormonally inactive storage site for triglycerides. Only in recent years, paralleling the emergence of obesity and associated disorders such as diabetes, cardiovascular disease, and cancer as leading causes of morbidity and mortality has adipose tissue achieved recognition as an active endocrine organ playing key roles in maintaining homeostasis and involved in the pathogenesis of a variety of diseases [3–5].

The adrenal gland is composed of an outer cortex producing steroid hormones including mineralocorticoids, glucocorticoids, and sex steroids and an inner medulla of neuroectodermal origin producing catecholamine hormones. Each of these hormones acts on adipose tissue, which is readily apparent from the well-described clinical manifestations of primary adrenal endocrinopathies such as Cushing syndrome, Addison disease, and pheochromocytoma.

Adipose tissues are a heterogeneous group of tissues classified based on histology and function as white adipose tissue (WAT) and brown adipose tissue (BAT). Adipose tissues can be further categorized by anatomical location as visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) [6]. While rodents maintain both BAT and WAT in distinct depots through adult life, BAT in humans is found mainly in the neck and thorax in neonates and is mostly replaced by WAT by adulthood [6].

The adipose tissues are a source of several hormones termed "adipokines" or "adipocytokines" including adipocyte-derived factors such as leptin, adiponectin, resistin, and proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), C-reactive protein (CRP), serum amyloid A (SAA), and interleukin-6 (IL-6) which are secreted not only from adipocytes, but also from myeloid-derived cells such as macrophages located in the adipose tissue stroma

[7, 8]. While adipokine roles in regulating systemic energy homeostasis, body weight, vascular biology, inflammation, and glucose and lipid metabolism have been well defined, more recently their regulation by adrenal hormones and effects on adrenal function has become a "hot topic" with increased representation in the scientific literature [9–11].

The growing body of knowledge describing two-way interactions between adrenal hormones and the "endocrine adipocyte" has resulted in the proposal of the existence of an "adipose-adrenal axis" [12]. In this review we critically examine the available published data investigating the interplay between the adrenals and adipose tissue with an emphasis on novel mechanistic insights into roles in pathophysiology of obesity and primary diseases of the adrenals.

2. Mineralocorticoid-Adipose Interactions

Aldosterone is the primary mineralocorticoid hormone produced by the adrenal glands in humans and exerts its effects via the mineralocorticoid receptor (MR). While the well-described "classical" effects of aldosterone on transepithelial Na transport are mediated by MR present in epithelial cells, MR has been shown to be present in a number of other cell types including adipocytes [13].

Activation of MR appears to have important roles in adipose tissue including differentiation of preadipocytes to mature adipocytes and promotion of a proinflammatory state via induction of cytokines including TNF-α, monocyte chemotactic protein-1 (MCP-1), and IL-6 in WAT, while decreasing the thermogenic activity and lowering uncoupling protein 1 (UCP1) transcription of BAT [14]. MR mRNA expression was shown to correlate with increasing body mass index (BMI) in humans and to be increased in obese db/db mice [15].

2.1. Mineralocorticoid Receptor Effects in Adipocytes Are Regulated Primarily by Glucocorticoids. Since glucocorticoids (cortisol in humans and corticosterone in rodents) and mineralocorticoids (aldosterone) both bind to MR with relatively high affinity, the MR specificity of aldosterone in epithelial cells in humans is due to the intracellular inactivation of cortisol to cortisone (and corticosterone to 11-dehydrocorticosterone in rodents) by the enzyme 11-beta-hydroxysteroid dehydrogenase type 2 (11HSD2) (Figure 1). Adipocytes do not exhibit significant 11HSD2 activity, allowing glucocorticoids (GC), which circulate at 10-to-100-fold higher concentrations than mineralocorticoids (MC), to be the main ligand for adipocyte-MR [16]. Further enhancing the role of glucocorticoid hormones on adipose tissue MR activity is the increased 11-beta-hydroxysteroid dehydroxygenase type 1 (11HSD1) present in adipocytes, which results in increased conversion of cortisone to cortisol (Figure 1). While this balance of HSD enzyme activity explains the increased exposure of adipocyte-MR to endogenous glucocorticoids, the fact that glucocorticoid induced alteration in adipocyte function is an MR mediated phenomenon was demonstrated by studies in which adipocyte dysfunction induced by cortisol and corticosterone treatment in cultured murine adipocytes was reversed by the MR antagonist eplerenone but not by glucocorticoid receptor (GR) antagonism with mifepristone [15]. Therefore it is likely that many of the functions of glucocorticoids in regulation of adipocyte biology may be mediated via MR rather than GR.

2.2. Aldosterone in Obesity. Increased serum aldosterone levels have been observed with both obesity-associated hypertension and the metabolic syndrome. While several mechanisms may be at play, this finding is most likely due in large part to the upregulation of the renin-angiotensin-aldosterone system (RAAS) in obesity, a proposed mechanism linking hypertension with obesity that is reversed by weight loss [17].

A second, more novel mechanism of increased aldosterone production in obese individuals, exemplifying the concept of adrenal-adipose "cross talk," is the identification of adipose-derived factors able to directly stimulate adrenal aldosterone production [12]. This finding raises the prospect of a "vicious cycle" seen in obese states between the adipocyte and adrenal zona glomerulosa in which adipose tissue derived factors could stimulate aldosterone production, leading to hypertension, inflammation, and endothelial dysfunction. The enhanced MR activation that results would in turn promote adipocyte differentiation and inflammation, promulgating the cycle. The exact nature of the adipocyte-derived factors is unknown, yet certain candidate fatty acids have been proposed, particularly oxidized derivatives of linoleic acid [18].

A third candidate mechanism contributing to our understanding of the proposed role of MR activation in adipose tissues was brought up by a recent report demonstrating both in vitro production and in vivo production of aldosterone by adipocytes, resulting in both autocrine and paracrine regulatory effects on adipocyte function [19].

Clinical implications of these insights into the role of adipocyte MR in obesity include whether MR blockade would be of benefit in treating obesity-associated morbidities such as hypertension, diabetes, and the metabolic syndrome. While studies of MR blockade in obese mice have shown benefits on adipose tissue dysfunction [15, 20], clinical trials in humans of MR blockers specifically investigating its effects in obesity do not exist. In clinical trials assessing effects on hypertension, RAAS blockade has been linked to decreased risk of new onset diabetes [21]. While the exact mechanisms that explain this observation are unknown, effects on adipocyte differentiation and inflammation have been proposed. Studies of MR blockade in cardiovascular disease have shown benefits, though not specifically on BMI or metabolic syndrome per se [22, 23].

2.3. Adipose Tissue Dysfunction in Primary Hyperaldosteronism. Primary hyperaldosteronism (PA), known also by its eponymous name Conn syndrome, is a state of continuous aldosterone excess due to autonomous production of aldosterone by an adrenal adenoma or bilateral adrenal zona glomerulosa hyperplasia and thus provides a relevant model for investigating the results of systemic aldosterone excess on adipose tissue.

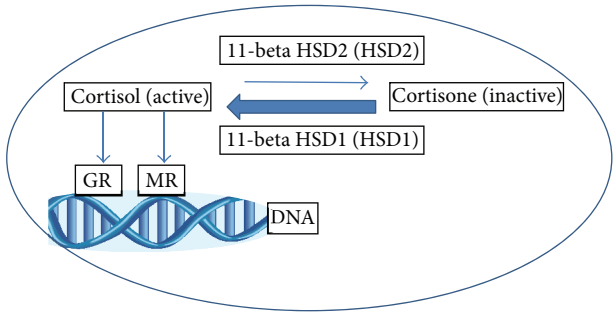

Figure 1: Adipocytes do not have significant HSD2 activity and yet maintain a relatively high level of HSD1 activity, allowing for increased intracellular active cortisol relative to inactive cortisone. Cortisol exerts its effects in adipocytes by binding to both glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) with similarly high affinity. Obesity has been associated with increased adipose HSD1 activity, further increasing intra-adipocyte cortisol concentrations.

While continuous infusion of aldosterone over 12 days resulted in weight gain in adrenalectomized rats [24], both cross-sectional studies and longitudinal studies of PA in humans, including those assessing changes following surgical cure of PA, report no consistent effect on body weight [14]. Though a relationship with increased insulin resistance and hyperglycemia has been described in older reports [25], several recent studies have not confirmed this finding [14]. While most studies of PA have not reported changes in circulating leptin and adiponectin, levels of the proinflammatory adipokine resistin were significantly increased in PA when compared to hypertensive controls and correlated with presence and severity of metabolic syndrome in PA subjects [26].

3. Glucocorticoid-Adipose Interactions

Glucocorticoids have effects on adipose tissue development, metabolism, and adipocyte secretory function. Studies investigating the effects of GC on adipogenesis and adipocyte lipid metabolism provide discordant results, while the anti-inflammatory nature of GR activation in adipocytes is a consistent finding [27, 28]. Glucocorticoids promote differentiation of preadipocytes to mature adipocytes acting synergistically with insulin [29]. In BAT, GC decrease UCP1 expression and increase lipid storage, in effect mediating a phenotypic conversion of BAT to WAT [30]. The effects of GC on adipose tissues particularly on lipid synthesis and lipolysis appear to depend on the physiologic context in which they occur as well as the specific depot, with predominantly lipogenic effect in VAT and lipolytic role in SAT [28]. Particularly important in determining the net effect of GC exposure on adipose lipid metabolism are nutritional and hormonal milieu. For instance while cortisol synergistically may stimulate adipocyte expansion during energy surplus and abundant insulin supply, such as what would occur in Cushing syndrome, during catabolic states cortisol production increases as part of the stress response and has a largely lipolytic role, mobilizing vital energy stores. This paradigm may partially explain the observation that hypercortisolism is associated with increased adiposity in Cushing syndrome and paradoxically with decreased adiposity in states of undernutrition such as anorexia and acute illness.

Alterations of circulating cortisol dynamics, mainly as hypothalamic-pituitary-adrenal (HPA) axis dysfunction, as well as local metabolism of glucocorticoids in adipose tissue, have been linked to obesity and the metabolic syndrome.

3.1. Adipocyte Cortisol Metabolism in Obesity. The dramatic changes in fat distribution characterized by central adiposity with wasting of subcutaneous fat depots, typical of Cushing syndrome, have led to the hypothesis that alterations in GC action may play a role in more common forms of visceral obesity. While circulating cortisol levels are not increased in obese individuals, increased net cortisol production occurs locally in the adipose tissue due to the above-described alterations in adipocyte HSD enzymes, and thus obesity has been described as "Cushing syndrome of the omentum" [31] (Figure 1).

An important confounder in interpreting studies of GC action on adipose tissue is the fact that GC effects in adipose tissue are mediated via the MR receptor as well as the GR receptor. Due to the lack of adipocyte activity of 11HSD2, an enzyme which inactivates endogenous GC in mineralocorticoid target tissues, much of the observed effect of naturally occurring GC in adipocytes is due to its effect on MR, rather than GR [32] (Figure 1).

Many studies have suggested increased 11HSD1 activity in obesity [33, 34]. Transgenic mice overexpressing 11HSD1 develop metabolic syndrome, while mice deficient in 11HSD1 are protected from metabolic syndrome and associated cardiovascular disease [35, 36]. A variety of studies in humans have found increased 11HSD1 in both VAT and SAT with obesity [37]. Several naturally occurring and synthetic HSD1 inhibitors have been investigated in regard to their effects on metabolic disorders [38]. One such compound, INCB13739, when given to subjects with T2DM improved HbA1c, insulin resistance, and lipid parameters. The effects were more pronounced in the obese group (BMI > 30) when compared with the overweight group (BMI > 25) [39]. These findings point to the importance of HSD enzymes in determining the effect of GC in adipose tissue and make HSD modification an attractive candidate for pharmacological intervention in the treatment of obesity.

While studies of the effects of GC on adipogenesis and adipocyte lipid metabolism have yielded inconsistent results, the effects of GC in suppressing inflammatory signaling from adipocytes are remarkably consistent. Experiments of selective GR stimulation with dexamethasone have generally resulted in decreased expression of proinflammatory cytokines by adipocytes, while GR knockdown with siRNA has the opposite effect [40]. Investigations of GC effects on adipocytokine production have yielded consistent findings

of increased leptin expression [41] and inconsistent results regarding effects on adiponectin [42].

3.2. Obesity and the HPA Axis. Obesity and metabolic syndrome are associated with alterations in the HPA axis including changes in adrenal cortisol production, peripheral cortisol metabolism, and dynamic tests of the HPA axis. Whether these changes play a role in the development and pathophysiology of obesity and related comorbidities or represent an adaptation to the obese state is unknown. Basal levels of ACTH and cortisol appear to be undisturbed in obesity. While 24-hour urine-free cortisol generally has not been found to be increased in obese subjects, one study described significantly elevated night time (7 pm–7 am) urine-free cortisol in women with abdominal obesity [43]. The most convincing evidence of abnormal HPA axis function in obesity comes from dynamic studies of stimulation or suppression of the axis. Obese individuals exhibit greater ACTH and cortisol responses to AVP and CRH [44, 45] and increased cortisol response to both low dose and high dose ACTH stimulation tests [46, 47]. Though most studies have demonstrated normal cortisol suppression after 1 mg overnight dexamethasone suppression in obese subjects compared to lean controls, one study positively correlated higher postdexamethasone serum cortisol levels with increased waist-to-hip ratio in women, but not in men [48].

4. Adrenal Androgens and Adipose Tissue

The adrenal cortex produces androgen hormones in both sexes, including dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEA-S), and androstenedione. In humans DHEA-S circulates at higher concentrations than any other steroid hormone and levels decline substantially with age, paralleling the changes in body composition characteristic of aging [49]. Of particular relevance is the fact that DHEA is present at nearly 10-time higher concentration in adipose tissue than in the systemic circulation [50]. A number of epidemiologic studies have shown correlations between circulating DHEA-S and obesity and insulin resistance and cardiovascular disease [51]. Both in vitro and in vivo studies have demonstrated antiadipogenic effects of DHEA on animal adipocytes [52]. DHEA effects favorable changes in adipocyte insulin sensitivity and adipokine profile [53, 54]. While several studies of DHEA supplementation in adults with various disease states have not shown consistent changes in body weight or body fat percentage [55], one study of DHEA administration in the elderly resulted in a significant decrease in abdominal fat after 6 months of treatment [56].

A potential confounder in interpreting these data is the fact that DHEA acts as a precursor hormone and is converted into both testosterone and estrogens, which have independent effects on adipose tissue and body composition [57]. Receptors for sex steroids are distributed differently among the genders and have important effects in regulating both lipoprotein lipase and leptin production [58, 59].

5. Adipose Tissue and the Adrenal Medulla

The adrenal medulla functions similarly to a sympathetic ganglion, secreting catecholamine hormones, primarily epinephrine, into the bloodstream under the control of the sympathetic nervous system (SNS). Epinephrine has potent lipolytic effects on adipose tissue [60] and impairment in catecholamine-dependent lipolysis has been implicated in the pathogenesis of obesity [61].

There is increasing data indicating cross talk between catecholamines and adipokines, with evidence supporting stimulation of adrenal medullary function by leptin, while resistin may suppress medullary catecholamine secretion [62]. Closing this adrenal-adipose feedback loop, catecholamines have been shown in vitro to modulate adipocyte endocrine function, promoting expression of proinflammatory cytokines by adipocytes and reducing leptin and resistin expression [62].

5.1. Adrenal Medullary Function in Obesity. Obesity is associated with overactivity of the sympathetic nervous system. This SNS "overdrive" has been implicated as a possible contributor to the pathogenesis of obesity-associated morbidities and end-organ damage attributed to the obese state. One theory explains the increased SNS output of obesity as a homeostatic counterregulatory mechanism aimed at redirecting excess energy supply into adrenergic thermogenesis, thus protecting from the harms of further fat storage [63]. Adipose tissue may play an important role in coordinating this response by secreting adipokines which stimulate SNS and adrenal medullary function [64]. Adipokines that may directly or indirectly modulate SNS activity include leptin, nonesterified-free fatty acids, angiotensinogen, TNF-α, IL-6,and adiponectin.

The relationship between leptin and catecholamine production may be of particular significance in the discussion of obesity as leptin has been implicated as a direct inducer of increased SNS outflow in obesity [65]. Conversely, acute activation of beta-adrenergic receptors has been shown to decrease leptin production by adipocytes in both humans and rodent models, though in a study of pheochromocytoma (PHEO) circulating leptin levels were not suppressed [66].

5.2. Catecholamines and BAT. Recent reports have highlighted the importance of catecholamines in regulating BAT in adults. Several observational studies of patients harboring PHEO describe that active BAT, as evidenced by increased uptake on 18-fluorodeoxyglucose-positron emission tomography (FDG-PET) by BAT, is diminished following surgical treatment of pheochromocytoma [67, 68]. Intriguing findings of a recent study were that adiponectin expression was increased from BAT in patients with PHEO while serum adiponectin levels decreased after surgical removal of PHEO and its surrounding BAT [69].

6. Nonfunctioning Adrenal Adenomas and Adipose Tissue

Adrenal masses are discovered in approximately 4% of CT or MRI studies performed for reasons other than suspicion

of adrenal disorder [70]. Such incidentalomas are most often nonfunctioning adenomas (NFA) of the adrenal cortex, though 5–9% may result in mild hypercortisolism not resulting in clinically obvious Cushing syndrome, an entity defined as subclinical Cushing syndrome (SCS) [70]. NFA has been associated with obesity as well as insulin resistance and the metabolic syndrome. In reports comparing persons with NFA to obese controls matched for BMI, NFA subjects typically exhibit a more central distribution of fat associated with decreased insulin sensitivity [71]. In line with these findings, we previously reported increased epicardial fat thickness and left ventricular mass in NFA subjects [72]. Careful analysis of many of these studies reveals statistically significant higher indices of cortisol production, particularly late night serum cortisol and dexamethasone suppressed cortisol levels, but not to the degree meeting accepted criteria for SCS [71, 73]. Since cortisol production can be best understood as a continuous spectrum, one explanation for the abnormalities in adiposity and metabolic parameters observed in NFA patients could be subtle hypercortisolism. Others have proposed that NFA may be the result, rather than the cause, of insulin resistance [74].

7. Adipocytokine Effects on Adrenal Function

While the primary regulation of adrenal hormone production, classical glucocorticoids and androgens by ACTH, aldosterone by the RAAS, and catecholamines by the SNS, has been well described, it has more recently been proposed that adipokines may have a role in modulating adrenal function. While a comprehensive review of the effects of all adipokines on adrenal function is beyond the scope of this review, we will summarize the effects of two adipocytokines of particular relevance, leptin and adiponectin, as follows.

8. Leptin-Adrenal Interactions

Since its discovery as a WAT-derived circulating satiety factor in 1994 [75], the effects of leptin on adrenal function have been studied extensively. In vitro experiments have elucidated the direct effects of leptin on adrenal steroidogenesis. In bovine adrenal cell culture, leptin administration downregulated expression of several steroidogenic enzymes and blunted the cortisol response to ACTH [11, 76]. Similar results were found in studies of human and rat primary adrenal cell culture in which leptin inhibited ACTH-stimulated cortisol production but had no effect on basal cortisol production [77]. Leptin also appears to have effects at higher levels of the HPA axis. Leptin deficient ob/ob mice exhibit increased HPA activity [78]. In vivo experiments of chronic leptin administration in ob/ob mice demonstrate a blunting of the corticotropin-releasing hormone (CRH) response to stress and decreased basal corticosterone levels [78]. However, a study of short term administration of leptin in rhesus macaques yielded conflicting results demonstrating no effect of leptin on either basal or stimulated cortisol levels [79].

While the reciprocal relationship between leptin and sympathetic nervous system activity has been detailed above, leptin has also been shown to exert direct effects on the adrenal medulla. Leptin stimulates secretory activity of porcine chromaffin cells [80], while both resistin and leptin promoted secretion of catecholamines from rat chromaffin cells [62]. The same investigators demonstrated evidence of a reciprocal regulation of leptin by the adrenal medulla, providing experimental proof of catecholamine inhibition of secretion of leptin from cultured adipocytes, once more supporting the existence of an adipoadrenal axis.

9. Adiponectin-Adrenal Interactions

Adiponectin is the most abundant adipokine in the circulation with insulin-sensitizing, antiatherogenic and anti-inflammatory properties mediated mainly by the adenosine monophosphate-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor gamma (PPARγ) signaling pathways. Adiponectin receptors are present in both human and mouse adrenals [81, 82]. Paschke and colleagues reported that adiponectin receptors were present in all layers of the adrenal cortex and medulla in rats, while adiponectin mRNA expression was demonstrated in the rat adrenal zona glomerulosa only [10]. Short term exposure of the murine adrenocortical Y-1 cell line to adiponectin downregulated key enzymes of steroidogenesis, resulting in decreased corticosterone and aldosterone production [82]. In turn, both glucocorticoids and ACTH are known to decrease adiponectin production in WAT [83, 84]. In a primary culture of rat adrenocortical cells, treatment with adiponectin enhanced adrenocortical cell proliferation and increased corticosterone secretion in a dose-dependent manner while aldosterone secretion was unaltered [10].

10. Conclusions and Future Perspectives

The emergence of obesity as a worldwide epidemic has brought the study of adipocyte biology and secretory functions of adipose tissue to the forefront of biomedical and endocrine research. The interaction between adipokines and other endocrine organs is an area of recent intensive investigation. In this regard, the hormones of the adrenal glands are particularly relevant given their well-established effects on lipid metabolism, inflammation, and the "stress" response. Here we have discussed the physiologic and pathophysiologic actions of each of the adrenal hormones in the adipose tissues with focused discussions of the specific alterations observed in obesity and metabolic syndrome as well as mechanistic considerations of their roles in obesity and associated comorbidities. We have also examined the evidence linking adipocyte dysfunction to primary adrenal disorders and elucidated several examples of two-way interactions or cross talk between hormones of the adrenals and adipokines. Further research is needed to improve our collective understanding of the significance of the described changes in adipocyte function in primary adrenal diseases and particularly of the changes in adrenal function resulting from the adipokine milieu characteristic of obesity; a fundamental concept in need of further exploration is whether these are adaptive

mechanisms representing a protective response to the obese state or, conversely, contributors or "drivers" in the morbidity associated with excess adiposity. Lastly, we have provided a theoretical basis for potential future pharmacological interventions aimed at adrenal hormone targets in the adipose tissue.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Atil Y. Kargi wrote the paper. Gianluca Iacobellis edited the paper.

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Review Article

Paragangliomas/Pheochromocytomas: Clinically Oriented Genetic Testing

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Paragangliomas are rare neuroendocrine tumors that arise in the sympathetic or parasympathetic nervous system. Sympathetic paragangliomas are mainly found in the adrenal medulla (designated pheochromocytomas) but may also have a thoracic, abdominal, or pelvic localization. Parasympathetic paragangliomas are generally located at the head or neck. Knowledge concerning the familial forms of paragangliomas has greatly improved in recent years. Additionally to the genes involved in the classical syndromic forms: *VHL* gene (von Hippel-Lindau), *RET* gene (Multiple Endocrine Neoplasia type 2), and *NF1* gene (Neurofibromatosis type 1), 10 novel genes have so far been implicated in the occurrence of paragangliomas/pheochromocytomas: *SDHA, SDHB, SDHC, SDHD, SDHAF2, TMEM127, MAX, EGLN1, HIF2A,* and *KIF1B*. It is currently accepted that about 35% of the paragangliomas cases are due to germline mutations in one of these genes. Furthermore, somatic mutations of *RET, VHL, NF1, MAX, HIF2A,* and *H-RAS* can also be detected. The identification of the mutation responsible for the paraganglioma/pheochromocytoma phenotype in a patient may be crucial in determining the treatment and allowing specific follow-up guidelines, ultimately leading to a better prognosis. Herein, we summarize the most relevant aspects regarding the genetics and clinical aspects of the syndromic and nonsyndromic forms of pheochromocytoma/paraganglioma aiming to provide an algorithm for genetic testing.

1. Introduction

Paragangliomas are neuroendocrine tumors that can originate in either the parasympathetic or sympathetic nervous system. Most parasympathetic paragangliomas are chromaffin-negative (meaning that they do not stain brown when exposed to potassium dichromate) and do not secrete catecholamines. Sympathetic paragangliomas (including those derived from the adrenal medulla) are chromaffin-positive tumors that generally secrete catecholamines [1].

The designation of pheochromocytoma appears in the literature associated with different meanings. The World Health Organization (WHO) Tumor Classification defines pheochromocytoma as a paraganglioma derived from the adrenal medulla [2], whilst some authors use the term pheochromocytoma to refer to catecholamine-producing paragangliomas independently of being adrenal or extra-adrenal. In the present revision, we will use the WHO classification.

Sympathetic paraganglia are mainly found in the adrenal medulla but also in the axial regions of the trunk along the prevertebral and paravertebral sympathetic chains and in the connective tissue within/near pelvic organs. In contrast, parasympathetic paraganglia are almost exclusively confined to the head and neck in the vicinity of major arteries and nerves [3]. Paragangliomas can be categorized into functioning/nonfunctioning according to their ability to secrete catecholamines. Sympathetic tumors (including pheochromocytomas) tend to hypersecrete catecholamines (up to 90%), whereas only about 5% of parasympathetic paragangliomas secrete catecholamines [4, 5]. Among the functioning paragangliomas, the pheochromocytomas are the most frequent (80–85% of the cases) followed by the extra-adrenal abdominal paragangliomas [4–6].

The clinical presentation of these patients is highly variable, with most symptoms being nonspecific and mimicking other clinical conditions. Headaches, hypertension,

tachycardia, diaphoresis, pallor, anxiety, and panic attacks are the most frequent signs and symptoms at presentation [6]. The classic triad of palpitations, headaches, and profuse sweating altogether can provide a specificity of more than 90% [7]. Paroxysmal hypertension is frequent, either in patients with sustained hypertension or normal blood pressure. In fact, these patients typically present paroxysmal signs and symptoms (lasting less than an hour) that result from episodic release of catecholamines usually due to a triggering factor (surgery, stress, exercise, certain foods, medications, alcohol, etc.) [6]. Signs and symptoms in patients harboring parasympathetic paragangliomas are related to their mass effect causing compression of adjacent tissues and nerves, such as cranial nerves IX–XII [8, 9].

Paragangliomas are rare tumors occurring with an overall estimated incidence of 1/300 000, with an average age at diagnosis of around 40 years and no gender differences [2, 4, 10, 11]. However, the incidence of these tumors is much higher at autopsy ($\approx 0.05\%$), probably due to the often-asymptomatic clinical course of these tumors that, on the other hand, may result in premature mortality [12–14]. In hypertensive patients' series, the prevalence of paragangliomas/pheochromocytomas ranges from 0.1 to 0.6% [15–17].

Although most tumors are benign, about 10% of pheochromocytomas and 15% to 35% of extra-adrenal paragangliomas are malignant [18]. Prior to the appearance of distant metastases, commonly found in lungs, bone, or liver, there are no reliable histological, genetic, or imaging markers to predict malignancy of these tumors [18]. The histological PASS (Pheochromocytoma of the Adrenal gland Scaled Score) system was developed to predict the risk of malignant pheochromocytomas; however, the high interobserver and intraobserver variations make this score of limited clinical use [19–21]. Some studies have also pointed out that the size and location of the tumor, the downregulation of metastasis suppressor genes, early onset postoperative hypertension, high levels of plasma/urine metanephrines, immunochemical expression of the angiogenesis-related genes, and high levels of serum chromogranin A at the time of diagnosis, amongst many others, increase the likelihood of malignant pheochromocytoma [18, 22–25]. Of particular importance are the germline mutations in the *SHDB* gene (discussed in detail later), which have been associated with up to 72% of malignant tumors [26].

Paragangliomas can be classified into either sporadic or familial. In the last years, our knowledge concerning the familial forms of paragangliomas has greatly improved. Additionally to the genes involved in the classical syndromic forms: *VHL* gene in von Hippel-Lindau disease, *RET* gene in Multiple Endocrine Neoplasia type 2 (MEN 2), and *NF1* gene Neurofibromatosis type 1, 10 novel genes have so far shown to be implicated in the occurrence of paragangliomas/pheochromocytomas [27–29]. Amongst these, the most relevant are those of the mitochondrial succinate dehydrogenase (SDH) complex subunits genes (*SDHA*, *SDHB*, *SDHC,* and *SDHD*) and one complex cofactor, *SDHAF2*, mainly involved in head and neck and abdominal paragangliomas and initially discovered by Baysal et al. [30–34]. More recently, the *TMEM127*, *MAX*, *HIF2A*, *EGLN1*, *KIF1B,* and *H-RAS* complete the list of susceptibility genes implicated in the development of paragangliomas/pheochromocytomas [35–40]. So far, *H-RAS* mutations have been identified only at a somatic level.

Pheochromocytoma (here meaning catecholamine-secreting paraganglioma) was known as the 10% tumor, meaning that 10% of cases were familial, 10% bilateral, 10% malignant, and 10% extra-adrenal [1]. The 10 percent dogma concerning the hereditary forms of these tumors was completely discarded by a study in 2002 by Neumann et al. [41]. In this study, it was found that 24% of the patients who presented with nonsyndromic pheochromocytoma and without family history of the disease had mutations in *VHL*, *RET*, *SDHD,* and *SDHB* genes. Younger age at presentation (24.9 *versus* 43.9 years of age), multiple tumors (32% *versus* 2%), and presence of extra-adrenal tumors (28% *versus* 8%) were significantly associated with the presence of a mutation [41]. In 2006, a study comprising a larger number of patients with pheochromocytoma/paraganglioma showed that 33% of the patients carried germline mutations in one of the following genes: *VHL*, *RET*, *NF1*, *SDHB,* and *SDHD* [42]. So, it is currently accepted that up to 35% of paragangliomas/pheochromocytomas are associated with an inherited mutation [43, 44].

In this review, we summarize the clinical and genetic aspects of the syndromic and nonsyndromic forms of pheochromocytoma/paraganglioma. The risk of developing pheochromocytoma/paraganglioma will be addressed for each gene. A clinically oriented strategy for genetic testing will be discussed.

2. Genetics of Paragangliomas/ Pheochromocytomas

2.1. Syndromic Forms

2.1.1. von Hippel-Lindau. von Hippel-Lindau (VHL) disease is an autosomal dominant syndrome characterized by a variety of benign and malignant tumors including retinal and central nervous system hemangioblastomas, clear renal cell carcinoma and renal cysts, pheochromocytomas, pancreatic islet cell tumors and pancreatic cysts, epididymal cystadenomas, and endolymphatic sac tumors [27].

This disease affects about 1 in 36 000 live births and is divided into 2 clinical categories according to absence (type 1) or presence (type 2) of pheochromocytomas, respectively [45, 46]. VHL type 2 is further divided in type 2A, identifying patients with low risk of developing clear renal cell carcinoma, type 2B, for patients with high risk of developing clear renal cell carcinoma, and type 2C, for patients that only present pheochromocytomas without the other classical lesions of VHL disease [27]. Pheochromocytomas occur in 10–20% of VHL patients, typically around 30 years, but rare cases have been described below the age of 10. About 5% of pheochromocytomas in VHL disease are malignant [27, 47, 48]. Due to the early onset of these tumors and frequent absence of signs and symptoms, it has been proposed that catecholamine screening should begin at the age of 2,

especially in patients with a familial history of pheochromocytomas [47]. VHL-associated pheochromocytomas secrete mostly norepinephrine due to low or absent expression of phenylethanolamine N-methyltransferase; thus, patients present with increased plasma and urinary normetanephrine [49]. The adrenal medulla is the most common paraganglia affected in VHL type 2 patients but rare sympathetic and parasympathetic paragangliomas have also been described [27, 47, 50]. Pheochromocytomas are often bilateral and generally have a good prognosis [51, 52].

von Hippel-Lindau protein (pVHL) is a tumor suppressor protein that regulates the activity of hypoxia-inducible factor alpha (HIFα) and several other proteins involved in tumorigenesis [53]. In normoxic conditions, pVHL binds to the α subunits of HIF1 and 2, targeting it for ubiquitination and proteasomal degradation. Conversely, in hypoxic conditions or when *VHL* gene is mutated, HIFα is able to interact with HIFβ, inducing the transcription of hypoxia-inducible genes, leading to an increased expression of angiogenic growth and mitogenic factors [53–55]. This disruption of pVHL-mediated degradation of HIF will ultimately contribute to tumor formation through multiple mechanisms [53].

VHL gene was mapped to the short arm chromosome 3 (3p25), it comprises 3 exons that encode for the 2 isoforms of the pVHL protein [56]. More than 150 *VHL* germline mutations have been associated to the VHL disease. These mutations are missense, deletion, nonsense, or frameshift mutations and are distributed throughout the coding sequence [57, 58]. Although genetic testing studies have been able to identify mutations in virtually every VHL-affected family, diagnosis is still challenging in up to 20% of affected kindreds in which a *de novo* mutation occurs [58, 59]. Genotype-phenotype correlation studies have shown that VHL type 1 families frequently harbor *VHL* deletions or nonsense mutations, whereas families at risk for developing pheochromocytoma (type 2 families) almost invariably present with *VHL* missense mutations [57, 58, 60]. Particularly, missense mutations at codon 167 were associated with a high risk of developing pheochromocytoma (53% and 82% at ages 30 and 50 years, resp.) [60]. *VHL* mutations associated with the phenotype 2A or 2B have been shown to affect the proteasomal degradation of HIF1, whereas type 2C mutations do not disrupt the ability of pVHL to downregulate HIF1, suggesting that pheochromocytoma formation is not related with HIF1 expression levels [61, 62]. It has been proposed that VHL-associated pheochromocytoma tumorigenesis is related with an abnormal extracellular matrix formation and to upregulation of tyrosine hydroxylase, leading to increased catecholamine synthesis [61, 63, 64].

2.1.2. Multiple Endocrine Neoplasia Type 2. Multiple endocrine neoplasia type 2 (MEN 2) is an autosomal dominant cancer syndrome characterized by the association of medullary thyroid carcinoma (MTC), pheochromocytoma, and hyperparathyroidism [29]. Depending on the most frequent manifestations, there are three subtypes MEN 2A, MEN 2B, and Familial MTC (FMTC): in MEN 2A patients, MTC is present in virtually all patients, unilateral or bilateral pheochromocytoma in 50% of cases, and multigland parathyroid tumors in 20–30% of cases; in MEN 2B patients, the third component (hyperparathyroidism) is not present, MTC has an earlier onset, and there are developmental alterations such as multiple mucosal ganglioneuromas and a "marfanoid" habitus; in FMTC patients, MTC is the single manifestation [65–67]. MEN 2A is the most frequent subtype representing over 75% of MEN 2 cases [66]. It is now accepted that FMTC might be a variant of MEN 2A with a lower clinical penetrance of pheochromocytoma [67, 68].

The genetic basis for MEN 2 syndrome lies within the long arm of chromosome 10 (10q11.2), where the *RET* (REarranged during Transfection) protooncogene is located. It comprises 21 exons that encode for a tyrosine transmembrane receptor with three domains: extracellular, transmembrane, and intracellular. When a ligand of the glial-derived neurotropic factor (GDNF) family binds to RET protein, it triggers RET dimerization and autophosphorylation, inducing a signaling phosphatidylinositol 3′-kinase- (PI3K-) mediated cascade that regulates cell proliferation and apoptosis. This requires the presence coreceptors of the GDNF family receptor-α1–4 (GFR-α1–4) at the cell surface [69].

MEN 2 subtypes have been associated with specific *RET* mutations. More than 98% of MEN 2A families present with missense mutations in one of five codons: 609, 611, 618, 620 (exon 10), or 634 (exon 11). Codon 634 mutations represent almost 90% of MEN 2A cases and a cysteine to arginine substitution at this codon (p.Cys634Arg) is found in more than 50% of cases [70–73]. All these mutations affect cysteine residues in the RET extracellular domain and induce a ligand-independent dimerization of RET, leading to a constitutive activation of its intrinsic tyrosine kinase [74–76]. About 80% of patients with FMTC present with a similar mutational spectrum of MEN 2A, but mutations are relatively evenly distributed among codons 618, 620, and 634 [70–73]. Interestingly, the p.Cys634Arg mutation is almost never found in FMTC families [67]. Generally, MEN 2B tumors are a consequence of mutations in the substrate binding pocket of the RET tyrosine kinase. A single missense mutation in codon 918 (p.Met918Thr) is responsible for over 90% of MEN 2B cases, whereas other rare mutations have been described in exons 14 and 15 [69, 71–73]. The American Thyroid Association (ATA) proposed the categorization of patients into four risk levels (A to D) based on the mutation identified and on the genotype-phenotype correlation. Clinical recommendations concerning prophylactic surgeries in asymptomatic individuals depend on the attributed risk level [67].

Pheochromocytomas in MEN 2A and B syndromes are generally benign tumors and bilateral in >50% of the patients [29]. Extra-adrenal paragangliomas have been described but are very rare [29, 77]. The biochemical phenotype of these tumors is increased plasma and urinary levels of metanephrine as a result of epinephrine hypersecretion, possibly due to overexpression of phenylethanolamine N-methyltransferase [49]. Large cohort series show that malignancy affects less than 5% of MEN 2-associated pheochromocytomas [78, 79].

Based on a large study enrolling 323 MEN 2A patients, Quayle et al. reported an overall penetrance of pheochromocytoma of 32%, with a median age at diagnosis of

34 years; the earliest pheochromocytoma was observed at 15 years; bilateral pheochromocytomas were observed in 66% of patients; the following codon-specific expression of pheochromocytoma was observed: codon 634 was expressed in 50%, codon 618 was expressed in 22%, codon 620 was expressed in 9%, and codon 609 was expressed in 4%. The mean age at diagnosis did not differ amongst these codon-grouped patients [80].

Childhood pheochromocytoma is rare in MEN 2, but reports at 12 years of age have occurred for both the 918 and 634 *RET* mutations [79, 81]. Therefore, the ATA recommends that pheochromocytoma screening (by plasma or 24-hour urine fractionated metanephrines) should begin by age 8 in carriers of *RET* mutations associated with MEN 2B and mutated *RET* codons 634 and 630 and by the age 20 years in carriers of other MEN 2A *RET* mutations. Patients with *RET* mutations associated only with FMTC should be screened at least periodically from the age of 20 years [67].

2.1.3. Neurofibromatosis Type 1. Neurofibromatosis type 1 (NF 1), or von Recklinghausen's disease, is an autosomal dominant disorder clinically diagnosed by six or more *cafe au lait* macules; two or more cutaneous/subcutaneous neurofibromas or a single plexiform neurofibroma; axillary or inguinal freckling; optic nerve glioma; two or more Lisch nodules (iris hamartomas); dysplasia of long bones or pseudarthrosis; and a first degree relative with NF1 [28, 82]. Patients with NF 1 are also at higher risk than general population of developing various tumors such as peripheral nerve sheath tumors, gastrointestinal stromal tumors, rhabdomyosarcoma, breast cancer, and pheochromocytomas [83, 84]. Worldwide birth incidence of NF 1 is 1 in 2 500–3 000 and prevalence is at least 1 in 4 000 [84].

NF 1 is caused by loss of function mutations in the tumor-suppressor *NF1* gene [85]. This gene is located on chromosome 17q11.2 comprising 60 exons that encode for neurofibromin, a negative regulator of RAS proteins. Neurofibromin is a GTPase activating protein that promotes the conversion of active RAS-GTP to its inactive form, RAS-GDP. Mutations in *NF1* gene result in constitutive activation of RAS activity triggering a kinase cascade and the activation of mitogen-activated protein kinases (MAPK), mammalian target of rapamycin (mTOR), and PI3 K pathways, therefore regulating the transcription of genes associated with cell proliferation, cell death, differentiation, and migration [86]. Mutational analysis in NF 1 patients remains a considerable challenge due to the occurrence of different types of mutations (nonsense, missense, or deletions) that span the entire length of the *NF1* gene, the presence of 36 pseudogenes, and the fact that nearly half of NF 1 cases present *de novo* mutations [87, 88].

Pheochromocytomas are a rare feature in NF 1, affecting approximately 0.1% to 6% of all patients [83, 89]. A prevalence rate as high as 13% has been reported in autopsy series, suggesting that the diagnosis of pheochromocytoma may be missed in some NF 1 patients [89]. The mean age at presentation of pheochromocytoma is 42 years. The majority of patients have unilateral adrenal tumors, whereas 10% of patients present with bilateral and 6% abdominal tumors. Malignant pheochromocytomas were identified in 12% of the NF 1 patients [42, 89]. Similarly to MEN 2-associated pheochromocytomas, in NF 1 these tumors have been shown to produce more epinephrine and less norepinephrine, resulting in increased levels of metanephrine [49]. Although pheochromocytoma NF 1-associated is rare, due to the risk of malignancy, it has been proposed that any patient with hypertension/paroxysmal hypertension or with symptoms of catecholamine excess, such as headache, sweating, palpitations, or anxiety, should undergo measurement of 24-hour urine or plasma metanephrines [28].

Unlike mutations in VHL or MEN 2 disorders, NF 1 mutations that offer an increased risk in pheochromocytoma remain to be identified. A study carried out by Bausch et al. in NF 1 patients with associated pheochromocytoma showed that the cysteine-serine rich domain was affected in 35% of the cases whereas the Ras GTPase activating protein domain in only 13%, suggesting that the cysteine-serine rich could play a role in the formation of NF1-associated pheochromocytoma. Moreover, in accordance with the Knudson's two-hit theory that states that pheochromocytoma development requires biallelic inactivation, loss of heterozygosity (LOH) was shown in NF 1-related pheochromocytoma. No association was found between *NF1* mutational genotype and the clinical features of pheochromocytoma [90].

2.2. Familial Paraganglioma Syndromes (SDHx and SDHAF2). Familial paraganglioma syndromes (PGLs) are a group of autosomal dominant disorders responsible for the development of paragangliomas/pheochromocytomas caused by mutations in the genes encoding for the succinate dehydrogenase (SDH) mitochondrial complex. SDH or respiratory complex II is an enzyme complex that catalyses the oxidation of succinate to fumarate in the Krebs cycle and participates in the electron transport chain [91]. SDH is composed of 4 subunits encoded by the corresponding genes: *SDHA*, *SDHB*, *SDHC,* and *SDHD*. Complex subunits A (flavoprotein) and B (iron-sulfur protein) constitute the catalytic core of the enzyme, while subunits C and D anchor the complex to the inner mitochondrial membrane. In general, inactivating mutations in one of the *SDHx* genes leads to accumulation of succinate and formation of reactive oxygen species, stabilizing HIFα and activating hypoxia-dependent pathways [91]. Four PGL syndromes have been described: types 1, 2, 3, and 4, caused by mutations in the *SDHD*, *SDHAF2* (responsible for the flavination of subunit A), *SDHC,* and *SDHB*, respectively [30–33]. Immunohistochemistry can be used to triage genetic testing of paraganglioma/pheochromocytoma. Particularly for SDHB immunohistochemistry, a negative staining is more commonly found associated with *SDHB* mutation, whereas a weak diffuse staining often occurs with *SDHD* mutation [92, 93]. Functioning *SDHx* paragangliomas sometimes release dopamine and/or norepinephrine, originating raised plasma levels of methoxytyramine, contributing to distinguish *SDHx* patients from those with *VHL*, *RET,* or *NF1* mutations [49, 94]. However, methoxytyramine should be regarded as a useful biomarker of malignancy in the setting of paraganglioma/pheochromocytoma independent of the underlying gene. Penetrance and clinical presentation of PGL syndromes varies significantly with the underlying mutation [95].

2.2.1. PGL 1 Syndrome. PGL 1 syndrome is caused by mutations in *SDHD* gene, which are inherited in an autosomal dominant fashion with a predominant paternal transmission, suggesting a maternal imprinting of this gene [30, 34, 44, 96]. However, rare cases of maternal transmission have been described and the precise mechanism responsible for this parent-of-origin effect remains to be elucidated [97–99]. A three-hit model has been hypothesized requiring a *SDHD* mutation, loss or mutation of the wild-type *SDHD* allele, and loss of a further imprinted (paternally silenced and maternally active) tumor suppressor gene from chromosome 11 [99, 100]. PGL 1 patients generally present with multiple benign parasympathetic head and neck paragangliomas, but multiple sympathetic and adrenal tumors are also very frequent. In fact, Neumann et al. have shown that among 34 patients with mutations in *SDHD* gene, 79% had head and neck paraganglioma, 53% had pheochromocytoma, and 39% thoracic/abdominal paraganglioma, whereas 74% of the patients presented with multiple tumors [96]. Mean age at presentation is around 30 years [43, 96, 101, 102]. Ricketts et al. estimated the risk of developing head and neck paragangliomas at 71% and the risk of pheochromocytoma at 29%, at age 60 [102]. Malignancy has rarely been found in *SDHD*-derived sympathetic or parasympathetic paragangliomas [43, 96, 101–105]. Several different mutations have been described in exons 2–4 of *SDHD*, mainly nonsense, missense, and frameshift, but its relation with the phenotypic expression of the disease is still unclear [43, 96, 101, 102].

2.2.2. PGL 2 Syndrome. Familial PGL 2 syndrome is a very rare condition characterized by multiple head and neck paragangliomas, of which only few cases have been reported [106, 107]. It happens as a consequence of mutations in *SDHAF2* gene (also known as *SDH5*) that encodes for a succinate dehydrogenase complex assembly factor 2 (SDHAF2), which is responsible for the flavination of SDHA enabling SDH complex activity [31]. To our knowledge, only two apparently unrelated kindreds (of Dutch and Spain origin) have been described as carriers of a missense mutation in this gene, c.232G > A (p.Gly78Arg) [31, 106, 107]. Both kindreds show a high penetrance for this mutation, which has a paternal mode of transmission. Among the 16 mutations carriers of the largest branch of the Dutch family, considered as at-risk patients, 11 patients had head and neck tumors, out of which 10 had multiple tumors (91%). The mean age of diagnosis was 33 years [107].

The scarcity of *SDHAF2* mutations was reinforced by the failure to document mutations in this gene among 315 patients with paraganglioma and without mutations in the *SDHD*, *SDHC,* or *SDHB* genes. Nonetheless, it is justified to screen for *SDHAF2* mutations in young patients with isolated head and neck paragangliomas or in individuals with familial antecedents who are negative for other risk genes [106].

2.2.3. PGL 3 Syndrome. Mutations in the *SDHC* gene are causative for familial PGL syndrome 3, which has an autosomal dominant mode of transmission without a parent-of-origin effect [32]. This is a rare condition characterized by benign parasympathetic head and neck tumors, but rare cases of sympathetic paragangliomas and pheochromocytomas have been described [44, 108–111]. In the studies by Burnichon et al. [44] and Schiavi et al. [112], the mean age at presentation was 38 (17–70) and 46 years (13–73), respectively.

About 4% of paraganglioma patients carry mutations in the *SDHC* gene [44, 112]. Different types of mutations (missense, nonsense, splicing, deletions, and insertions) encompassing the whole *SDHC* gene have been found [44, 105, 109, 112]. Malignancy associated with *SDHC* gene is extremely rare with only two cases described so far, with distinct causal mutations [113, 114].

2.2.4. PGL 4 Syndrome. Familial PGL 4 syndrome is characterized by abdominal and pelvic catecholamine-secreting paragangliomas, which can also be present in adrenal medulla and head and neck [94, 102, 112, 115]. Symptoms are those classically associated with paraganglioma/pheochromocytoma (headache, palpitations, and diaphoresis) but can also be due to a mass effect rather than catecholamine secretion [94]. Mean age at diagnosis is around 32 years [94, 96, 102, 115]. Primary tumors are usually large and associated with a high rate of malignancy ranging from 31 to 72% of patients [26, 94, 96, 115].

Germline mutations in *SDHB* gene, which encodes for the iron sulfur subunit of the SDH complex (subunit B), are responsible for PGL 4 familial syndrome [33]. Functional assays have shown that these mutations lead to stabilization of HIF1α, causing overexpression of hypoxia-induced angiogenic pathway genes, such as VEGF (vascular endothelial growth factor) and EPAS1 (endothelial PAS domain protein 1), providing therefore support for tumor growth [116–118]. Loss of heterozygosity has been shown to occur as a consequence of *SDHB* mutations [33, 116]. Of interest, mutations in *SDHB* gene have also been associated with an increased susceptibility to develop other neoplasms, namely, renal cell carcinoma, gastrointestinal stromal tumors, papillary thyroid cancer, and neuroblastoma [96, 102, 115, 119].

A wide spectrum of *SDHB* mutations have been found associated with PGL 4, namely, missense, frameshift, splicing, nonsense, and large deletions. However, several studies have failed to unveil genotype-phenotype correlations, particularly in what concerns tumor location, age of presentation, and aggressiveness of the tumor [94, 120]. Since mutations in *SDHB* gene are the most frequent cause of metastatic paraganglioma tumors, it has consistently been proposed that all patients presenting with malignant paraganglioma/pheochromocytoma should be tested for *SDHB* gene mutations.

2.2.5. SDHA. The long-sought link between *SDHA* gene and paraganglioma development was only unveiled in 2010, when a patient with an extra-adrenal paraganglioma was found to have an *SDHA* missense mutation [34]. Functional studies show that *SDHA*, like other *SDHx* genes, operates as tumor suppressor gene and activates the pseudohypoxic pathway leading to tumorigenesis. Furthermore, in accordance with Knudson's two-hit hypothesis, it was shown that the *SDHA*-mutated tumors have lost the wild type allele

[34, 121]. The few *SDHA*-affected individuals described so far have presented with distinct phenotypic characteristics: pheochromocytoma, sympathetic (abdominal and thoracic), and parasympathetic head and neck paragangliomas [34, 121–123]. The reported age at diagnosis is highly variable. Missense and nonsense mutations have been found, without any genotype-phenotype correlation [34, 121–123]. Recently, *SDHA* gene mutations have also been implicated in the development of gastrointestinal stromal tumors [124, 125].

2.3. Other Susceptibility Genes

2.3.1. TMEM127. *TMEM127* is a tumor suppressor gene initially identified as a pheochromocytoma susceptibility gene [37] and later also associated with the development of paragangliomas of head and neck and extra-adrenal abdominal paragangliomas [126–130]. *TMEM127* gene encodes a highly conserved transmembrane protein, transmembrane protein 127, which is associated with several cellular organelles and thought to limit mTORC1 activation thus controlling protein synthesis and cell survival [37]. Mutations in this gene are inherited in an autosomal dominant fashion and induce tumor development by enhancing the kinase-dependent signaling pathways, similarly to mutated *RET* and *NF1* genes [37]. Patients may present either unilateral or bilateral pheochromocytomas. The mean age at diagnosis is around 42 years and the risk of malignancy is very low (≈1%). The prevalence of *TMEM127* mutations in patients with paraganglioma/pheochromocytoma varies between 0.9 and 2%. Different missense, frameshift, or nonsense *TMEM127* mutations have been found across the three exons of the gene [126, 129].

2.3.2. MAX. Comino-Méndez et al. identified mutations in *MAX* gene as responsible for the development of bilateral pheochromocytoma in eight index patients [38]. This association was further confirmed by another study comprising 1,694 patients with paraganglioma/pheochromocytoma [131]. The latter study documented *MAX* germline mutations in 23 nonrelated patients, all with adrenal tumors; among the 19 patients considered for phenotypic associations, 13 (68%) presented with bilateral or multifocal pheochromocytoma and 16% developed additional thoracoabdominal paragangliomas [131]. Median age at diagnosis was 34 years and 37% of the patients had familial antecedents. Overall, *MAX* germline mutations were found in 1.12% of patients without other mutations [131]. Both studies presented patients with metastatic disease, but further research is required to ascertain the risk of malignancy associated with *MAX* mutations [38, 131]. *MAX* tumors have an intermediate biochemical phenotype with a predominant normetanephrine release [131, 132].

MAX (myc-associated factor X) gene is a tumor suppressor gene that encodes for MAX protein, which is a component of the MYC-MAX-MXD1 complex that regulates cell proliferation, differentiation, and apoptosis [38, 133]. Mutations in *MAX* gene have a paternal mode of transmission and are responsible for the loss of the wild type allele with consequent abrogation of protein expression. Consequently, inhibition of MYC-dependent cell transformation by MAX protein is disrupted, causing tumor development [38].

2.3.3. HIF2A and EGLN1. As stated before in the context of VHL disease, HIFα proteins (HIF1α, HIF2α, and HIF3α) are transcription factors that respond to oxygen concentrations in tissues. Under hypoxic conditions, stabilization of HIFα proteins occurs, allowing transcription of genes involved in angiogenesis, glycolysis, erythropoiesis, apoptosis, proliferation and growth [134]. Mutations in *VHL* and *SDHx* genes have been shown to induce pseudohypoxic states that induce the development of paragangliomas/pheochromocytomas. In 2012, Zhuang et al. described two somatic mutations in the gene encoding of the hypoxia-inducible factor 2α (*HIF2A*) in two patients with polycythemia and multiple paragangliomas (one of the patients also presented with somatostatinomas). Functional assays show that both mutations affected pVHL hydroxylation, impairing HIF2α degradation leading to an intact/increased transcriptional activity of genes downstream of HIF2α, such as *VEGFA* and erythropoietin [39]. These findings were further corroborated by other recent studies that confirmed somatic *HIF2A* gain-of-function mutations as causative for the development of polycythemia and multiple paragangliomas/pheochromocytomas and somatostatinomas in patients, corresponding to a novel syndrome [135–142]. The occurrence of multiple tumors presenting the same somatic mutations without familial history suggests the occurrence of a *de novo* postzygotic event early in the embryogenesis [39, 138]. Somatic mutations in *HIF2A* have also been identified in sporadic pheochromocytomas/paragangliomas in the absence of erythrocytosis [137].

EGLN (egg-laying-defective nine) family of proteins (also called PHD or HPH) are responsible for hydroxylation of prolyl residues of HIFα under normoxic conditions, allowing pVHL binding and proteosomal degradation of HIFα proteins [134]. The association between EGLN proteins and paraganglioma development was first established by Ladroue et al., by reporting a patient presenting with erythrocytosis and recurrent abdominal paragangliomas who carried a germline mutation in the *EGLN1* gene (formerly known as *PHD2*) [35]. Loss of heterozygosity involving the tumor wild type *EGLN1* allele suggests that *EGLN1* may act as tumor-suppressor gene. Functional studies indicate stabilization of HIF2α in the presence of EGLN1 mutant protein [35]. Additional research is required to disclose the role of *EGLN1* mutations in paragangliomas.

2.3.4. H-RAS. The RAS-ERK pathway has long been associated with the development of cancer [143]. Regarding paragangliomas/pheochromocytomas, it is currently accepted that there are 2 distinct tumorigenesis clusters according to their transcriptional profile: a pseudohypoxic cluster (associated with mutations in *VHL/SDHx/EGLN1* genes) and a kinase receptor-signaling cluster (associated with *RET/NF1/TMEM127/MAX/KIF1B* mutations) [144]. Evidence for a novel link between the latter cluster and paraganglioma development has been provided by Crona et al., through

the identification of somatic mutations in *H-RAS* gene in four male patients presenting with pheochromocytoma (3 patients) and paraganglioma (1 patient) [40]. Very recently, the same authors have described an additional *H-RAS* somatic mutation in a patient with unilateral pheochromocytoma [145].

2.3.5. KIF1B. Kinesin family member 1B (*KIF1B*) gene expression results in two protein isoforms, KIF1Bα and KIF1Bβ, which are motor proteins involved in the anterograde transport of mitochondria and synaptic vesicle precursors, respectively [146, 147]. Schlisio et al. firstly associated two *KIF1B* missense mutations as causative of pheochromocytoma in two tumor samples [36]. It was also shown that KIF1Bβ acts downstream from oxygen-dependent prolyl hidroxylase EGLN3 (or PHD3) to induce apoptosis. These loss of function mutations in KIF1Bβ could therefore protect neuroblasts from apoptosis, leading to tumor development [36]. This study was further extended to five relatives of a patient harboring a germline *KIF1B* mutation. These individuals presented unilateral or bilateral pheochromocytoma and other nonneural crest-derived malignancies, such as ganglioneuroma, leiomyosarcoma, and lung adenocarcinoma [148]. Transcriptional analysis of KIF1Bβ mutant pheochromocytomas showed that these tumors are transcriptionally related to *RET* and *NF1*-associated tumors.

3. Genetic Testing Strategy in Paraganglioma/Pheochromocytoma

According to the general recommendation for genetic screening of the American Society of Clinical Oncology, all patients with a risk of at least 10% of carrying a genetic mutation should be offered genetic testing [149]. It is currently accepted throughout the literature that about 35% of paraganglioma/pheochromocytoma cases are due to germline mutations in one of the formerly described genes [41–43, 150, 151]. Therefore, it has been proposed by several authors that genetic testing should be performed to all paraganglioma/pheochromocytoma patients [6, 41, 96, 151, 152].

In the clinical setting, hereditary paraganglioma/pheochromocytoma syndromes should be considered in all individuals with paragangliomas and/or pheochromocytomas, particularly those with tumors that are multiple and recurrent and have an early onset (age < 45 years). Absence of a known family history is not enough to exclude this hypothesis. However, there are subgroups of patients with a very low risk as it is the case for those with apparently sporadic paraganglioma/pheochromocytoma after age 50 [65, 151].

There are several meaningful motives in favor of offering the genetic testing to paraganglioma patients. The inherited syndromic forms caused by mutations in *VHL*, *RET,* and *NF1* genes are associated with other malignant tumors. Thus, an early diagnosis of patients will allow an improved lifelong surveillance and forehand treatment with a consequent improved prognosis [27–29]. On the other hand, patients with germline mutations are more likely to have multiple and recurrent paragangliomas/pheochromocytomas, which requires a more close follow-up [6]. Molecular testing of relatives will clarify their genetic status allowing excluding those who did not inherit the mutation from unnecessary and costly diagnostic procedures.

Advanced techniques like whole genome sequencing or next-generation sequencing appear to be promising genetic strategies for testing paraganglioma patients [153–155]. Nevertheless, these techniques are still unavailable for many genetic laboratories or remain far from being cost-effective. To overcome a time consuming gene-after-gene analysis and the difficulties associated with the overlapping clinical features of several syndromic and sporadic forms, different algorithms have been proposed based on a sequential approach and taking into account the patient's family history and clinical presentation. Particular aspects such as the localization of the primary tumor, the biochemical profile, and age at diagnosis are considered of extreme relevance to orient the genetic study. Herein, we propose an algorithm (Figure 1) for genetic testing of paraganglioma/pheochromocytoma patients that incorporates clinical data as well as information derived from previous analytical reviews [48, 65, 150, 151, 156].

Patients presenting specific syndromic features or a positive familial history should be considered for the analysis of the specific genes: *VHL*, *RET*, *SDHB*, *SDHD*, *NF1,* or *HIF2A* [27–30, 33, 39]. For instance, the presence of hemangioblastomas (suggestive of von Hippel-Lindau) or medullary thyroid carcinoma along with pheochromocytoma (suggestive of MEN 2A) strongly implies mutations in *VHL* or *RET* gene, respectively [27, 29]. The coexistence of pheochromocytoma with interscapular pruritic lesions strongly suggests a mutation in codon 634 of the *RET* gene [157]. Expression of disease associated with a paternal transmission mode, consistent with maternal imprinting, orients genetic testing towards specific genes such as *SDHD* or, more rarely, *SDHAF2* [30, 106]. Adrenal pheochromocytomas (unilateral or bilateral) are more frequently associated with *VHL* and *RET*. Thus, *SDHB*, *SDHD*, *TMEM127,* or *MAX* genes should be considered only in a second step [37, 47, 80, 112, 131]. Extra-adrenal sympathetic paragangliomas (abdominal or thoracic) are more frequently caused by *SDHB*, *SDHD,* and *VHL* mutations [47, 77, 112]. Head and neck tumors are more frequently caused by *SDHD* (especially in presence of multiple tumors) and *SDHB* gene mutations and less often by *VHL* and *SDHC* gene mutations [44, 50, 96]. If patients are negative for mutations in these genes, *SDHAF2* might be considered for analysis [106]. Malignant tumors have been strongly associated (>30%) with germline mutations in *SDHB*, so initial analysis should address this gene [96]. If negative, then *VHL*, *NF1*, *SDHD,* or *MAX* genes can be considered for investigation [48, 96, 131]. It should be emphasized that mutations in the above-described genes may result in atypical phenotypes therefore rendering oriented genetic testing more complex.

A few and quick ways are likely to improve cost-effectiveness of molecular genetic testing. For instance, in VHL disease-associated pheochromocytoma, codon 167 of *VHL* gene appears as a first target, since missense mutations in this codon are strongly associated with pheochromocytoma development [60]. In addition, we should be aware

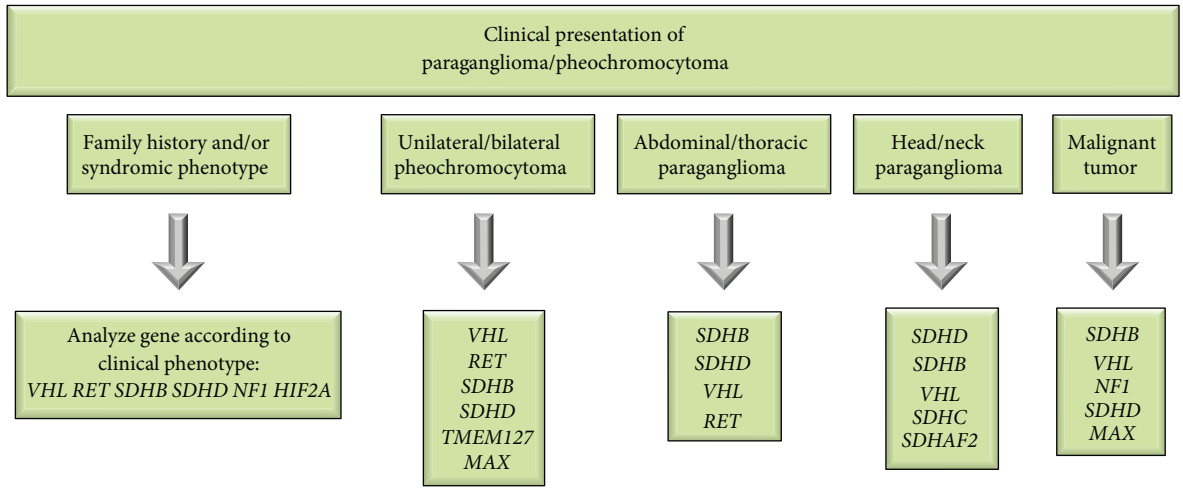

Figure 1: Proposed algorithm for molecular genetic testing of paraganglioma/pheochromocytoma patients. The genes depicted in the boxes are most likely to account for the clinical phenotype and should be analyzed in the proposed order. Mutations in *TMEM127*, *MAX*, *HIF2A*, and *SDHAF2* are extremely rare, so they should only be analyzed when patients are negative for the other gene mutations.

of founder mutations already reported for *SDHx* genes in different countries such as the Netherlands, Poland, Italy, Spain, and Portugal in order to develop effective screening protocols [158–162].

Partial or large deletions may respond for false-negative results, when using conventional PCR followed by automatic sequencing techniques. Large deletions account for about 10% of the cases of *SDHx*-related paragangliomas [44]. Ideally, laboratories would routinely use methods for searching large genomic deletions such as quantitative multiplex PCR of short fluorescent fragments (QMPSF) or multiplex ligation-dependent probe amplification (MLPA) in order to minimize the risk of false-negative results.

Identification of a mutation allows tailoring treatment and follow-up therefore contributing to a better prognosis. The same holds true for the patients' relatives. In the specific cases of *RET*-associated pheochromocytoma, young relatives carriers of *RET* mutations may undergo prophylactic thyroidectomy to prevent the development of medullary thyroid carcinoma [67]. On the other hand, due to the higher risk of malignancy in patients carrying *SDHB* gene mutations, a closer biochemical and imaging follow-up might be provided in order to prevent the development of metastatic disease [95].

4. Conclusions and Future Perspectives

A great deal of knowledge has been added to the genetics of paragangliomas since the beginning of the millennium. Until then, the genes responsible for inheritable forms of paragangliomas were restricted to those underlying the syndromic forms of the disease; *RET* gene in multiple endocrine neoplasia type 2, *VHL* in von Hippel-Lindau disease; and *NF1* in neurofibromatosis type 1. The discovery of the succinate dehydrogenase genes associated with the development of familial paraganglioma syndromes, in particular the *SDHB* gene, frequently associated with malignant tumors, brought new insights into the management and prognosis of paragangliomas.

So far, at least 14 genes (*RET*, *VHL*, *NF1*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *TMEM127*, *MAX*, *EGLN1*, *HIF2A*, *H-RAS*, and *KIF1B*) have been associated with the development of paragangliomas. These genes have been divided into two tumorigenesis clusters: a pseudohypoxic cluster (associated with mutations in *VHL*/*SDHx*/*EGLN1*/*HIF2A* genes) and a kinase receptor-signaling cluster (associated with *RET*/*NF1*/*TMEM127*/*MAX*/*KIF1B* gene mutations). Functional studies involving these genes and paraganglioma-associated mutations as well as gene expression profiles of tumor samples have greatly contributed to our understanding of tumorigenic pathways of paragangliomas. Progresses in genetic knowledge and the evidence for genotype-phenotype correlations have largely influenced the care of patients with positive impact.

In this review, we summarized the most relevant aspects regarding the genetics and clinical aspects of the syndromic and nonsyndromic forms of pheochromocytoma/paraganglioma aiming to provide an algorithm for genetic testing. Recent comprehension of the molecular pathways involved in the tumorigenesis of paragangliomas is likely to be improved by further functional assays, possibly hinting novel molecular-targeted therapy approaches.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Adrenal Incidentalomas: Should We Operate on Small Tumors in the Era of Laparoscopy?

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Tumor size smaller than 4 cm as an indication for surgical treatment of incidentaloma is still a subject of discussion. Our aim was the estimation of the incidence of malignancy and analysis of treatment outcomes in patients with incidentaloma smaller than 4 cm in comparison to bigger lesions. 132 patients who underwent laparoscopic adrenalectomy for nonsecreting tumors were divided into two groups: group 1 (55 pts., size < = 40 mm) and group 2 (77 pts., size > 40 mm). Operation parameters and histopathological results were analyzed. No differences in group characteristics, mean operation time, and estimated blood loss were noted. Complications in groups 1 and 2 occurred in 3.6% and 5.2% of patients, respectively ($P = 0.67$). Malignancy in groups 1 and 2 was present in 1 and 6 patients, respectively ($P = 0.13$). Potentially malignant lesions were identified in 4 patients in group 1 and 4 patients in group 2 ($P = 0.39$). The results do not allow for straightforward recommendations for surgical treatment of smaller adrenal tumors. The safety of laparoscopy and minimal, but impossible to omit, risk of malignancy support decisions for surgery. On the other hand, the risk of malignancy in smaller adrenal tumors is lower than surgical complications, which provides an important argument against surgery.

1. Introduction

Incidentalomas are a group of hormonally inactive adrenal tumors incidentally found during imaging studies performed in patients due to symptoms unrelated to adrenal tumors. Patients diagnosed and treated for neoplastic disorders of other organs are excluded as metastasis to the adrenal glands should be suspected at first place in that group of patients [1]. The prevalence of incidentaloma grows with the patients' age [2]. A steady increase in this diagnosis can also be observed over the last few decades. According to the latest data such lesions can be found in 4–10% of patients [1, 3, 4]. This greater incidence of hormonally inactive tumors results at least partially from the higher quality and precision of imaging techniques available today compared to those at the end of the previous century. Nowadays imaging is based on high resolution computed tomography (CT) or magnetic resonance imaging (MRI) as opposed to ultrasound (US), which was used for diagnosis in the past. However, it also seems impossible to exclude the fact that there is a real increase in the incidence of incidentaloma in the general population [1, 5].

Even though current guidelines clearly list the indications for adrenalectomy (tumor size, abrupt growth found in follow-up studies, or radiologic appearance suggesting a malignant lesion), the final decision whether to operate or to follow up belongs to the surgeon [6]. The greatest concern is the potentially malignant character of an adrenal tumor. Adrenal cortical carcinoma, which is quite rare, is, like other even more rare malignancies, found mainly in large tumors [3]. Despite this fact, evidence of possible malignancy in smaller adrenal tumors also exists [2]. The poor prognosis associated with this type of tumors can prompt a more radical approach also in the case of smaller lesions [7–9]. Different values of tumor size as indication for surgical treatment can

Table 1: Characteristics of the study groups of patients.

	Group 1 (tumor < 4 cm), $n = 55$	Group 2 (tumor ≥ 4 cm), $n = 77$	P value
Female/male	39 (71%)/16 (29%)	52 (68%)/25 (32%)	0.69
Left side/right side	29 (53%)/26 (47%)	40 (52%)/37 (48%)	0.93
Mean age	57 years (±11.4) (24–76)	52.9 years (±14.1) (19–81)	0.15
Average tumor size	3.13 cm (±0.57) (1.2–3.8)	5.63 cm (±2.2) (4–16)	<0.001

be found in various sources in the literature. The size quoted usually ranges from 3 to 5 cm, and one of the most commonly used is 4 cm [6, 10, 11]. With an increase in the upper range of tumor size the risk of missed potentially resectable malignancy increases. On the other hand, an overconfident belief in the safety of minimally invasive procedures may lead to an unjustified broadening of the inclusion criteria for surgery in the case of smaller tumors. This can expose a larger number of patients to postoperative complications without obvious benefits.

2. Aim

The aim of this study is the evaluation of the incidence of malignant or potentially malignant lesions in patients with hormonally inactive adrenal tumors smaller than 4 cm in diameter in comparison to the group of patients with lesions greater than 4 cm in diameter. The second aim was the analysis of early treatment results in relation to the tumor size.

3. Materials and Methods

The study focused on the retrospective analysis of patients who underwent laparoscopic lateral transperitoneal adrenalectomy between 2003 and 2013. The inclusion criterion was the presence of an adrenal tumor incidentally discovered in imaging studies (computed tomography, CT; magnetic resonance imaging, MRI; ultrasound, US). During diagnostic workout hormonal activity and active malignant processes of other origins were excluded. Patients with a previous history of malignancies were excluded as well. The main indication for adrenalectomy in case of hormonally inactive tumors was size above 40 mm. In case of smaller lesions the decision on surgery was based on significant growth in follow-up CT/MRI or a so-called malignant phenotype discovered in imaging.

In the analyzed period 468 adrenalectomies were performed at our center. In 319 patients the indication for the surgical treatment was the presence of a hormonally active adrenal tumor, in case of 17 patients, metastasis from another source. The study group consisted of the 132 remaining patients with hormonally inactive and incidentally discovered tumors. Patients were divided into two groups, according to tumor size. Group 1 included 55 patients (39 females and 16 males) with a tumor not larger than 40 mm in diameter. Group 2 included 77 patients (52 females and 25 males) with a tumor larger than 40 mm in diameter. Table 1 presents the characteristics of the study groups. Operation time, conversion rate, perioperative complications, and histopathological results were analyzed. Blood loss was measured by volume in the suction container. This method was sufficiently accurate, as irrigation was never used in case of laparoscopic adrenalectomy. During the evaluation of the pathological results, the lesions were classified into one of 3 categories: benign, potentially malignant, and malignant. Tumors were classified as potentially malignant if they did not present overt features of malignancy intraoperatively or histologically; however, as we have known from the literature, they occasionally show malignant behavior [12].

Chi-square, Student's t-, and Pearson correlation tests were used in statistical analysis. $P < 0.05$ was considered significant.

4. Results

The characteristics of the study groups are presented in Table 1. There were no statistically significant differences between groups in regard to the gender, age, or the side where the tumor was located.

There were no conversions to open surgery in group 1 and in group 2 conversions were necessary in two cases ($P = 0.34$). Reasons for conversions included adhesions after previous surgeries in one patient and tumor capsule rupture in a patient with an 8 cm tumor highly suspected to be malignant. Mean operation time in group 1 was 77 min., while in group 2 81 min. ($P = 0.713$; Figure 1).

Mean estimated blood loss in group 1 was 37 mL and 81 mL in group 2. No statistically significant difference was shown between the groups ($P = 0.136$; Figure 2).

Complications occurred in two (3.6%) patients in group 1 (wound infection and pleural effusion, respectively; 1st and 2nd grade in the Clavien-Dindo classification). Complications in group 2 occurred in four (5.2%) patients. They included diaphragm injury, inferior vena cava injury that was sutured laparoscopically during the primary procedure (3rd grade in the Clavien-Dindo classification), hematoma in the removed adrenal tumor's bed (2nd grade), and wound infection (1st grade in the Clavien-Dindo classification). The difference between the number of complications in the study groups is not statistically significant ($P = 0.67$). None of the patients required reoperation. There were no deaths in the 30-day postoperative period.

During the histopathological analysis of the results in group 1 only one malignant lesion was found (1.8%) and in four patients (7.2%) lesions that were regarded potentially malignant were found. Group 2 had six cases (7.8%) of malignant lesions and in an additional four patients tumors that were considered potentially malignant were found. Table 2

Table 2: Histological types of removed lesions.

Histological type		Group 1		Group 2		*P* value
Benign	Cortical adenoma	**50 (91%)**	42 (76.4%)	**68 (88.3%)**	43 (55.8%)	0.72
	Adrenal cyst		3 (5.4%)		12 (15.6%)	
	Angiomyolipoma		2 (3.6%)		9 (11.7%)	
	Cavernous haemangioma		1 (1.8%)		0 (0%)	
	Schwannoma		1 (1.8%)		2 (2.6%)	
	Ganglioneuroma		1 (1.8%)		2 (2.6%)	
Potentially malignant	Pheochromocytoma	**4 (7.2%)**	2 (3.6%)	**3 (3.9%)**	3 (3.9%)	0.39
	Oncocytic adrenal adenoma		2 (3.6%)		0 (0%)	
Malignant	Adrenal cortex cancer	**1 (1.8%)**	1 (1.8%)	**6 (7.8%)**	5 (6.5%)	0.13
	Primary neuroectodermal tumor		0 (0%)		1 (1.3%)	

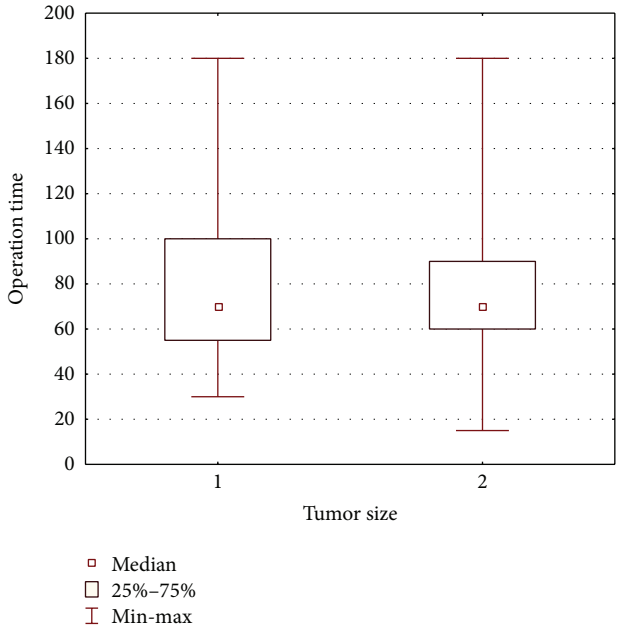

Figure 1: Mean operation time in studied groups.

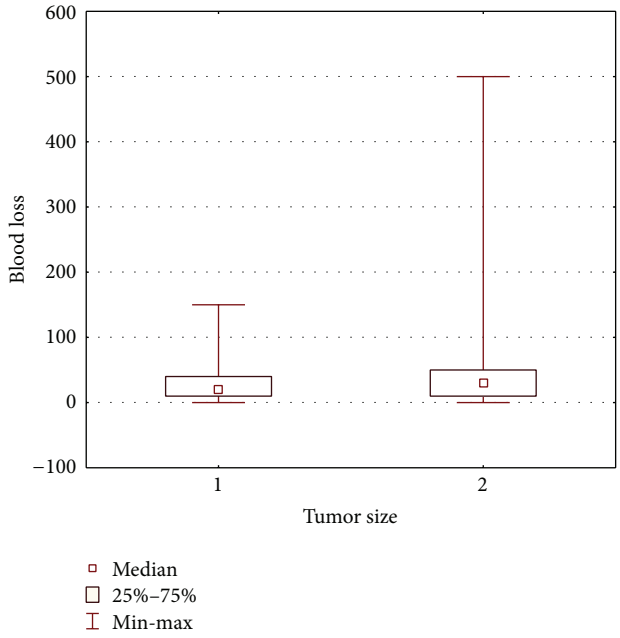

Figure 2: Mean estimated blood loss in studied groups.

presents the histological types of the removed adrenal tumors.

5. Discussion

Until the mid-1990s adrenalectomies were performed in highly specialized endocrinological surgery hospitals. The procedure was considered difficult and risky, with high morbidity and mortality rates. In 1992 Gagner was the first to report laparoscopic adrenalectomy and this significantly influenced the development of endocrine surgery [13, 14]. Even though the introduction of minimally invasive techniques theoretically should not influence the indications for the surgery, an appreciable increase in the number of adrenalectomies in many centers can be observed. A low risk of complications, shorter hospital stays, and more rapid recovery contributed to high popularity of laparoscopic adrenalectomy. Endocrinologists refer their patients more willingly to centers where laparoscopic surgery is successfully performed [15, 16]. There is now a general assumption that minimally invasive techniques provide a relatively safe and predictable way to treat adrenal tumors. There is, however, a risk that this situation can prompt surgeons to operate on patients that potentially could instead be safely followed up.

One of the greatest concerns here is the borderline tumor size that constitutes an indication for surgery [17, 18]. The upper limit of the size of tumors that can be removed by minimally invasive technique has not been clearly established. However, this problem is less controversial as there are many reports on successful laparoscopic adrenalectomy for large tumors [19–21]. The question concerning the lower limit of tumor size that constitutes an indication for the operative treatment seems to be more important. This criterion has changed several times, ranging from 2 to 6 cm. The majority of authors advocate surgery for tumors between 3 and 5 cm in diameter. It seems that most surgeons, though certainly not

all of them, accepted a lower limit of the tumor size of 4 cm as an indication for the surgical treatment [3, 11].

The greatest concern in the case of hormonally inactive adrenal tumors is the potential for malignancy, which can occur even in relatively small tumors. It is well known that the incidence of cancer is associated with the size of the tumor. In lesions over 6 cm it can vary between 5% and 25% [7]. Yet, it is strikingly lower in the case of tumors smaller than 4 cm (0.8–2%) [13]. In our group, adrenal cortex cancer was found in one patient (1.8%) in group 1 and in five patients (6,5%) in group 2. These data are relevant to the reports from the literature and confirm the hypothesis that adrenal malignancy in case of small incidentalomas is rare, but still possible. However, the concern about missed malignancy may lead to a potential risk of overtreatment, as the objective incidence of these lesions is relatively low [3].

The extended indications for surgery in the case of small tumors should include their characteristics in commonly used imaging techniques. The sensitivity and specificity of CT in case of typical adrenal adenoma are 71% and 98%, respectively [22–24]. Unfortunately, even up to 30% of adenomas do not present a typical image in CT and cannot be differentiated from malignant lesions [8]. Similarly to CT, MRI in up to 10–30% of cases cannot distinguish the character of the adrenal tumor [8, 25]. The inability to reliably assess up to 1/3 of all incidentalomas presenting the so-called radiologically malignant phenotype is one of the most common factors influencing the decision about surgery. Other imaging techniques, like scintigraphy or scintigraphy with metaiodobenzylguanidine, are used rather in the case of hormonally active tumors, while PET CT may be of some value in the case of suspected metastases in patients with a history of malignancy [1]. These methods, however, are not routinely used in the diagnostics of incidentalomas and have little influence on the decision about surgery.

The outcome of the follow-up in patients with lesions that were not operated on is another important issue. The review of 21 studies by Kapoor et al. included 1690 patients with incidentalomas smaller than 4 cm treated conservatively. It was noticed that, in follow-up (1.5 to 7 years), progression in size was present in 12.5% of patients, and a decrease in tumor size was observed in 4.3% of cases. Additionally, hormonal activity appeared in 1.2% of patients. The overall risk of growth of the adrenal tumors at surveillance in a 5-year period was estimated to be 18–29% [1]. The change in the size or character of the tumor is a significant criterion indicating the need for surgery. It can be assumed that every third patient with a tumor smaller than 3 cm will eventually require surgical treatment in the future. Unfortunately, it is impossible to predict in whom changes in tumor size or character will occur.

Another factor favoring the removal of adrenal tumors smaller than 4 cm is the safety profile provided by minimally invasive techniques. It is represented by a low complication rate, short hospital stay, and an absence of adverse long-term outcomes. In our group of patients the complication rate was 3.6% and 5.1% in groups 1 and 2, respectively. There were no complications higher than grade 3 in the Clavien-Dindo classification. Conversions, similarly to complications, were not related to the size of the adrenal tumor. Additionally, the operation time and mean estimated blood loss were similar in both groups. None of the patients required reoperation. Therefore, we can assume that tumor size within the range discussed in this paper does not affect the results of surgical treatment. We may thus conclude that laparoscopic adrenalectomy is safe regardless of tumor size, and during the qualification process one should not be guided by tumor size. All these factors may lead to an increase in the overall number of adrenalectomies for reasons poorly supported by scientific evidence.

6. Conclusions

This study does not provide unequivocal conclusions regarding the indications for and safety of laparoscopic adrenalectomy in tumors smaller than 4 cm. The safety of laparoscopic surgery and the minimal, though impossible to omit, risk of development of malignancy provide an argument for surgical treatment. On the other hand, the fact that the risk of malignancy in adrenal tumors smaller than 4 cm is lower (1.8%) than the risk of complications related to laparoscopic adrenalectomy (3.6%) provides an important counterargument against surgery.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Bone and Mineral Metabolism in Patients with Primary Aldosteronism

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Primary aldosteronism represents major cause of secondary hypertension, strongly associated with high cardiovascular morbidity and mortality. Aldosterone excess may influence mineral homeostasis, through higher urinary calcium excretion inducing secondary increase of parathyroid hormone. Recently, in a cohort of PA patients a significant increase of primary hyperparathyroidism was found, suggesting a bidirectional functional link between the adrenal and parathyroid glands. The aim of this study was to evaluate the impact of aldosterone excess on mineral metabolism and bone mass density. In 73 PA patients we evaluated anthropometric and biochemical parameters, renin-angiotensin-aldosterone system, calcium-phosphorus metabolism, and bone mineral density; control groups were 73 essential hypertension (EH) subjects and 40 healthy subjects. Compared to HS and EH, PA subjects had significantly lower serum calcium levels and higher urinary calcium excretion. Moreover, PA patients showed higher plasma PTH, lower serum 25(OH)-vitamin D levels, higher prevalence of vitamin D deficiency (65% versus 25% and 25%; $P < 0.001$), and higher prevalence of osteopenia/osteoporosis (38.5 and 10.5%) than EH (28% and 4%) and NS (25% and 5%), respectively. This study supports the hypothesis that bone loss and fracture risk in PA patients are potentially the result of aldosterone mediated hypercalciuria and the consecutive secondary hyperparathyroidism.

1. Introduction

Primary aldosteronism (PA) is a condition caused by overproduction of aldosterone and is a major cause of secondary hypertension accounting for 0.5–13% of all hypertensive subjects [1]. In a large prospective study of 1.180 Italian patients with newly diagnosed arterial hypertension (known by the acronym PAPY), primary aldosteronism was diagnosed in 11% of patients [2]. The two main causes of PA are aldosterone-producing adenoma (APA) and bilateral adrenal hyperplasia, so called idiopathic hyperaldosteronism (IHA) [3].

Patients with PA typically present with hypertension, high plasma aldosterone concentrations (PAC) that are typically associated with a low plasma rennin activity levels (PRA), and varying degrees of hypokaliemia and metabolic alkalosis. PA is strongly associated with an excess of cardiovascular morbidity and mortality risk that cannot be explained only by arterial hypertension [4].

In recent decades, dynamic studies have demonstrated multiple biological properties of aldosterone, exceeding its classic effect on the water and electrolyte balance. In fact, excess of aldosterone secretion exerts proinflammatory effects, vascular and renal fibrosis, and actions of some cytokines and influences the immune system [5, 6].

Besides cardiovascular and metabolic alterations experimental studies in rats showed that aldosterone excess may also impact mineral homeostasis. In particular, hyperaldosteronism is reported to elevate urinary calcium excretion [7], and urinary calcium correlates with sodium excretion;

each 100 mEq/dL increment sodium excretion promotes an increase of 40 mg/dL in calcium excretion [8]. Increased urinary calcium excretion in hyperaldosteronism could be due to the reduced reabsorption on sodium in aldosterone-insensitive tubular sites [9]. Moreover, prolonged ipercalciuria in PA can determine secondary increase of parathyroid hormone (PTH), by the chief cells of the parathyroid gland, a principal regulatory of calcium and phosphate homeostasis.

Recently, in a relative cohort of patients with unequivocally confirmed PA due to APA, Maniero et al. [10] showed a highly significant 31% increase in the number of cases of hyperparathyroidism, thus suggesting that there is a bidirectional functional link between the adrenocortical zona glomerulosa and the parathyroid gland. Moreover, these researchers demonstrated the expression of the mineral corticoid receptors (MR) in both PTH secreting adenoma and in parathyroid tissue [11], and the MR was predominantly located in the nucleus of the parathyroid cells, indicating that aldosterone participate in a "tonic" regulation of PTH synthesis and secretion. Finally, Tomaschitz et al. [7] showed that patients with PA are with secondary hyperparathyroidism that can be successfully treated with either mineral corticoid receptor antagonists or adrenal surgery.

The aim of this study was to evaluate the impact of aldosterone excess on mineral metabolism and bone mass density (BMD) in patients with PA.

2. Materials and Methods

2.1. Subjects. We enrolled 73 consecutive PA patients referred to the Internal Medicine and Secondary Hypertension Unit, Department of Internal Medicine and Medical Specialties, University of Rome La Sapienza, Italy, from 2009 to 2012.

Patients were divided according to the subtypes into 2 subgroups: APA (35 pts) and IHA (38 pts), matched for age, sex, and blood pressure values.

Patients with renal failure were not included in this study, and all patients were on a normal sodium/potassium restrictions. Control group consists of patients with essential hypertension (EH) and healthy subjects (HS). Previous antihypertensive therapy was withdrawn in all hypertensive patients at least two weeks (in case of spironolactone at least 4 weeks) before the investigation. To standardize the treatment and to eliminate the interference of antihypertensive drugs, all patients were switched to alpha-blocker (doxazosin) and slow-releasing calcium channel blocker (verapamil). Patients with hypokaliemia have continued with oral potassium supplementation.

The suspicion of PA was based on the findings of aldosterone-renin ratio (ARR) $\geq$ 30 (ng/dL)/(ng/mL/h), plasma renin activity (PRA) $\leq$ 0.2 ng/mL/h, and plasma aldosterone >15 ng/dL, and the diagnosis of PA was confirmed by the lack of aldosterone suppression (<7 ng/dL) following intravenous saline load (2 lt of 0.9% saline infused over 4 hours).

Differential diagnosis of PA forms (APA and IHA) was supported by a computed tomography scan (CT) or magnetic resonance imaging (MRI), and by a selective adrenal venous sampling (AVS). We used AVS criteria according to previously published guidelines [12]; selectivity was defined as adrenal vein/inferior vena cava cortisol gradient >2 and the lateralization was considered to be present when the aldosterone/cortisol ratio at one side was 2 times greater than in contralateral vein [13]. In addition, the diagnosis of APA was confirmed when successful laparoscopic adrenalectomy was done with histological verification of adrenocortical adenoma. Immunohistochemistry was not performed because it is not necessary for the diagnosis.

EH was established after exclusion of secondary hypertension on the basis of appropriate clinical and laboratory evaluation, including PAC/PRA $\leq$ 30 (ng/dL)/(ng/mL/h) Table 4.

2.2. Anthropometric Parameters. All subjects underwent assessment of weight (kg), height (cm), and body mass index (BMI) calculated by the formula (kg/m^2), waist circumference (WC, cm) measured to a minimum of inspiration to the mid-point of the line joining the last rib and the iliac crest. Office blood pressure (BP) was measured with a standard aneroid manometer with subjects sitting for 5 minutes, systolic blood pressure (SBP) was taken as the first sound was on of the cuff (Korotkoff phase I), and diastolic blood pressure (DBP) was taken on the complete disappearance of Korotkoff sounds (phase V). Hypertension was confirmed by repeated BP measurements SBP $\geq$ 140 mmHg and DBP $\leq$ 90 mmHg.

2.3. Biochemical Parameters. Biochemical variables were determined after an overnight fast by anaerobic sampling and evaluating calcium metabolism, renal function, and lipid-glucose metabolism. Ionized calcium was measured with an analyzer: the range of this method was 1.17–1.33 mmol/l. Intact serum PTH (i-PTH) was measured using a radioimmunoassay method (RIA commercial kits: PTH, Still Water, MN, USA).

Measurement of 25-hydroxyvitamin D [25(OH)D] was performed by means of a chemiluminescence assay (IDS-iSYS 25-hydroxyvitamin D; Immunodiagnostic systems Ltd., Boldon, UK) on an IDS-iSYS multidiscipline automated analyzer. PRA, PAC, and plasma cortisol (PC) levels were measured as previously described [14].

2.4. Bone Mineral Density. Bone mineral density (BMD) at lumbar spine (L1–L4) and femoral neck (FN) was obtained in all subjects using dual-energy X-ray absorptiometry (DEXA) using Hologic QDR-4800 device (Hologic Inc., Walstrom, MA, USA) according to WHO recommendations. The assessment of BMD was expressed as g/m^2 and as standard deviation from the mean peak bone mass revealed in healthy subjects adults of the same sex (T-score).

The diagnosis of osteoporosis was made in the case of T-score $\leq$ 2.5, osteopenia if T-score was between 2.5 and 1, and normal bone mass was made with superior T-score of 1. Regarding the precision of BMD evaluation, the coefficient variation was 1% at the lumbar spine and 1.2% at the femoral neck site.

Table 1: Demographic and anthropometric parameters in all subjects enrolled.

Patient	Years (yrs)	BMI (Kg/m^2)	Waist circumference (cm)	SBP (mmHg)	DBP (mmHg)
PA (n.73)	52.5 ± 11.2	28.2 ± 4.7*	99.8 ± 13.1*	138.3 ± 16.8*	85.9 ± 11.4*
EH (n.73)	55.6 ± 12.4	29 ± 5*	100.5 ± 11.2*	131 ± 18.8*	82.4 ± 11.2*
HS (n.40)	55.7 ± 6.1	25.1 ± 2.2	95.5 ± 6.8	119.1 ± 4.2	77.2 ± 5.1
P	ns	<0.002 versus HS	<0.003 versus HS	<0.01 versus HS	<0.01 versus HS
APA (n.35)	52.8 ± 11.5	27.6 ± 4.8	100.4 ± 12.9	138.8 ± 19.1	88.3 ± 9.6
IHA (n.38)	52.5 ± 11.2	28.6 ± 4.6	99.3 ± 13.6	137.3 ± 14.5	83.4 ± 9.6
P	ns	ns	ns	ns	ns

PA: primary aldosteronism; EH: essential arterial hypertension, HS: healthy subjects; APA: aldosterone-producing adrenal adenoma; IHA: idiopathic bilateral hyperplasia; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure.
* *P* value.

Table 2: Biochemical parameters of all subjects enrolled.

Patient	Serum creatinine (mg/dL)	K (mEq/L)	Ca (mg/dL)	Ca^{2+} (mmol/L)	Ca-Ur (mg/24 h)	P (mg/dL)	PTH (pg/mL)	ALP (UI/L)	25-OH vitamin D (ng/mL)
PA (n.73)	0.9 ± 0.2	3.8 ± 0.5*	9.2 ± 0.4*	1.2 ± 0.09	242.8 ± 116.7*	3.5 ± 0.6	48.9 ± 19.9*	163.3 ± 33.9*	17.8 ± 12.5*
EH (n.73)	1.02 ± 0.2	4.2 ± 0.4	9.7 ± 0.3	1.2 ± 0.03	164.1 ± 84*	3.4 ± 0.4	30.7 ± 11.9	87.4 ± 46.7	32.9 ± 16
HS (n.40)	0.88 ± 0.2	4.17 ± 0.4	9.4 ± 0.3	1.21±0.02	154.6 ± 17.3	3.4 ± 0.3	29.1 ± 2.4	100.3 ± 52.8	23.8 ± 12.8
P	ns	<0.001 versus EH-HS	<0.001 versus EH-HS	ns	<0.001 versus EH-HS	ns	<0.001 versus EH-HS	<0.001 versus EH-HS	<0.001 versus EH-HS
APA (n.35)	0.9 ± 0.2	3.7 ± 0.7	9.2 ± 0.5	1.2 ± 0.07	222.5 ± 100.7	3.4 ± 0.7	46 ± 20.1	179.1 ± 27.4	21.3 ± 16.6
IHA (n.38)	0.8 ± 0.2	3.9 ± 0.3	9.2 ± 0.4	1.2 ± 0.12	274.7 ± 140.4	3.6 ± 0.6	50.6 ± 20.2	135.7 ± 26.9	18.5 ± 17.8
P	ns	ns	ns	ns	ns	ns	ns	ns	ns

K: serum potassium; Ca: serum total calcium; Ca^{2+}: ionized serum calcium; Ca-Ur: 24-hour urinary calcium excretion; P: serum phosphorus; PTH: parathyroid hormone; ALP: alkaline phosphatase.
* *P* value.

This study was conducted in accordance with the Declaration of Helsinki guidelines and also approved by a local ethical committee. All subjects gave their informed consent before the study begun.

2.5. Statistical Analysis. Analysis was performed using Sigmastat Program (Jandel Corporation, USA). All data are expressed as mean ± standard deviation (±SD). Comparison for variables between the groups of subjects was performed using Student's *t*-test and Mann-Whitney test for non-parametric variables. Correlations between variables were assessed by simple linear regression analysis. Linear regression analysis was performed to evaluate the relationship among variables in all subjects. *P* value < 0.05 was considered statistically significant.

3. Results

The main characteristics of study subjects are summarized in Tables 1, 2, and 3. Seventy-three subjects were affected by PA (51 males, 22 females; mean age 52.5 ± 11.2 yrs); among these, 35 PA patients (48%) had an APA, whereas 28 (52%) patients had an IHA. Seventy-three subjects were affected by EH (35 males, 38 females; mean age 55.6 ± 12.4 yrs), and 40 subjects were normotensive and otherwise healthy (HS) (16 males, 24 females; mean age 55.7 ± 6.1 yrs).

Clinical and biohumoral parameters for each study group are reported in detail in Tables 1 and 2. In particular, PA and EH subjects showed highest BMI and WC ($P < 0.002$; $P < 0.003$, resp.) compared to HS. For these anthropometric parameters, no difference was found in PA patients subgroup (APA and IHA).

As expected, serum potassium, PAC, PRA values, and ARR were significantly different in PA patients when compared with EH and HS subjects ($P < 0.001$ for all).

3.1. Mineral Metabolism. All subjects with PA had significant lower serum calcium levels ($P < 0.001$) associated to higher calcium excretion values ($P < 0.001$), compared to EH and HS subjects (Table 2). Moreover, PA patients showed lower serum 25(OH)-vitamin D levels and higher plasma PTH values with respect to EH and HS subjects ($P < 0.001$ and $P < 0.001$, resp.) (Table 2). No statistically significant difference for these mineral parameters was showed in PA subtypes patients (APA and IHA).

Several studies have shown that vitamin D scoreis easily assessed by serum 25(OH)-vitamin D. Concentration less than 20 ng/mL is generally considered vitamin D deficiency whereas between 20 and 30 ng/mL vitamin insufficiency. In

Table 3: Renin-angiotensin-aldosterone system parameters in all subjects enrolled.

Patient	PAC (ng/dL)	PRA (ng/mL/h)	PA/PRA ratio (ng/mL : ng/mL/h)	PAC postinfusion test (ng/dL)	AUR (μg/24 h)
PA (n.73)	37 ± 25.1*	0.9 ± 0.7*	41.1 ± 11.5*	115.9 ± 78.7*	31.6 ± 18.1*
EH (n.73)	22.5 ± 13	1.4 ± 1.6	16.7 ± 7.3	24.5 ± 8.7	16.3 ± 4.5
HS (n.40)	9.2 ± 1.7	1.1 ± 0.4	8.4 ± 2.8	—	18.3 ± 5.3
P	<0.001 versus EH-HS	<0.001 versus EH	<0.001 versus EH-HS	<0.001 versus EH	<0.001 versus EH-HS
APA (n.35)	39.8 ± 25.6	0.7 ± 0.6	56.9 ± 15.2	148.1 ± 95.7	34.3 ± 22.8
IHA (n.38)	34.4 ± 24.6	1.1 ± 0.8	31.3 ± 5.6	85.1 ± 40.5	29.4 ± 12.8
P	ns	ns	ns	<0.001	ns

PA: primary aldosteronism; EH: essential arterial hypertension, HS: healthy subjects; APA: aldosterone-producing adrenal adenoma; IHA: idiopathic bilateral hyperplasia; PAC: plasma aldosterone concentration; PRA: plasma renin activity; AUR: 24-hour aldosterone urinary excretion.
* *P* value.

Table 4: Bone mineral density (BMD) evaluated by dual-energy X-ray absorptiometry (DXA) in all subjects enrolled.

Patient	*T*-score L1–L4	BMD L1–L4 (g/cm^2)	*T*-score FN	BMD FN (g/cm^2)
PA (n.73)	−0.28 ± 1.3*	1.01 ± 0.17*	−0.67 ± 1.1*	0.84 ± 0.16
EH (n.73)	0.03 ± 0.6	1.11 ± 0.17	−0.29 ± 0.7	0.84 ± 0.12
HS (n.40)	0.027 ± 0.8	1 ± 0.09	−0.30 ± 0.6	0.81 ± 0.08
P	0.06* versus EH-HS	0.06* versus EH-HS	0.06* versus EH-HS	ns
APA (n.35)	−0.30 ± 1.3	1 ± 0.18	−0.7 ± 1.05	0.82 ± 0.14
IHA (n.38)	−0.25 ± 1.4	1.02 ± 0.17	−0.63 ± 1.3	0.85 ± 0.19
P	ns	ns	ns	ns

PA: primary aldosteronism; EH: essential arterial hypertension, HS: healthy subjects; APA: aldosterone-producing adrenal adenoma; IHA: idiopathic bilateral hyperplasia; L1–L4: lumbar spine side; FN: femoral neck side.

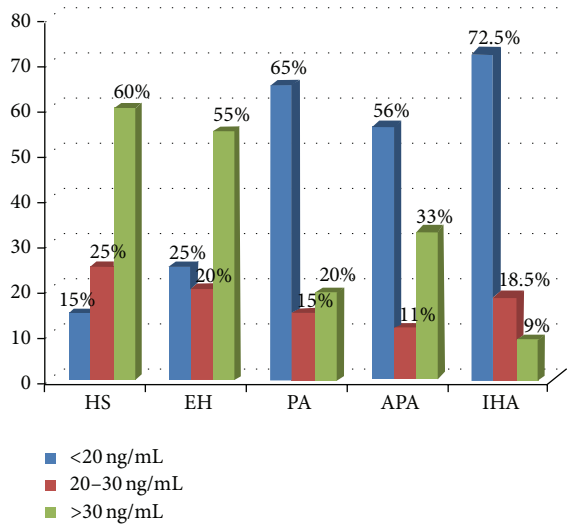

Figure 1: Plasma levels of 25(OH)-vitamin D in all subjects enrolled. HS: healthy subjects; EH: essential arterial hypertension; PA: primary aldosteronism; APA: aldosterone-producing adrenal adenoma; IHA: idiopathic bilateral hyperplasia.

Figure 1, we reported the behavior of serum 25(OH)-vitamin. D levels in all study groups. In particular the prevalence of vitamin D deficiency was significantly higher in patients with PA than in EH and HS subjects (65% versus 25% and 25%, resp.; $P < 0.001$). Among PA subjects the prevalence of vitamin D deficiency was higher in subjects with IHA when compared with those with APA (72.5% versus 56%; $P < 0.01$, resp.).

3.2. Bone Densitometry (BMD). Bone densitometric parameters are reported in Table 4. In PA patients we found high prevalence of osteopenia and osteoporosis (38.5 and 10.5%) than EH (28% and 4%) and NS (25% and 5%), respectively. Moreover, PA patients with APA showed more bone remodeling with respect to IHA patients (Figure 2). Study of correlations revealed in all patients with PA a negative correlation with PAC and BMD Neck ($r = -0.27$; $P < 0.05$) and with the *T*-score Neck ($r = -0.28$; $P < 0.04$) (Table 5).

4. Discussion

PA is the most common form of hormone related arterial hypertension, representing a curable disease [1]. PA is characterized by autonomous overproduction of adrenal aldosterone with suppression of PRA, sodium retention, and consequent hypertension. Various primary adrenal processes cause this syndrome. Some of them are best treated by surgery and others by medicine [3]. In the past it had been documented that PA contributed to the development of cardiovascular disease [15], and the metabolic alterations caused by inappropriate secretion of aldosterone are being

Table 5: Study correlation in PA subjects.

Parameters	P	r
24 h calcium excretion		
Serum calcium	<0.01	−0.56
Age	<0.001	−0.75
PTH		
BMD FN	<0.02	−0.461
T-score FN	<0.01	−0.2
Serum phosphorus		
BMD L1–L4	<0.03	−0.403
24 h aldosterone urinary excretion	<0.03	−0.37
Plasma aldosterone		
BMD FN	<0.05	−0.27
T-score FN	<0.04	−0.28

BMD: bone mineral density; FN: femoral neck side; L1–L4: lumbar spine side.

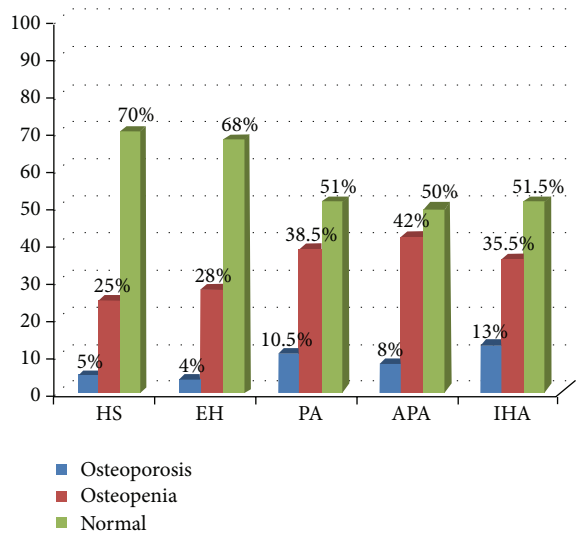

Figure 2: Prevalence of osteoporosis in in all subjects enrolled. HS: healthy subjects; EH: essential arterial hypertension; PA: primary aldosteronism; APA: aldosterone-producing adrenal adenoma; IHA: idiopathic bilateral hyperplasia.

recognized such as metabolic syndrome [16]. However, only recently the calcium metabolism alterations and hyperaldosteronism have been systematically recognized [11]. In particular, studies aimed to verify the hypothesis postulating the effect of aldosterone on the secretion of PTH are also worth mentioning [10]. In this study we examined the calcium mineral metabolism and BMD in PA patients, (both APA and IHA), compared to EH patients. Our results showed that all PA patients had significant lower serum calcium levels associated to higher calcium excretion compared to EH. Moreover, PA patients showed lower serum 25(OH)-vitamin D levels and higher plasma PTH values with respect to EH. No statistically significant differences for these mineral parameters were showed in PA subtypes patients (APA versus IHA). These data confirmed and extend other results reported in the literature [17–19].

In particular, in a study firstly conducted in humans, patients with PA were detected to have significantly higher concentrations of PTH compared to both normal and hypertensive subjects [20]. In Graz Endocrine Causes of Hypertension (GECOH) study, Pilz and coworkers [17] reported in 10 PA patients (5 APA and 5 IHA) lower serum calcium and higher plasma PTH levels compared to EH patients; however, serum 25(OH)-vitamin D concentrations were similar in both groups. These authors hypothesized that PA contributes to secondary hyperparathyroidism.

Recently, Ceccoli and coworkers [18] in 116 PA patients (40 with APA and 70 with IHA) compared with 110 EH patients showed an increase of PTH levels and urinary calcium excretion, while serum calcium decreases with comparable vitamin D levels. Moreover, this PTH increase, more evident in patients with APA than in those with IHA, is reversible after appropriate treatment of aldosterone excess. These data supported the hypothesis that secondary hyperparathyroidism in PA seems to be due to the presence of aldosterone excess with an increased urinary excretion of calcium and consequent hypocalcaemia. In fact, renal hypercalciuria in these patients may be due to aldosterone excess combined with high salt retention. Expansion of effective circulating value in PA patients decreases proximal tubule reabsorption of calcium as well as sodium because calcium reabsorption is coupled to transtubular sodium uptake [18].

Although the distal nephron can reabsorb calcium, excessive delivery can overwhelm this absorptive capacity. In addition, potassium depletion causes intracellular acidosis [9]; aldosterone, by augmenting sodium chloride cotransfer in the distal nephron [21], can lead indirectly to impaired calcium reabsorption. The reduction in urine calcium excretion in response to specific treatment (adrenalectomy or MR-antagonists) reported by Ceccoli et al. [18] in patients with PA supported the hypothesis that hyperaldosteronism is the main cause of the hypercalciuria in these patients. Another data shown in our study is the alterations of BMD evaluated as T-score value for two skeletal sites (the lumbar spine and femoral neck) and higher prevalence of osteoporosis and osteopenia in PA patients with respect to EH and HS. Our data are in line with previous studies [17, 22].

In the setting of PA, bone metabolism might be affected (1) directly by aldosterone-MR-mediated effects on osteoblasts and osteoclasts and (2) indirectly by PTH levels and increased bone resorption [20]. Salcuni et al. [19] showed higher 24 h urinary calcium and elevated PTH levels in patients with PA compared to patients with adrenal incidentaloma without aldosterone excess. Moreover, these authors documented significantly lower bone mineral density (measured at the lumbar spine, total, and femoral neck) in PA patients compared to patients without hyperaldosteronism. Moreover, Ceccoli et al. [18] reported in 40 patients with PA (16 APA and 24 IHA), available both at baseline and after adrenalectomy (APA) and treatment with MR antagonists (IHA), an improvement of BMD and suggested that these changes seem to be due to target treatment and not the vitamin D status, because there were no significant changes in serum 25(OH)-vitamin D before and after treatment of aldosterone excess.

In our study, whereas, we found lower serum 25(OH)-vitamin D levels associated at the higher available phosphate levels in PA patients compared with EH patients and a higher percentage of vitamin D deficiency (<20 ng/dL). This is an important finding, because the hypovitaminosis D can favor bone abnormalities and reduced bone mass and associated a significant increase in renal excretion and increased circulating levels of PTH, leading to typical feature of osteomalacia.

In conclusion, these observations support the hypothesis that bone loss and potentially fracture risk in PA patients are potentially the result of aldosterone mediated hypercalciuria and the consecutive secondary hyperparathyroidism.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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Research Article

Prevalence of Nonclassic Congenital Adrenal Hyperplasia in Turkish Children Presenting with Premature Pubarche, Hirsutism, or Oligomenorrhoea

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Background. Nonclassic congenital adrenal hyperplasia (NCAH), caused by mutations in the gene encoding 21-hydroxylase, is a common autosomal recessive disorder. In the present work, our aim was to determine the prevalence of NCAH presenting as premature pubarche (PP), hirsutism, or polycystic ovarian syndrome (PCOS) and to evaluate the molecular spectrum of *CYP21A2* mutations in NCAH patients. *Methods*. A total of 126 patients (122 females, 4 males) with PP, hirsutism, or PCOS were included in the present study. All patients underwent an ACTH stimulation test. NCAH was considered to be present when the stimulated 17-hydroxyprogesterone plasma level was >10 ng/mL. *Results*. Seventy-one of the 126 patients (56%) presented with PP, 29 (23%) with PCOS, and 26 (21%) with hirsutism. Six patients (4,7%) were diagnosed with NCAH based on mutational analysis. Four different mutations (Q318X, P30L, V281L, and P453S) were found in six NCAH patients. One patient with NCAH was a compound heterozygote for this mutation, and five were heterozygous. *Conclusion*. NCAH should be considered as a differential diagnosis in patients presenting with PP, hirsutism, and PCOS, especially in countries in which consanguineous marriages are prevalent.

1. Introduction

Nonclassic congenital adrenal hyperplasia (NCAH), caused by 21-hydroxylase deficiency, is one of the most common autosomal recessive disorders in humans, attributable to mutations in the *CYP21A2* gene and the related pseudogene *CYP21A1P*.

Genetic defects in the *CYP21A2* gene are classified into three categories depending on the extent of residual enzymatic activity. In vitro studies have shown that mutations causing complete inactivation of 21-hydroxylase activity are associated with the salt-wasting (SW) form of the condition, those that reduce 21-hydroxylase activity to about 2% of the normal value with the simple-virilising (SV) form of the disease, and those that reduce 21-hydroxylase activity to 10–75% of the normal value with the nonclassical (NC) form [1, 2].

The prevalence of NCAH varies among ethnic groups, and the condition has been estimated to occur in 0.1% of the general worldwide population but in 1-2% of Hispanics and Yugoslavs and 3-4% of Ashkenazi Jews [3]. Patients with NCAH exhibit variable clinical presentations, but all have signs and symptoms of excess androgen synthesis. Children and adolescents may present with premature pubarche (PP), hirsutism, polycystic ovarian syndrome (PCOS), menstrual dysfunction, severe cystic acne, male-pattern alopecia, advanced bone age, accelerated linear growth velocity, and decreased fertility [4].

PP is diagnosed based on exclusion of precocious puberty and NCAH. Most children with PP exhibit idiopathic premature adrenal androgen secretion. However, in 5–20% of such children, PP is caused by NCAH [5]. If it is desired to control the final height attained, early diagnosis is important and treatment should be commenced before signs of

virilisation become obvious. At adolescence, females are more symptomatic than are males and may present with hirsutism, menstrual irregularity, or PCOS. These symptoms are thought to be caused by androgen excess impairing hypothalamic sensitivity to progesterone and in turn creating persistent GnRH pulsing and LH hypersecretion [4]. The gold standard test for diagnosis of NCAH is assay of stimulated adrenocorticotropic hormone (ACTH) level [6].

In the present study, we evaluated 126 Turkish children and adolescents with suspicious symptoms to determine the prevalence of NCAH. We assayed stimulated ACTH levels in patients presenting with PP, hirsutism, or PCOS and evaluated the molecular pattern of *CYP21A2* gene mutations in such patients.

2. Subjects and Methods

A total of 126 patients 3–17.8 years of age admitted to our Paediatric Endocrinology Department because of PP, PCOS, or hirsutism were studied. All patients underwent physical examination. Height was measured in the standing position, without shoes, using a stadiometer with a sensitivity of 0.1 cm. Weight was measured using a portable scale (sensitivity, 0.1 kg) with the patient dressed in light clothing. Body mass index (BMI) (weight (kg)/height $(m)^2$) was recorded. Weight, height, and BMI standard deviations (SD) were calculated using reference curves for Turkish children [7, 8]. Pubertal status was evaluated using the criteria of Marshall and Tanner [9]. Bone-age SD was measured according to Greulich and Pyle [10]. Gender, chronological age, age at symptom onset, birth weight, weight SD, height SD, BMI SD, and bone-age SD were recorded, respectively. Small-for-gestational age (SGA) status was defined as a birth weight below the 10th percentile; children with birth weights between percentiles 10–90 were defined as appropriate-for-gestational age (AGA); and those with birth weights above the 90th percentile were considered to be large-for-gestational age (LGA) [11]. Precocious pubarche was defined as onset of pubic hair growth before the age of 8 years in females and 9 years in males [12]. Prospective subjects were excluded if they exhibited clinical signs of central precocious puberty. PCOS was diagnosed using the criteria of the National Institutes of Health (NIH), thus by clinical and biochemical evidence of hyperandrogenism and ovulatory dysfunction (menstrual irregularity) that could not be explained by the presence of another disorder [13]. The extent of hirsutism was evaluated in nine body areas using a modified Ferriman-Gallwey scoring system, and patients scoring eight or over on the scale were considered to be hirsute [14]. Idiopathic hirsutism was diagnosed in patients with hirsutism who had normal menstrual cycles, normal serum androgen concentrations, and no identifiable cause of hirsutism [15]. All patients underwent ACTH stimulation testing. Female adolescents were tested during the follicular phase of the menstrual cycle. After overnight fasting, 0.25 mg of ACTH (Synachten, Ciba-Geigy, Basel, Switzerland) was injected as an intravenous bolus between 08:00 a.m. and 10:00 a.m.; and cortisol, 17-hydroxyprogesterone (17-OHP), and dehydroepiandrosterone sulphate (DHEAS) levels were measured 0 (basal) and 60 min after ACTH administration. Cortisol and DHEAS levels were analysed using electrochemiluminescence assays (Roche E-170; Basel, Switzerland). 17-OHP levels were determined by ELISA. NCAH was considered present if the stimulated 17-OHP plasma level was greater than 10 ng/mL. Molecular analysis of the *CYP2A2* gene was performed on all patients diagnosed with NCAH based on the ACTH stimulation test.

3. Molecular Analysis of *CYP21A2*

Peripheral blood samples and genomic DNA from peripheral blood leukocytes were prepared using Roche MagNa Pure Compact Nucleic Acid Isolation Kits and Magna Pure Compact System Kits, respectively (Roche Diagnostics, Indianapolis, IN). Genetic analysis was conducted using a reverse-hybridisation strip-based assay (the CAH StripAssay) that explored the presence of the 11 *CYP21A2* mutations most prevalent in European populations: P30L, IVS2 splice (IVS2 G), Del 8bp E3 (G110del8nt), I172N, Cluster E6 (I236N, V237E, and M239K), V281L, L307 frameshift (F306+T), Q318X, R356W, P453S, and R483P.

4. Statistical Analysis

SPSS for Windows 20.0 was used for analysis of raw data. The Shapiro-Wilk test was used to confirm that sample data were normally distributed. The parametric Independent Samples *t*-test was used to compare normally distributed data among groups. The nonparametric version of the Mann-Whitney *U*-test was used to perform comparisons among groups exhibiting nonnormal distribution of data. Pearson's chi-squared and Fisher's exact tests were used to conduct paired categorical data analysis. All data are expressed as means ± SD or median values obtained at percentiles 25 and 75 (Q1 and Q3). A P value <0.05 was considered to reflect statistical significance.

5. Results

Seventy-one of the 126 patients (56%) presented with PP, 29 (23%) with PCOS, and 26 (21%) with hirsutism. Mean age at symptom onset was 6.6 ± 1.2 years in PP patients, 12.6 ± 1.3 years in PCOS patients, and 11.5 ± 2.8 years in patients with hirsutism. Birth weight data were available for 108 patients; 18 children scored as SGA, four as LGA, and 86 as AGA. Ferriman-Gallway scores were available only for PCOS patients (mean: 19.7 ± 5) or those with hirsutism (mean: 20.6 ± 4). Clinical and biochemical data on all study subjects are shown in Table 1. Examination of 17-OHP values after ACTH stimulation revealed that nine patients (7.1%) had NCAH. Based on the genetic analysis, six patients (4.7%) were diagnosed with NCAH. The NCAH prevalence was 4.2% ($n = 3$) in PP patients, 6.8% ($n = 2$) in PCOS patients, and 3.8% ($n = 1$) in patients with hirsutism.

The clinical characteristics of and laboratory findings of all patients with or without NCAH are shown in Table 1. Basal and stimulated levels of 17-OHP and bone-age SD were significantly higher in those with than without NCAH ($P < 0.05$ for all comparisons).

Table 1: Clinical characteristics and laboratory findings of study subjects.

	Subjects without NCAH	Subjects with NCAH	*P* value
Age (year)	8,80 (7,30–14,60)	7,80 (6,80–13,30)	0, 336
Gender (female/male)	113/4	9/0	0,741
BMI SDS	0,82 ± 0,09	1,03 ± 0,36	0,572
Height SDS	0,33 ± 1,10	0,49 ± 0,39	0,685
Bone Age SDS	1,02 ± 0,09	2,07 ± 0,12	**0,002**
Ferriman Gallway Score	20,18 ± 4,65	23,00 ± 5,00	0,594
T.Testosterone (ng/dL)	16,50 (0,69–38,37)	7,05 (0,79–20)	0,752
$Cortisol_0$ (μg/dL)	14,41 ± 5,72	13,75 ± 5,68	0,740
$Cortisol_{1\,hour}$ (μg/dL)	28,69 ± 5,66	32,47 ± 5,30	0,055
$17\text{-}OHP_0$ (ng/mL)	1,20 (0,90–2)	3,90 (2,15–9,50)	<**0,001**
$17\text{-}OHP_{1\,hour}$ (ng/mL)	3,10 (2,25–4,40)	21,60 (9,80–34,05)	<**0,001**
$DHEA\text{-}S_0$ (μg/dL)	103 (53,50–181,50)	128 (128–331,50)	0,619
$DHEA\text{-}S_{1\,hour}$ (μg/dL)	102 (53,70–188,50)	130 (50,50–312,50)	0,622

Data are expressed as the means ± standard deviations or medians (Q1–Q3) as appropriate. NCAH: nonclassic congenital adrenal hyperplasia; BMI-SDS: body mass index standard deviation score; 17-OHP: 17-hydroxyprogesterone; DHEA-S: dehydroepiandrosterone sulphate.

Table 2: Comparisons of clinical and laboratory findings between patients with nonclassic congenital adrenal hyperplasia and premature pubarche.

	IPP ($n = 65$)	NCAH ($n = 6$)	*P* value
Gender (female/male)	61/4	6/0	0,697
Age (year)	7,50 (6,95–8,20)	6,90 (6,60–8,85)	0,400
BMI SDS	0,85 ± 0,99	0,94 ± 1,17	0,827
Height SDS	0,71 ± 1,11	0,91 ± 1,15	0,676
Bone Age SDS	1,12 ± 1,02	2,11 ± 0,43	**0,022**
T.Testosterone (ng/dL)	9,47 (0,30–20)	20 (4,82–45,50)	0,258
$Cortisol_{0\,hour}$ (μg/dL)	13 ± 5,36	11,86 ± 3,14	0,594
$Cortisol_{1\,hour}$ (μg/dL)	29,20 (24,30–32,20)	31,70 (29–35)	0,121
$17\text{-}OHP_{0\,hour}$ (ng/mL)	1,18 ± 1,01	6,33 ± 4,96	0,052
$17\text{-}OHP_{1\,hour}$ (ng/mL)	3 (2,20–4)	25,30 (9,97–39,32)	<**0,001**
$DHEA\text{-}S_{0\,hour}$ (μg/dL)	65,20 (43,70–107)	62,40 (40,77–128,50)	0,960
$DHEA\text{-}S_{1\,hour}$ (μg/dL)	67,70 (43,45–101)	64,50 (45,70–131,25)	0,848

Data are expressed as means ± standard deviations or medians (Q1–Q3) as appropriate. IPP: idiopathic premature pubarche; NCAH: nonclassic congenital adrenal hyperplasia; BMI SDS: body mass index standard deviation score; 17-OHP: 17-hydroxyprogesterone; DHEA-S: dehydroepiandrosterone sulphate.

Data on patients with or without NCAH were compared with those of PP patients. Nonpathological exaggerated secretion of androgen was defined as idiopathic PP (IPP). The clinical characteristics of and laboratory findings on IPP and NCAH patients are shown in Table 2. The stimulated levels of 17-OHP, and bone-age SD, were higher in children with NCAH ($P < 0.05$). However, no significant difference in any of chronological age, BMI SD, height SD, baseline or stimulated cortisol level, or baseline 17-OHP or DHEAS level was evident between children with PP and NCAH. Birth weight data were available for 60 patients; 13 children were of SGA status, two of LGA, and 45 of AGA. Birth weight did not differ between patients diagnosed with or without NCAH ($P > 0.05$).

The clinical characteristics of nine patients diagnosed with NCAH according to the ACTH stimulation test are shown in Table 3. Six of these patients presented with PP, two with PCOS, and one with hirsutism. Four different mutations (Q318X, P30L, V281L, and P453S) were found in six of the nine patients diagnosed with NCAH. One patient with PP had compound heterozygous mutations (V281L and P30L). Five patients were heterozygous for disease-related mutations. V281L and P453S mutations were detected in PP patients, P30L was detected in PCOS patients, and Q318X in patients with both PCOS and hirsutism. Consanguinity was in play in three of nine NCAH patients.

6. Discussion

Nonclassic congenital adrenal hyperplasia should be considered in the differential diagnosis of PP, PCOS, and IH, because neither clinical presentation nor androgen level is a reliable predictor of this disease. In the present study, we determined the prevalence of NCAH in children and adolescents presenting with symptoms of androgen excess and evaluated the clinical characteristics of such patients.

Table 3: Clinical characteristics and genotypes of patients with nonclassic congenital adrenal hyperplasia.

Patient	Genotype	Clinical feature	Age	Gender	Consanguinity	BA-SDS	17-OHP$_0$	17-OHP$_{peak}$
1	NA	PP	6,8	F	No	+1,5	13	40
2	V281L/P30L	PP	6,0	F	No	+2,6	2,1	10
3	NA	PP	7,8	F	No	+1,7	10,6	39
4	NA	PP	6,8	F	No	+2,4	8,4	21,6
5	Q318X	Hirsutism	13,1	F	Yes	+2,3	2,3	10,5
6	Q318X	PCOS	13,5	F	No	+2,0	3,9	10,8
7	P30L	PCOS	15,9	F	Yes	+1,7	8,1	26,5
8	V281L	PP	7,0	F	Yes	+2,4	2,2	29
9	P453S	PP	12,0	F	No	+2,1	1,7	10

NA: not applicable; BA-SDS: bone age-standard deviation score; 17-OHP: 17-hydroxyprogesterone; PP: premature pubarche; PCOS: polycystic ovarian syndrome.

The prevalence of NCAH in children with PP varies between 0 and 40% among different populations [16–19], and the prevalence of NCAH causing hirsutism or PCOS is up to 20%, varying with both ethnicity and geographical area [20–22]. Few data have been gathered on the prevalence of NCAH in Turkish PP patients. Erdeve et al. [23] studied 159 patients, and Gonc et al. [24] reported 186 patients; the prevalences of non-classical CAH were 5.7% and 3.2%, respectively. In our cohort, six patients (4.7%) were diagnosed with NCAH based on the genetic analysis. The prevalence of NCAH was 4.2% ($n = 3$) in PP patients, 6.8% ($n = 2$) in PCOS patients, and 3.8% ($n = 1$) in patients with hirsutism. Unluhizarci et al. [25] conducted an extensive study of hirsute and hyperandrogenic females from various regions of Turkey. The cited authors concluded that NCAH was not prevalent in the Turkish population (the incidence was 2.1%) [25]. Akinci et al. [26] found that the prevalence of NCAH in hirsute adolescent females was 3.1%, and Yarman et al. [27] found an NCAH prevalence of 33% in Turkish females with hirsutism and PCOS; the data were confirmed by genotyping and HLA typing. The high prevalence noted in the cited report was considered to be associated with ethnic diversity in Istanbul. In another Turkish study, the prevalence of NCAH was 6.9% in Turkish woman with PCOS and 15% in those with IH [28].

The limitations of the present study include the small number of patients (three) diagnosed with NCAH causing hirsutism or PCOS; we were unable to compare adolescents with hirsutism to those with PCOS and NCAH. However, we have described the clinical and laboratory findings of patients exhibiting androgen excess and we compared data on all patients with those of nine patients with NCAH. We examined all IPP patients, and the six patients for whom NCAH was diagnosed as the cause of PP, in an effort to identify factors predictive of NCAH development. Most prior studies have proposed that patients with NCAH exhibit accelerated bone-age maturation [23, 29–31] and increased basal or stimulated levels of 17-OHP [29, 32, 33]. In the present study, as expected, the basal and stimulated levels of 17-OHP were higher ($P < 0.001$), and the bone-age SD was more advanced ($P < 0.05$), in children with NCAH. However, we found no between-group difference in any of current age, age at onset of symptoms, BMI, height, baseline and stimulated levels of cortisol, or DHEAS ($P > 0.05$ for all comparisons). Birth weight did not differ between patients diagnosed with or without NCAH ($P > 0.05$). Similarly, neither birth weight nor testosterone level was a predictor of NCAH development in the study of von Oettingen et al. [29], which involved 122 patients of varied ethnicity presenting with premature adrenarche. This result is in contrast with previous reports that elevated basal testosterone is a potential predictor of NCAH development [5, 33]. Ghizzoni et al. [34] studied the clinical and biochemical profiles of 152 Italian children with PP and genotypically screened all subjects. The cited authors could not define any diagnostic clinical characteristic of NCAH patients; even bone-age was not informative. Similarly, Ibáñez et al. [17] were unable to identify any clinical parameter differentiating PP patients into those with or without NCAH.

In our patient cohort, two of 29 PCOS patients and one of 26 patients with hirsutism were diagnosed with NCAH. Adolescent females with NCAH may suffer from gonadal dysfunction, menstrual disorders, or PCOS. These conditions are thought to be caused by conversion of adrenal androgens to oestrogens, altering the level of gonadotropin secretion or disrupting the cyclicity of gonadotropin release, thus directly affecting the ovary and ultimately leading to formation of androgen-producing cysts. Many authors have examined the similarity between NCAH and PCOS and the differences that might differentiate these two diseases. Obesity, insulin resistance, hirsutism, and polycystic ovarian morphology may be present in both NCAH and PCOS. The only exception is that the 17-OHP level is not elevated significantly in PCOS, at least not to the levels seen in NCAH. It has been suggested that NCAH should be excluded in patients presenting with hirsutism, oligomenorrhoea, and polycystic ovarian morphology. Alternations in *CYP21A2* gene transcription should be kept in mind as a cause of PCOS [22, 28, 35].

Molecular analyses of the *CYP21A2* gene were performed on nine patients diagnosed with NCAH according to the ACTH stimulation test. The V281L and P453S mutations were detected in PP patients; P30L was detected in PCOS patients; and Q318X was detected in patients with either or both of PCOS and hirsutism. Consanguinity was in play in three patients. Two PP patients in the V281L-heterozygous

group had bone-age SD ≥2, and the basal levels of 17-OHP were 2.1 and 2.2 ng/mL, respectively. Consanguinity was in play in one patient. Both patients presented with hirsutism and PCOS were heterozygous for Q318X and had bone-age SD and peak 17-OHP levels of +2.3 and 10.8 ng/mL, respectively. The bone-age SD was <2 in a PCOS patient in whom P30L heterozygosity was recorded. In patients with relevant mutations, the maximum stimulated 17-OHP level was 29 ng/mL. It is surprising that the basal and peak levels of 17-OHP were higher in patients in whom we did not detect any mutation. This may indicate that it is important to evaluate other regions of the *CYP21A2* gene to detect rare mutations causing disease.

Previous studies have shown that mutations exerting mild phenotypic effects (the V281L, P30L, and P453S mutations) result in retention of 20–50% of enzymatic activity, and mutations exerting severe phenotypic effects (I2 splice, I172N, and Q318X) 0–2% [3, 36–38]. An association is evident between genotype and phenotype. Homozygous mutations exerting mild phenotypic effects and heterozygous mutations with mild or severe phenotypic effects trigger the nonclassical form of the disease [38]. Our results are consistent with these earlier data.

The phenotypic heterogeneity of NCAH patients can be explained by the simultaneous expression of different mutations. Such patients may carry an unidentified mutation in the other *CYP21A2* allele or may carry a mutation exerting a mild phenotypic effect in one allele only [3]. This suggests that patients who are compound heterozygotes for one mutation exerting a mild phenotypic effect and another exerting a severe phenotypic effect may be younger have a greater height SD, exhibit a more advanced bone age, and express a higher 17-OHP level at presentation [2, 36]. However, in the absence of full sequences of the *CYP21* gene and regulatory elements thereof, we cannot presently draw such conclusions. The V281L mutation was more common than the P30L mutation in NCAH patients presenting with PCOS and hirsutism [21, 39, 40]. As found previously, the Q318X mutation was also noted in patients presenting with PCOS [22, 41]. Erdeve et al. [23] detected the V281L mutation in all PP patients of a Turkish population and suggested that the condition was caused by this mutation rather than other known mutations. Moreover, it has been reported that the V281L mutation is the most common mutation in Turkish patients with PP, PCOS, and hirsutism [25, 27]. In the present study, the Q318X, P30L, and P453S mutations were detected, in addition to the V281L mutation, in four patients presenting with PP, PCOS, or hirsutism. The P453S mutation was previously noted in Turkish children with NCAH [42, 43]. In other countries, the P453S and P30L mutations were found in 23.1% and 10.3%, respectively, of NCAH patients [3]. Helmberg et al. [38] identified R339H and P453S mutations associated with NCAH and proposed that each mutation caused a 50% reduction in normal *CYP21A2* activity. In another study, P453S was found in 46.2% of NCAH patients [44].

In conclusion, NCAH is a heterogeneous disorder in terms of age of onset, symptomology, and causative mutation. NCAH should be a differential diagnosis in patients presenting with PP, hirsutism, PCOS, and/or advanced bone age, especially in countries in which consanguineous marriages are prevalent. Investigating the molecular genetic mechanism of NCAH should resolve the diagnostic difficulties encountered with hormone testing. Genotyping should be considered for defining biological cohorts and it should shed light on whether the genetic variation is related to the disease in question. Of our nine NCAH patients, five had heterozygous and one had compound heterozygous mutations. Other disease-related mutations may also be present in such patients. Therefore, sequencing is the gold standard for identification of disease-associated mutations.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Bone Mineral Density in Children and Adolescents with Congenital Adrenal Hyperplasia

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Chronic glucocorticoid therapy is associated with reduced bone mineral density. In paediatric patients with congenital adrenal hyperplasia, increased levels of androgens could not only counteract this effect, but could also advance bone age, with interference in the evaluation of densitometry. We evaluate bone mineral density in paediatric patients with classic congenital adrenal hyperplasia taking into account chronological and bone ages at the time of the measurement. Patients aged between 5 and 19 years underwent radiography of the hand and wrist followed by total body and lumbar spine densitometry. Chronological and bone ages were used in the scans interpretation. In fourteen patients, mean bone mineral density Z-score of total body to bone age was −0.76 and of lumbar spine to bone age was −0.26, lower than those related to chronological age (+0.03 and +0.62, resp.). Mean Z-score differences were statistically significant ($P = 0.004$ for total body and $P = 0.003$ for lumbar spine). One patient was classified as having low bone mineral density only when assessed by bone age. We conclude that there was a reduction in the bone mineral density Z-score in classic congenital adrenal hyperplasia paediatric patients when bone age was taken into account instead of chronological age.

1. Introduction

The risks of osteoporosis and bone fractures are frequently studied in elderly people. It is becoming clear, however, that the amount of bone that is gained during growth is an important determinant of future fracture resistance, since 90% of bone mass is acquired in the first two decades of life [1, 2].

Osteoporosis, which is consequent to low bone mass, deterioration of bone tissue, and disruption of bone architecture, has various origins. Hypogonadism, low calcium intake, vitamin D insufficiency, and the use of certain drugs such as anticonvulsants and glucocorticoids are the main risk factors [3]. The glucocorticoids are considered to be an important component of therapy for several conditions including autoimmune, rheumatic, pulmonary, gastrointestinal, and endocrine disorders [4, 5].

Congenital adrenal hyperplasia (CAH) is an endocrine disorder caused by a deficiency in enzymes responsible for the synthesis of cortisol. With the increase of corticotropin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH), stimulated in response to cortisol deficit, adrenal hyperplasia and overproduction of androgens occur, giving the virilised phenotypic characteristics of the disease. Treatment with corticosteroids increases patient survival, but the optimal dose for the control can be difficult to achieve. High doses may be required, which can compromise bone health [6, 7].

Several studies have examined the status of bone mineral density (BMD) in patients with CAH. Some have shown no difference in the BMD of CAH patients compared with healthy patients, measured by dual-energy X-ray absorptiometry (DXA). Other studies have shown low BMD in all or

some subsets of patients with CAH [8–18]. Few studies have evaluated only children [19, 20].

A normal BMD in CAH patients, despite chronic use of glucocorticoids, can be plausible by an androgen excess particular to the disease [7, 21], leading to increased peripheral conversion to oestrogens, opposing the deleterious effect on bone architecture described earlier. Sexual steroids increase osteoblast activity, inhibit the removal of calcium from the body to decrease the formation and activity of osteoclasts, stimulate longitudinal growth of long bones puberty while glucocorticoids promote osteoblast and osteocytes apoptosis, and increase bone resorption by a direct effect on osteoclasts as well as indirect effects that interfere with the metabolism of calcium and vitamin D.

The excessive oestrogen action in paediatric CAH patients causes advanced maturation of the epiphyseal plate, culminating with the usual finding of increased bone age (BA) [22]. We think this could advance the acquisition of peak bone mass compared with healthy children and distort the assessment of DXA, overestimating evaluation of BMD in these patients.

The aim of this study was to evaluate the BMD of total body and lumbar spine by DXA in paediatric patients with CAH classic form, taking into account the chronological age (CA) and BA at the time of the measurement.

2. Materials and Methods

Between August 2011 and October 2012, all patients with classic CAH being monitored at the Endocrinology Clinic of Instituto de Puericultura e Pediatria Martagão Gesteira (IPPMG), the paediatric hospital of the Universidade Federal do Rio de Janeiro (UFRJ), were identified and selected to participate in the study. The inclusion criteria were patients with a clinical laboratory diagnosis of classic CAH (salt-wasting or simple-virilising) and age between 5 and 19 years. Exclusion criteria were the presence of other diseases or associated treatment that could interfere with the assessment of BMD in this population including precocious puberty and GnRH analogue use. This study was approved by the Research Ethics Committee of the institute in November 27, 2011, and all parents of patients provided written informed consent to the study.

The medical records of each patient were reviewed to confirm the unequivocal diagnosis of CAH and ascertain associated diseases or treatments. Glucocorticoid therapy in use (converted to hydrocortisone as 10 mg of hydrocortisone is the same of 2 mg of prednisone/prednisolone or 0.375 mg of dexamethasone) [6] was noted. All patients then underwent a complete physical examination by the researcher, including assessment of height, weight, and Tanner puberty stage.

On the date scheduled as routine followup for patients with CAH, radiography of the hand and wrist was performed. BA was evaluated by the researcher and another professional experienced in BA assessment, using as a reference the *Greulich and Pyle* Atlas [23–25]. If there was a discrepancy in the evaluation of the BA, the mean value was used. Difference greater than one year from BA to CA was classified as advanced BA. Close to adult height was considered in patients with BA greater than 15 years in females or greater than 16 in males.

DXA (Lunar Prodigy Advance, GE Healthcare) was performed in a period of up to three months from obtaining the radiographs. All patients underwent the same paediatric protocol preparation, positioning, and image acquisition [26–31]. Patient data (name, birth date, weight, height, and BA) were included in the software (GE Lunar enCORE v.11.40.004). Evaluations of the patients DXA were made by a professional member of the International Society for Clinical Densitometry (ISCD). The results of densitometry were evaluated according to chronological and bone ages. To this end, the program only switches automatically between chronological age and bone age reported to the software, thus assessing bone mass obtained with the aimed age.

BMD assessment comprised total body (less head) densitometry and lumbar spine (L1–L4) densitometry. Data on absolute BMD (g/cm^2), total body BMD Z-score relative to CA (TB Z CA), lumbar spine BMD Z-score relative to CA (LS Z CA), total body BMD Z-score relative to BA (TB Z BA), and lumbar spine BMD Z-score relative to BA (LS Z BA) were analysed. Results for BMD Z-score less than −2.0 were regarded as low BMD [2].

The database was built with Microsoft Office Excel XP. Statistical analysis was done with XLSTAT software (v. 2012.6.08), including Student's t-test for two-paired samples and Pearson correlation for variables investigated, on the basis of a significance level of 5% ($P < 0.05$). Values are shown as mean ± standard deviation.

3. Results

Twenty-two patients were eligible for the study. Fourteen (one male patient) met the inclusion criteria and reached the final stage of the study. Six patients (four male patients) had precocious puberty and were treated with GnRH analogue; two patients (both female) had a history of seizures and anticonvulsant use. As these treatments can interfere with the densitometry analysis, these eight patients were excluded.

The age of the participants ranged from 6.9 to 18.5 years (mean 11.3 ± 3.6 years), and the BA ranged from 5.75 to 19 years (mean 12.9 ± 3.6 years). Nine children had advanced BA (BA − CA ≥ 1) with the largest difference being +4.8 years. Three patients (numbers 3, 12, and 14) were close to their adult height (Table 1).

Four patients (numbers 1, 2, 4, and 7) were at Tanner I stage, with three of these having BA advancement of at least two years. Regarding the use of glucocorticoids, two were being treated with hydrocortisone, eight with prednisolone, three with prednisone, and one with dexamethasone. All patients started glucocorticoid treatment before 4 months of life. The mean hydrocortisone equivalent dose was 12.9 mg/m^2 per day. Eight patients had salt-wasting form and received fludrocortisone at a dose of 0.1 mg/day (Table 2).

Regarding the assessment of BMD, Table 3 shows the data obtained from the total body and lumbar spine DXA of the 14 patients.

Table 3 also shows the conversion of total body and lumbar spine BMD to BMD Z-score to CA and BA. The conversion of these values using the software database for healthy

Table 1: Patient characteristics according togender, chronological age, bone age, difference between bone age and chronological age, and Z-score for height.

Patient	Gender	CA (years)	BA (years)	ΔBA − CA	Z height
1	F	6.9	5.75	−1.15	−0.29
2	F	7.4	11	3.6	−0.07
3	F	18.5	19	0.5	−0.47
4	F	7.5	10	2.5	0.16
5	F	9.2	14	4.8	1.88
6	M	15.4	15	−0.4	−1.34
7	F	8.1	11	2.9	−1.07
8	F	12.4	14	1.6	−0.25
9	F	12	14	2	−0.95
10	F	11.3	11	−0.3	−0.18
11	F	8.5	11	2.5	1.65
12	F	15.8	19	3.2	−4.11
13	F	10.9	10	−0.9	0.12
14	F	14.8	16	1.2	−2.13
Mean ± SD	—	11.3 ± 3.6	12.9 ± 3.6	1.57 ± 1.81	−0.50 ± 1.47

F: female, M: male, CA: chronological age, BA: bone age, Z height: height Z-score, and SD: standard deviation.

Table 2: Patient characteristics according to gender, congenital adrenal hyperplasia form, Tanner stage, type of corticosteroid treatment, hydrocortisone dose equivalent, and use of fludrocortisone.

Patient	Gender	CAH form	Tanner stage	Corticosteroid treatment	Hydrocortisone dose (mg/m^2/day)	Fludrocortisone dose (mg/day)
1	F	S.W.	M1P1	Prednisolone	4	Y (0.1)
2	F	S.W.	M1P1	Hydrocortisone	18	Y (0.1)
3	F	S.W.	M4P4	Prednisone	6	Y (0.1)
4	F	S.W.	M1P1	Hydrocortisone	15	Y (0.1)
5	F	S.W.	G4P4	Prednisolone	24	Y (0.1)
6	M	S.W.	M5P5	Dexamethasone	3.5	Y (0.1)
7	F	S.V.	M1P1	Prednisolone	14.8	N
8	F	S.V.	M2P2	Prednisolone	11.2	N
9	F	S.V.	M4P5	Prednisolone	18.8	N
10	F	S.W.	M2P4	Prednisolone	8	Y (0.1)
11	F	S.V.	M2P4	Prednisone	18	N
12	F	S.V.	M1P5	Prednisone	14.9	N
13	F	S.V.	M2P1	Prednisolone	3.6	N
14	F	S.W.	M5P5	Prednisolone	11.6	Y (0.1)

CAH: congenital adrenal hyperplasia, F: female, M: male, S.V.: simple-virilising, S.W.: salt-wasting, Y: yes, and N: no.

Table 3: Total body and lumbar spine BMD absolute values and conversion for Z-score to chronological and bone ages.

Patient	TB BMD	LS BMD	TB Z CA	TB Z BA	LS Z CA	LS Z BA
1	0.808	0.737	−0.3	0.5	0.8	1.5
2	0.849	0.648	0.4	−1.3	−0.3	−2.1
3	1.167	1.211	−0.5	−0.5	−0.2	−0.2
4	0.870	0.811	0.7	−0.5	1.5	−0.1
5	1.088	1.074	3.5	0.3	3.2	0.2
6	1.105	1.204	0.0	0.0	0.6	0.6
7	0.855	0.744	−0.5	−1.6	0.2	−1.4
8	1.020	1.190	−0.2	−1.1	1.7	0.5
9	0.936	1.001	−0.4	−1.3	0.6	−0.5
10	0.955	1.041	0.1	0.1	1.3	1.3
11	0.800	0.706	−0.6	−1.8	0.1	−1.5
12	1.125	1.339	−0.4	−1.5	1.1	0.5
13	0.912	0.727	−0.1	−0.1	−1.0	−1.0
14	1.028	0.998	−1.2	−1.9	−0.9	−1.5
Mean (±SD)			0.03 (±1.09)	−0.76 (±0.82)	0.62 (±1.11)	−0.26 (±1.11)

TB BMD: total body BMD absolute values, LS BMD: lumbar spine BMD absolute values, TB Z CA: total body BMD Z-score to CA, TB Z BA: total body BMD Z-score to BA, LS Z CA: lumbar spine BMD Z-score to CA, LS Z BA: total body BMD Z-score to BA, and SD: standard deviation.

Table 4: Pearson correlation between height Z-score, hydrocortisone dose and BA less CA, and BMD Z-scores with P value.

	TB Z CA	TB Z BA	LS Z CA	LS Z BA
Height Z-score	0,53 (0,046)	0,35 (0,208)	0,26 (0,357)	−0,10 (0,708)
Hydrocortisone dose	0,49 (0,086)	−0,48 (0,091)	0,44 (0,130)	−0,42 (0,145)
ΔBA − CA	0,48 (0,079)	−0,45 (0,099)	0,40 (0,148)	−0,41 (0,144)

TB Z CA: total body BMD Z-score to CA, TB Z BA: total body BMD Z-score to BA, LS Z CA: lumbar spine BMD Z-score to CA, LS Z BA: total body BMD Z-score to BA, BA: bone age, and CA: chronological age.

patients of the same CA gave a mean TB BMD Z-score of 0.03 ± 1.09 with a minimum and maximum value of −1.2 and +3.5, respectively, whereas the LS BMD Z-score average was 0.62 ± 1.11, with a lower limit of −1.0 and upper limit of +3.2. The analysis of these values, taking into account this time BA, gave a mean TB BMD Z-score of -0.76 ± 0.82 with minimum value of −1.9 and maximum value of +0.5. In LS BMD Z-score to BA; the mean values were -0.26 ± 1.11 ranging from −2.1 to +1.5.

All nine patients with advanced BA had lower values when BMD Z-score to BA was compared with BMD Z-score to CA. For total body, all patients had normal BMD Z-score when measured to CA and BA. For lumbar spine, all patients had normal BMD Z-score to CA, with one (patient 2) having low BMD to BA.

According to the Student's t-test for two-paired samples, the differences between the mean values of TB Z CA versus TB Z BA and of LS Z CA versus LS Z BA were statistically significant, with lower values for BA (P 0.004 and P 0.003, resp.) (Figure 1).

Using the Student's t-test and Pearson correlation, we found moderate statistically significant correlation between TB Z CA and height Z-score. Moderate correlation, but not significant, between height Z-score and TB Z BA, between hydrocortisone dose in use and BMD Z-scores assessed, and between BA − CA difference and BMD Z-scores assessed. Other correlations were assessed, but they were not significant as described in Table 4.

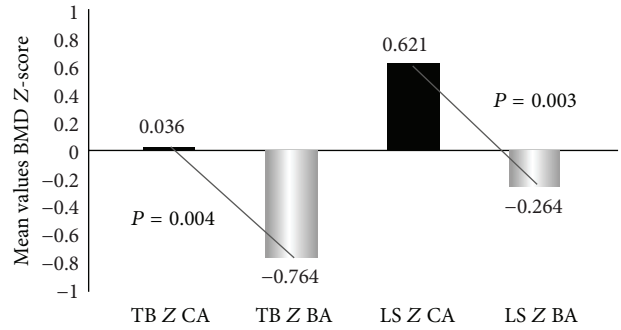

Figure 1: Total body and lumbar spine mean BMD Z-scores to chronological and bone ages. TB Z CA: total body BMD Z-score to CA, TB Z CA: total body BMD Z-score to BA. LS Z CA: lumbar spine BMD Z-score to CA, LS Z BA: lumbar spine BMD Z-score to BA.

4. Discussion

The studies evaluating BMD in patients with CAH are important since corticosteroid therapy is crucial in this type of disease. Reisch et al. [17] carried out an extensive review of bone health in patients with CAH and observed discrepant findings for BMD that ranged from normal to low. Two studies have included only children: Elnecave et al. [20] and de Almeida Freire et al. [19] found normal values of BMD Z-score to CA. de Almeida Freireet al. also assessed values of BMD Z-score to BA, finding lower values than those evaluated for CA. As this was, however, not the focus of their study, it was not discussed in detail.

In CAH, an increase in androgens circulation and their subsequent conversion into oestrogens influences bone maturation and closure of the growth plate in children and adolescents. This is easily verified by the detection of BA advancement, which is a common finding in CAH patients [32, 33]. The discrepancy between CA and BA can interfere with BMD measurement by DXA, since this examination, in the standard mode, only takes into account the CA of patients for analysis. Thus, the BMD of the patients should not be compared with that of a healthy population with the same CA as is usually done for evaluation of children DXA data.

One way to avoid this confounding factor in patients with advanced bone maturation would be the evaluation of the BMD with the BA. The inclusion of this analysis is done by simply adding the BA value into the software for the densitometer. This does not increase the cost and does not require an additional scan.

It should be noted that longer half-life glucocorticoids such as prednisone and prednisolone were commonly administered to our CAH patients as steroid replacement due to lack of availability of stable hydrocortisone oral formulation in our country. Leiteet al. [34] found no difference in the long-term use of these glucocorticoids by CAH patients.

On analysing the individual BMD data in our study, all patients evaluated by CA were classified as having normal density. The same occurred with the analysis of total body BMD to BA, but when it was assessed by lumbar spine, the LS Z BA of one patient (number 2) was less than −2, classified as having low BMD [24].

Moderate correlation, statistically significant, between height Z-score and TB Z CA, was found in our study. Other correlations evaluated were not significant, probably because the small number of participants, but we clearly demonstrated a reduction in BMD Z-score, both total body and lumbar spine, when we took into account BA instead of CA (mean BMD Z-score to BA was statistically lower than the mean BMD Z-score to CA).

Interestingly, only three of the 14 patients had reached almost adult height, giving a possible potential for worsening

of these BMD *Z*-scores in the patients with growth outlook with maintenance of current glucocorticoid treatment in the next few years.

The limitation of our study was the small number of participants limited to paediatric CAH classic form without use of GnRH analogue. We believe that these careful selection criteria have been a differential in the international literature and may stimulate research centers with more numbers of patients in using BA in DXA perform.

Unfortunately we could not assess the DXA according to Tanner stage of the participants because the limited number of patients included. Note that the evaluation of BMD stratified by stage of puberty finds several questions in the international literature as well as few comparative data. This assessment in CAH patients is even more difficult. The early adrenarche without necessarily developing central precocious puberty could classify patients with advanced pubertal stages. We believe that when comparing BMD with bone age, which has relationship with pubertal stage, we minimize errors of pubertal compared.

5. Conclusions

There was a reduction in the BMD *Z*-score in paediatric patients with classic CAH when BA was taken into account rather than CA. These results cause us concern about the real risk, only now viewed with the use of BA assessment by DXA methodology, of bone fractures and osteoporosis later in life in patients diagnosed with classic CAH. We have thus shown the importance of using the degree of maturation (BA) of children and adolescents for an accurate assessment of BMD by DXA in this subgroup of patients.

Abbreviations

BA:	Bone age
BMD:	Bone mineral density
CA:	Chronological age
CAH:	Congenital adrenal hyperplasia
DXA:	Dual-energy X-ray absorptiometry
ISCD:	International Society for Clinical Densitometry
LS *Z* BA:	Lumbar spine BMD *Z*-score relative to bone age
LS *Z* CA:	Lumbar spine BMD *Z*-score relative to chronological age
TB *Z* BA:	Total body BMD *Z*-score relative to bone age
TB *Z* CA:	Total body BMD *Z*-score relative to chronological age.

Disclosure

Laura Maria Carvalho de Mendonça is a member of International Society for Clinical Densitometry (ISCD).

Conflict of Interests

The authors declare that there is no conflict of interests that could be perceived as prejudicing the impartiality of the study. This research was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Authors' Contribution

All authors contributed to the preparation of this study, having read and approved the final paper. Paulo Alonso Garcia Alves Junior and Daniel Luis Gilban Schueftan conceived the study and participated in its design, analysis, and interpretation. Laura Maria Carvalho de Mendonça participated in the analysis and interpretation of DXA. Maria Lucia Fleiuss Farias and Izabel Calland Ricarte Beserra participated in its coordination.

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