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Pediatric adrenal neoplasms

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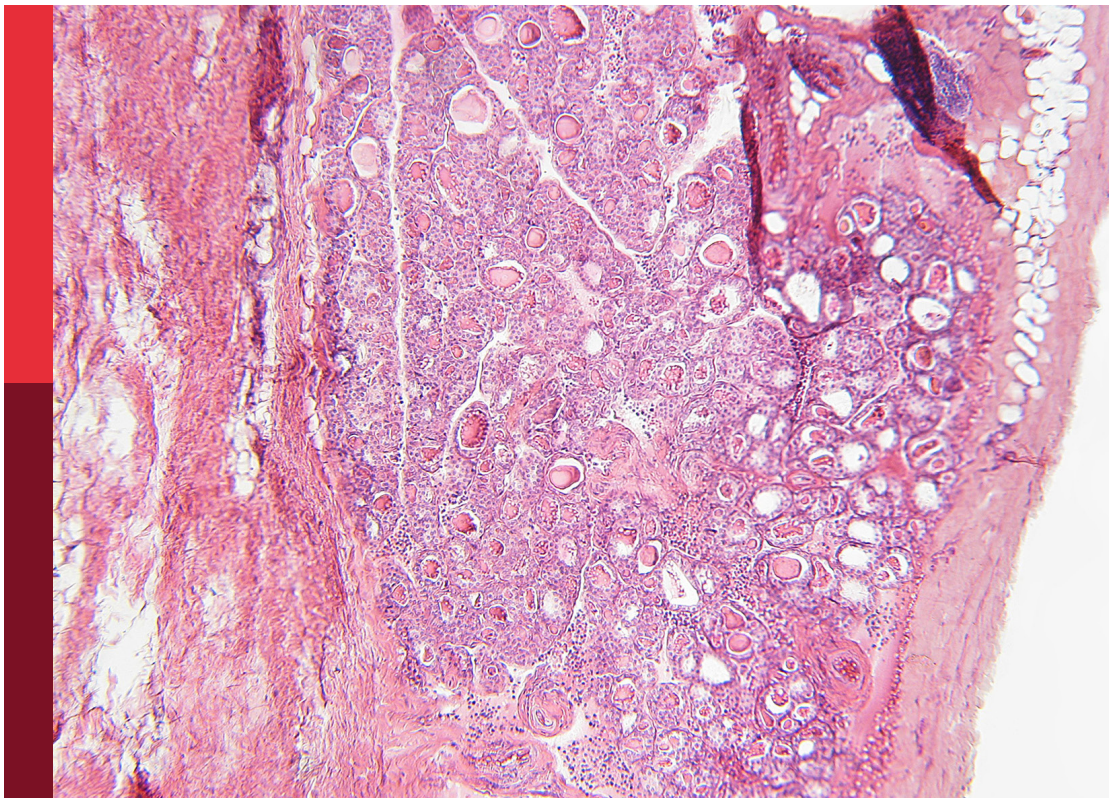

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Pediatric adrenal neoplasms

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Editorial: Pediatric adrenal neoplasms

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Editorial on the Research Topic
Pediatric adrenal neoplasms

Adrenal neoplasms present unique diagnostic and management challenges in pediatric patients related to both their rare and often highly aggressive nature. Although significant advances have been made during the last decades in the diagnosis and treatment of pediatric tumors, there is still no improvement of mortality in pediatric patients with adrenal neoplasms. One possible explanation could be the underestimation of these tumors due to their rare nature and the consequent lack of pediatric-based diagnostic and treatment protocols. Indeed, variable genomic alterations, clinical and biochemical presentations of adrenal tumors among different patient age groups often render the application of adult protocols in pediatric patients inappropriate. For example, pediatric patients with pheochromocytomas/paragangliomas (PPGLs) show significantly higher prevalence of extra-adrenal, multifocal, recurrent, and metastatic tumors compared to adults. As described by Bechmann et al. these findings can be partially explained by the higher prevalence of cluster 1 pathogenic variants in children than adults that activate pseudohypoxia-signaling pathways, and in turn lead to less differentiated tumor phenotypes. Similarly, Ilanchezhian et al. illustrated that children with adrenocortical carcinomas (ACC) present with higher hereditary predisposition, and in particular with higher overexpression of TP53 and insulin-like growth factor (IGFR) 2 pathogenic variants compared to adults, reflecting again different developmental origins between the two groups. Finally, illustration of the genetic background seems to be key for the management of more common and benign adrenal neoplasms in children, as discussed extensively in the draft of Pitsava and Stratakis.

Despite advances in the elucidation of the developmental origins of adrenal neoplasms, rare pediatric adrenal tumors are often misdiagnosed in daily clinical practice. A typical example is the misdiagnosis of PPGLs and ACCs for the more common neuroblastomas, resulting in worse patient outcomes as described in the case series of Kuhlen et al. Indeed, Kuo et al. well outlined that a biopsy of a PPGL falsely mistaken for neuroblastoma can lead to catecholamine excess crisis, whereas of an ACC to a high risk of tumor spillage, both situations dangerous or

detrimental to a patient. A detailed medical history and a proper clinical patient evaluation can assist in raising clinicians´ suspicion for rare adrenal tumors. ACCs in children are often functional, with most common manifestation that of virilization due to androgen excess followed by signs and symptoms of Cushing syndrome. Similarly, as indicated by Eisenhofer et al., signs and symptoms of catecholamine excess (e.g headache, palpitations, diaphoresis) should immediately raise suspicion of a PPGL in a patient with apparent working diagnosis of neuroblastoma, as neuroblastomas do not usually secrete catecholamines in sufficient amounts to justify such clinical manifestations. This is mainly due to the lower amounts of neurosecretory vesicles in the cytoplasm of neuroblastomas compared to PPGLs, as suggested by Mühlerthaler-Mottet et al., resulting in decreased catecholamine storage and rapid metabolism of catecholamines to their O-methylated metabolites, metanephrines.

After clinical evaluation, hormone profiles must be assessed in pediatric patients tested for adrenal tumors, in dedicated laboratories with appropriate analytical methods and reference intervals. Establishment of pediatric reference intervals may be though, challenging. Indeed, as illustrated by Eisenhofer et al., biochemists face difficulties in the establishment of pediatric reference intervals for plasma and urinary metanephrines, due to their dynamic changes during childhood and adolescence. Nevertheless, appropriate pediatric reference intervals are always essential to minimize false positive and negative results and laboratories should never use adult reference intervals for pediatric patients. Apart from analytical considerations, pre-analytical precautions should also be considered in the biochemical work up of patients with catecholamine producing tumors. Specifically, children should remain in supine position for at least 20 minutes before blood sampling for the measurements of plasma metanephrines, whereas blood should be ideally drawn *via* intravenous cannula. Such precautions are expected to minimize activation of the sympathetic nervous system, and thus false elevations of plasma normetanephrine concentrations.

Apart from diagnosis, disease stratification is also challenging among pediatric patients with adrenal neoplasms due to their heterogeneous nature, which can range from spontaneous remission of a relatively benign course to dangerous hormonal hyper-secretion or rapidly progressive metastatic disease. Thus, identification of reliable prognostic tools is essential. In this direction, apart from the role of overexpression of HIF-2A as predictor of more undifferentiated and aggressive phenotypes for catecholamine producing tumors elucidated by Bechmann et al., Lv et al. established that advanced tumor stage, lack of complete tumor resection and older age at diagnosis are independent predictors of poor prognosis for pediatric patients with adrenal malignancies. The above findings provide immediate guidance for stratification and further patient management in daily clinical practice. Finally, advances in molecular imaging have also contributed substantially to improved stratification of pediatric patients with adrenal neoplasms. As described by Fargette et al. (1) ^{123}I-MIBG and ^{68}Ga-DOTATATE PET/CT functional imaging are today routinely used for the staging of patients with neuroblastoma and PPGL respectively, whereas ^{18}F-FDG PET/CT has been effectively applied for the preoperative stratification of pediatric patients with ACC.

Illustration of underlying cellular and molecular mechanisms has proven beneficial also in the field of therapeutics. The role of HIF-2A in the HIF/MYC signaling has led to introduction of HIF-2α inhibitors for the treatment of metastatic/locally aggressive Cluster-1-related PPGLs. Importantly, findings from the pre-clinical study of Pacak et al. (2) in mice, indicate that intratumoral immunotherapy may constitute an effective future approach for the enhancement of the immune response of pediatric patients with PPGL. Finally, accumulating evidence, including the latest report by Urquhart et al., show that temozolamide may be a preferable alternative to systemic chemotherapy with cyclophosphamide/vincristine/dacarbazine in children with metastatic PPGL. With regard to pediatric patients with ACC, although the initially promising targeted therapies with the IGF1R-inhibitors failed expectations, Ilanchezhian et al. indicated that kinase inhibitors such as pembrolizumab or cabozantinib show promising results for the treatment of patients with advanced adrenocortical tumors.

Despite the aforementioned advances, there is need for multidisciplinary approaches and uniformly defined international diagnostic and therapeutic strategies for improved patient management. A solid body of literature has advocated the implementation of multidisciplinary approach for adherence to clinical guidelines, and improvement of patient outcomes. Indeed, findings from the study of Uttinger et al. indicate that surgical interventions of pediatric patients with adrenal neoplasms in high-volume referral centers is expected to minimize postoperative morbidity and increase treatment success rates. Finally, multidisciplinary approaches facilitate entry into transition and long-term surveillance programs, both crucial for the improvement of patient quality of life and the reduction of long-term mortality. Nevertheless, and despite the progress achieved during the last decades, transition care seems to be fragmented into mainstream oncology. Hence, future efforts should be focused on strengthening service coordination, infrastructure, resources, and finally, proper education of pediatric and adult endocrine oncologists in transition medicine.

Author contributions

CP has written the manuscript. All authors revised the article and approved the submitted version.

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Adrenocortical Tumors and Pheochromocytoma/Paraganglioma Initially Mistaken as Neuroblastoma—Experiences From the GPOH-MET Registry

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In children and adolescents, neuroblastoma (NBL), pheochromocytoma (PCC), and adrenocortical tumors (ACT) can arise from the adrenal gland. It may be difficult to distinguish between these three entities including associated extra-adrenal tumors (paraganglioma, PGL). Precise discrimination, however, is of crucial importance for management. Biopsy in ACT or PCC is potentially harmful and should be avoided whenever possible. We herein report data on 10 children and adolescents with ACT and five with PCC/PGL, previously mistaken as NBL. Two patients with adrenocortical carcinoma died due to disease progression. Two (2/9, missing data in one patient) patients with a final diagnosis of ACT clearly presented with obvious clinical signs and symptoms of steroid hormone excess, while seven patients did not. Blood analyses indicated increased levels of steroid hormones in one additional patient; however, urinary steroid metabolome analysis was not performed in any patient. Two (2/10) patients underwent tumor biopsy, and in two others tumor rupture occurred intraoperatively. In 6/10 patients, ACT diagnosis was only established by a reference pediatric pathology laboratory. Four (4/5) patients with a final diagnosis of PCC/PGL presented with clinical signs and symptoms of catecholamine excess. Urine tests indicated possible catecholamine excess in two patients, while no testing was carried out in three patients. Measurements of plasma metanephrines were not performed in any patient.

None of the five patients with PCC/PGL received adrenergic blockers before surgery. In four patients, PCC/PGL diagnosis was established by a local pathologist, and in one patient diagnosis was revised to PGL by a pediatric reference pathologist. Genetic testing, performed in three out of five patients with PCC/PGL, indicated pathogenic variants of PCC/PGL susceptibility genes. The differential diagnosis of adrenal neoplasias and associated extra-adrenal tumors in children and adolescents may be challenging, necessitating interdisciplinary and multidisciplinary efforts. In ambiguous and/or hormonally inactive cases through comprehensive biochemical testing, microscopical complete tumor resection by an experienced surgeon is vital to preventing poor outcome in children and adolescents with ACT and/or PCC/PGL. Finally, specimens need to be assessed by an experienced pediatric pathologist to establish diagnosis.

Keywords: childhood, adrenocortical carcinoma (ACC), pheochromocytoma, paraganglioma, differential diagnostics

INTRODUCTION

In children and adolescents, three different types of primary neoplasias can arise from the adrenal gland. Neuroblastoma (NBL) and pheochromocytoma (PCC) originate from the adrenal medulla. The third group, adrenocortical tumors (ACT), includes adrenocortical adenoma (ACA), adrenocortical tumor of undetermined malignant potential (ACx), and adrenocortical carcinoma (ACC)]. PCC and ACT are extremely rare.

ACT has an estimated annual incidence of 0.2–0.3 new cases per million children and adolescents (1, 2). The female-to-male sex ratio is approximately 2:1. It follows a bimodal age distribution, which peaks in early childhood (<3 years) and adolescence (2–4). The majority of ACT in childhood are related to cancer predisposition syndromes (CPS), most notably Li–Fraumeni syndrome and Beckwith–Wiedemann syndrome (3, 4).

PCCs are catecholamine-producing neuroendocrine tumors arising from chromaffin cells of the adrenal medulla. Extra-adrenal tumors are termed paraganglioma (PGL). The incidence of PCC/PGL is 0.8–1.6 cases per million children and adolescents per year; however, the incidence is likely underestimated since most PCCs/PGLs in children are not detected until adulthood (5, 6). A male predominance is reported inconsistently. The average age at diagnosis ranges between 11 and 15 years (6–8). Most PCCs/PGLs in childhood and adolescence are hereditary in nature including von Hippel–Lindau (*VHL*) syndrome, hereditary paraganglioma syndromes due to variants of the *SDHx* gene, multiple endocrine neoplasia type 2 (MEN2) due to variants of the rearranged during transfection protooncogene (*RET*), and neurofibromatosis type 1 (*NF1*) (6–9).

In contrast, NBL is the third most common pediatric malignancy and the most common extracranial solid tumor in children. Its incidence is 10.4 new cases per million children and adolescents per year (10). The male-to-female sex ratio is 1.1:1.0 in most large studies. The median age at diagnosis is reported to be ≤2 years (11). Germline chromosomal abnormalities or pathogenic variants are rarely reported in children with neuroblastoma. Prognosis is determined by stage, age, myc-n amplification status, and deletion of 1p, among others (11, 12).

In daily practice, it may be difficult to clearly distinguish between these three entities; however, precise discrimination is of crucial importance for correct patient management. Biopsy in ACT or PCC/PGL is potentially harmful and should be avoided whenever possible. In fact, complete surgical resection is a prerequisite for potentially curative treatment approaches. In contrast, tumor biopsy is recommended at the time of initial diagnosis in patients with (suspicion of) NBL as tumor histologyand molecular genetics are prerequisites for risk stratification (11).

We herein first report data on children and adolescents with ACT and PCC/PGL, previously suspected of having an NBL, as recorded in the German Pediatric Oncology Hematology-Malignant Endocrine Tumor (GPOH-MET) database. With respect to these cases, we then explore targeted diagnostics in children and adolescents with adrenal tumors.

PATIENTS

Information on children and adolescents reported to the GPOH-MET study center were analyzed retrospectively. Inclusion criteria were a histologically confirmed diagnosis of ACT or PCC/PGL following suspicion of NBL as documented in medical reports.

The GPOH-MET 97 and GPOH-MET 2013 databases were approved by the ethics committees of the University of Luebeck (Approval number 97-125) and Otto-von-Guericke-University Magdeburg (Approval number 174/12), Germany. Written informed consent was obtained from patients aged 15 years or older and/or their parents or legal guardians, as appropriate.

In the GPOH-MET 97 and GPOH-MET 2013 databases, 146 patients with ACT and 88 patients with PCC/PGL were registered between 1997 and December 2019. We identified 15 children and adolescents initially suspected to have NBL. The demographic details and clinical presentation as available are provided in **Tables 1**, **2**.

TABLE 1 | Demographic details and clinical presentation in 10 children and adolescents with adrenocortical tumors initially mistaken as neuroblastoma.

Patient	Presentation	Lab test	Imaging	Management		Final diagnosis	Outcome
				Tumor biopsy/ resection	*Consolidation of diagnosis*		
Female, 0.4 y	- Finding in surveillance for Beckwith–Wiedemann syndrome	- Blood: not done - Urine: catecholamines negative	- Sonography: suprarenal mass 20 ml	- Tumor resection (R_0)	Histology by reference pathologist	ACA	Alive
Male, 0.8 y	- Finding in surveillance for hemihypertrophy	- Not done	- Sonography: suprarenal mass 11 ml - mIBG: negative	- Tumor resection (R_0)	Histology by reference pathologist	ACA	Alive
Male, 1.0 y	- Finding in surveillance for hemihypertrophy	- Not done	- Sonography: suprarenal mass 3 ml	- Tumor resection (R_0)	Histology by reference pathologist	ACA	Lost to follow-up
Male, 0.2 y	- Incidental finding in sonography	- Blood: normal levels of endocrine hormones - Urine: not done	- Sonography: suprarenal mass 18 ml - mIBG: negative - Bone scan: negative	- Tumor resection (R_0)	Histology by reference pathologist	ACC	Death of disease
Male, 0.4 y	- Incidental finding in echocardiography - Perioral paleness	- Not done	- Sonography: suprarenal mass 7 ml	- Tumor resection (R_0)	Histology and reference pathology	ACA	Alive
Female, 8.3 y	- Abdominal and back pain for 2 months - Finding in diagnostic sonography	- Blood: normal levels of endocrine hormones - Urine: not done	- Sonography: suprarenal mass 168 ml	- Biopsy	Histology and reference pathology	ACC	Alive
Male, 3.2 y	- Fever - Painless abdominal mass - Hypertension	- Blood: androgens, testosterone, estrogen, progesterone levels increased - Urine: not done	- Sonography: abdominal mass 480 ml	- Biopsy	Histology and reference pathology	ACC	Alive
Female, 0.4 y	- Incidental clinical finding—virilization - Hypertrophy of clitoris - Hypertension - Sweating - Reduced performance - Tiredness - Weakness - Headache - Incidental clinical finding during presentation for another reason	- Blood: androgens, testosterone, DHEAS, androstenedione, estrogen, estriol, estradiol, progesterone, 17-OHP, glucocorticoids, cortisol, renin, aldosterone levels increased - Urine: not done	- Sonography: abdominal mass 304 ml	- Tumor resection - Intraoperative tumor spillage	Histology and reference pathology	Adreno-cortical tumor of unknown dignity	Alive
Female, 8.4 y	- Symptomatic therapy of endocrine signs for one year by non-specialist—virilization - Premature pubarche - Hypertrophy of clitoris - Acne - Sweating - Smelling perspiration - Greasy hair - Vertigo - Nausea and vomiting - Symptomatic therapy of endocrine signs for one year by non-specialist	- Blood: androgens, testosterone, DHEAS, progesterone, 17-OHP, glucocorticoids, cortisol levels increased - Urine: not done	- Sonography: abdominal mass 293 ml	- Tumor resection - Intraoperative tumor spillage	Histology by reference pathologist	ACC	Death of disease

(Continued)

TABLE 1 | Continued

Patient	Presentation	Lab test	Imaging	Management		Final diagnosis	Outcome
				Tumor biopsy/ resection	*Consolidation of diagnosis*		
Female, 15.8 y	- Patient from foreign country, no data available	No data	No data	- Tumor resection (R_0) - 8 cycles of chemotherapy	Histology by reference pathologist	ACC	Alive

ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; DHEAS, dehydroepiandrosterone sulphate; mIBG, meta-iodobenzylguanidine; NBL, neuroblastoma; NSE, neuron-specific enolase; R_0, microscopic complete tumor resection; y, years; 17-OHP, 17-hydroxyprogesterone.

Adrenocortical Tumor Cases Mistaken as Neuroblastoma

In 10/15 patients with suspected NBL, a histologically based diagnosis of ACT was subsequently established. The median age at presentation was 0.9 years (range, 0.2–15.8), and five patients were male (**Table 1**). At the last follow-up, 2/10 patients had died of disease.

In 3/10 patients (aged 0.4, 0.8, and 1.0 years), a small adrenal mass was detected by sonography for tumor surveillance because of Beckwith–Wiedemann syndrome and hemihypertrophy, respectively. Urine analysis showed catecholamines within the normal range (according to local reference values) in one-third of children, while for two-thirds of patients no measurements were performed. Meta-iodobenzylguanidine (mIBG) scintigraphy was negative in one-third of patients and not carried out in two-thirds. All three patients underwent tumor resection. Assessment by a local pathologist revealed NBL, which was subsequently revised to ACA by a reference pediatric pathologist in all three patients.

Two (2/10) patients (aged 0.2 and 0.4 years) underwent diagnostic sonography for other reasons. Blood analysis revealed normal levels of "endocrine" hormones (not further specified) in one. On suspicion of NBL, complete tumor resection was performed in both patients. In one patient, diagnosis of NBL was revised to ACA by a pediatric reference pathologist while ACC was diagnosed in the other by a local and reference pediatric pathologist.

In one 8.3-year-old patient, an adrenal mass was detected by diagnostic sonography for persisting abdominal and back pain. This patient also showed normal levels of "endocrine" hormones (not further specified). On suspicion of NBL, a biopsy was performed and revealed ACC.

One 3.2-year-old patient presented with fever and a palpable painless abdominal mass. Clinical assessment revealed hypertension raising suspicion of NBL; however, blood analysis demonstrated increased steroid hormone levels. Nevertheless, on suspicion of NBL, tumor biopsy was performed and revealed ACC.

Two (2/10) patients (aged 0.4 and 8.4 years) presented with virilization, hypertrophy of the clitoris, and inappropriate sweatiness. Abdominal sonography demonstrated an adrenal mass of approximately 300 ml in both patients. Blood tests determined increased levels of steroid hormones; however, NBL was still suspected, and tumor resection was performed. In both patients, tumor rupture occurred intraoperatively. ACx was diagnosed by a local pathologist and subsequently confirmed by a pediatric reference pathologist in the 0.4-year-old female. In the 8.4-year-old female, reference pathology assessment determined ACC.

One 15.8-year-old patient was diagnosed and treated for NBL abroad. Data on clinical presentation at diagnosis were not available. Once transferred to a German pediatric oncology clinic, histological specimen was reassessed by a pediatric pathologist and diagnosis revised to ACC.

In summary, seven (7/9; missing data in one patient) patients with ACT initially mistaken as NBL presented without obvious clinical signs and symptoms of steroid hormone excess while two of nine patients clearly did. Blood analysis determined increased levels of steroid hormones in one out of 10 additional patients; however, urine analysis demonstrated catecholamine levels within normal limits in one out of nine patients and was not performed in eight of nine. Two (2/10) patients underwent tumor biopsy, while in two out of 10 other patients tumor rupture occurred intraoperatively. In six out of 10 patients, ACT diagnosis was only established by reference pediatric pathology review.

Pheochromocytoma and Paraganglioma Mistaken as Neuroblastoma

In five out of 15 patients suspected to have NBL, subsequent histological findings confirmed a PGL in three out of five cases, PCC and PGL in another, and a composite PCC and NBL in the fifth case (**Table 2**). The median age at presentation was 9.6 years (range, 4.7–13.4). Three (3/5; 60%) patients were male. All patients were alive at the last follow-up.

Among five patients with PCC/PGL, four out five presented with an abdominal tumor and one out of five with a thoracic tumor. Daily activity was markedly reduced in four out five patients. Clear clinical signs and symptoms of catecholamine excess were reported in three out five patients. In addition, echocardiography demonstrated left ventricular load and hypertrophy in one out of five patients. Two (2/4) of those patients developed a hypertensive crisis. Tumors were detected by sonography in all patients. In two out of five patients, mIBG scintigraphy was positive (data missing for 3 patients). Urine analysis was performed in only two out of five cases; the first

TABLE 2 | Demographic details and clinical presentation in 5 children and adolescents with pheochromocytoma and paraganglioma initially mistaken as neuroblastoma.

Patient	Presentation	Lab tests	Imaging	Management		Final diagnosis	Outcome
				Tumor biopsy/ resection	*Consolidation of diagnosis*		
Male 4.7 y	- Reduced activity - Hypertension - Tachycardia - Diaphoresis - Palpitations - Headache - Swelling of hands - Tachypnea Acute condition: - Clouding of consciousness - Seizure	Urine: vanillylmandelic acids and metanephrine levels increased	- Sonography: thoracic mass 9,4 ml - MRI: suspicion of NBL - mIBG scintigraphy: positive - Bone scan: negative - ECG: left ventricular load and hypertrophy	- 1 cycle of NBL chemotherapy - Fine needle biopsy after first cycle of chemotherapy	Histology and reference pathology	PGL	Alive
Male 13.4 y	- Palpable abdominal mass - Reduced activity - Hypertension - Hypertensive crisis - Diaphoresis - Loss in weight - Abdominal pain - Nausea and vomiting	Urine: noradrenaline and normetanephrine levels increased	- Sonography: multifocal abdominal masses - Bone scan: negative - ECG: left ventricular load and hypertrophy	- Tumor resection on suspicion of NBL (R_0)	Histology and reference pathology	PCC and PGL	Alive
Female 8.2 y	- Reduced activity - Hypertension - Tachycardia - Palpitations - Diaphoresis - Headache - Flush - Weakness	Not done	- Sonography: adrenal mass 16,2 ml - mIBG scintigraphy: positive	- Tumor resection on suspicion of NBL (R_0)	Histology and reference pathology	Composite PCC and NBL	Alive
Male 11.2 y	- Palpable abdominal mass - Palpitations	Not done	- Sonography: abdominal mass 339 ml - CT: lung metastases - ECG: left ventricular load and hypertrophy	- Tumor resection (R_0) - 1 cycle of NBL chemotherapy	Histology by reference pathologist	PGL	Alive
Female 9.6 y	- Palpable abdominal mass - Reduced activity - Abdominal pain	Not done	- Sonography: abdominal mass 332 ml with tumor thrombus into the VCI - MRI: irregular roundish shape, heterogeneous structure, moderate heterogenous uptake of contrasting agent	- Unresectable - Fine needle biopsy	Histology and reference pathology	PGL	Alive

CT, computed tomography; ECG, echocardiography; mIBG, meta-iodobenzylguanidine; MRI, magnetic resonance imaging; NBL, neuroblastoma; PCC, pheochromocytoma; PGL, paraganglioma; R_0, microscopic complete tumor resection; VCI, vena cava inferior; y, years.

presented with increased levels of vanillylmandelic acid (VMA) and metanephrine and the second with increased levels of noradrenaline and normetanephrine (according to local reference values, information on assay methods was not available). Urine analysis was not done in three out of five patients.

Although presenting with serious clinical signs and symptoms of catecholamine excess including a tonic–clonic convulsive seizure, and the high metanephrine levels in urine, NBL-directed chemotherapy was administered in a 4.7-year-old male with a small thoracic mass. Fine-needle biopsy was performed only subsequent to the chemotherapy. Histology

revealed diagnosis of PGL further confirmed by genetic testing (identification of a pathogenic *VHL* variant).

Similarly, a 13.4-year-old male presented with serious clinical signs and symptoms of catecholamine excess confirmed by urine analysis (details not available). Sonography demonstrated adrenal and abdominal masses. On suspicion of NBL, tumor resection was performed. Histology confirmed PCC and PGL. A pathogenic *SDHB* variant was identified by genetic testing.

In the third patient (8.2-year-old female) with serious clinical signs and symptoms of catecholamine excess, sonography demonstrated a small, mIBG-positive adrenal mass. On suspicion of NBL, tumor resection was performed. The pathology report revealed a composite PCC and NBL. Genetic testing identified a pathogenic *VHL* variant.

An 11.2-year-old male presented with palpitations and a palpable abdominal mass. Computed tomography demonstrated lung metastases, echocardiography left ventricular load and hypertrophy. On suspicion of NBL, complete tumor resection was performed and one cycle of NBL-directed multidrug chemotherapy was administered. Reassessment of the tumor specimen by a pediatric reference pathologists ascertained a diagnosis of PGL.

A 9.6-year-old female with an abdominal mass and a tumor thrombus into the vena cava inferior (detected by sonography) presented with reduced activity and abdominal pain. Tumor resection was deemed impossible; thus, fine-needle biopsy was performed on suspicion of NBL. Histology revealed PGL.

In summary, four (4/5) patients with PCC/PGL initially mistaken as NBL presented with clinical signs and symptoms of catecholamine excess. Urine analysis indicated suspicious findings in two (2/5) patients, while testing was not done in three (3/5) patients. Importantly, measurements of plasma metanephrines were not performed in any patient. Despite signs and symptoms of catecholamine excess, accompanied in two (2/5) cases by evidence of catecholamine excess, no patient received adrenergic blocking drugs before surgery. In four (4/5) patients, PCC/PGL diagnosis was established by a local pathologist, while in one (1/5) patient the diagnosis was revised to PGL by a pediatric reference pathologist. Genetic testing indicated variants of PCC/PGL susceptibility genes in three (3/5) patients, whereas data were not available in two (2/5) patients.

CLINICAL PRESENTATION AND DIAGNOSTIC WORKUP IN CHILDREN WITH ACT AND PCC/PGL

In contrast to our case series that included 20% of patients with signs of virilization, ACT are associated with excessive androgen production and virilization in 80–95% of cases (2–4, 13). Signs of virilization are premature pubarche (early onset of pubic hair), hirsutism, and hypertrophy of the clitoris or penis. Typically, these signs show rapid progress. Often bone age is significantly accelerated. Further presentations in children with ACT comprise voice changes, accelerated growth, gynecomastia, or acne.

Hypercortisolism (Cushing's syndrome) is reported in up to 75% of patients (2–4, 13). Symptoms of Cushing's syndrome may include growth retardation, hypertension, central obesity, moon face, and buffalo hump. Hypertension without other symptoms of hypercortisolism was reported in only one (1/10) patient in our case series.

Typically, only few tumors are hormonally inactive and, thus, asymptomatic. The gradual onset of signs and symptoms may frequently be unrecognized for several months. In six (6/10) patients subsequently diagnosed with ACT, no signs and symptoms of excessive androgen production and/or hypercortisolism were reported.

The clinical presentation of PCC/PGL in children is dominated by manifestations of excessive catecholamine secretion, which can result in predominant symptoms such as palpitations, sweating, tremor, pallor, nausea, and vomiting (6, 14, 15). Other relevant symptoms include hypertension, headache, and visual impairment. These signs and symptoms often have a recurrent paroxysmal nature. This is particularly relevant for hypertension, which is less common in children than in adults. Therefore, paroxysmal hypertension should raise suspicion of catecholamine excess.

Also, NBL can present with hypertension due to excessive catecholamine secretion and/or renal vessel compression.

In fact, serious clinical signs and symptoms of catecholamine excess were present in three (3/5) patients in our case series, while one (1/5) additional patient presented with milder signs and symptoms. No typical manifestations of catecholamine secretion were reported in only one (1/5) patient. Although the basis for clinical suspicion of PCC/PGL depends usually on the presence of signs and symptoms of catecholamine excess, routine surveillance programs have indicated that in children as in adults, a catecholamine-producing tumor can be present without manifestations of hypertension or other signs and symptoms of catecholamine excess (16, 17). Thus, absence of hypertension or signs and symptoms of catecholamine excess cannot be used to exclude PCC/PGL. This is also born out from widespread use of imaging studies, which led to the incidental discovery of PCC/PGL without hypertension or other signs and symptoms of catecholamine excess.

ENDOCRINE ASSESSMENT FOR EXCESS HORMONE PRODUCTION

A detailed preoperative endocrine assessment is essential to establishing the origin of the neoplasia (cortex versus medulla versus others).

Biochemical Testing for ACT

In general, endocrine workup in a patient suspected of an adrenal tumor is complex and consultation of a pediatric endocrinologist should be sought. The biochemical diagnosis of ACT is based on

the analysis of steroid hormones in plasma or serum, as well as the determination of steroid hormone metabolites in urine (13). Currently, immunoassays or liquid chromatography–tandem mass spectrometry (LC-MS/MS) is the preferred technique for steroid hormone analysis in plasma or serum. However, due to specificity problems with direct immunoassays, clinicians must ensure that the methods applied are reliable. Thus, collaboration with specialized and experienced pediatric laboratories is highly advisable. The most important steroid hormone analytes in plasma or serum required for the diagnosis of ACT are the glucocorticoid marker cortisol, the main androgens DHEA-S and testosterone, and the mineralocorticoid aldosterone. The concentrations of steroid hormones in plasma or serum may vary with time over the day. A dexamethasone suppression test may be useful since autonomous cortisol production is typically not suppressible.

Urinary steroid metabolome analysis by gas chromatography–mass spectrometry (GC-MS) is useful for the delineation of adrenal tumors. The technique makes use of the fact that 90% of all steroid hormone metabolites are excreted into urine. Especially in children, urine is easy and non-invasive to obtain. As GC-MS is a non-selective (untargeted) analytical technique and has the highest separation power for steroid hormone metabolites, it permits characterization of the complete steroid metabolome (steroid metabolic fingerprint) that is unique for individuals with an adrenocortical tumor. The steroid metabolite pattern comprises glucocorticoids, mineralocorticoids, sex hormones, and intermediate metabolites (18).

The differential diagnosis of hyperandrogenism is complex. In this context, such a comprehensive GC-MS-based urinary steroid metabolome analysis provides the added benefit of discerning further diagnostically important entities such as the virilizing CAH forms of 21-hydroylase deficiency, 11-hydroxylase-deficiency, and 3β-hydroxysteroid dehydrogenase deficiency as well as other identifiable causes of hyperandrogenism such as 11β-hydroxysteroid dehydrogenase deficiency type 1 (19).

Biochemical Testing for Catecholamine-Producing Tumors

Despite the fact that four out of five pediatric patients with a final diagnosis of PCC/PPGL presented with signs and symptoms of catecholamine excess, a biochemical workup was incomplete or included analytes no longer recommended for biochemical testing, such as catecholamines or VMA, the final product of catecholamine metabolism. In addition, in three of five cases biochemical testing was not performed at all.

In contrast to chromaffin-cell-derived PCCs/PGLs that actively secrete catecholamines from storage vesicles by a process involving exocytosis, which only occurs episodically or at low or even negligible rates, NBLs display a relative lack of such catecholamine storage vesicles (Eisenhofer et al. 2022 submitted data, 20–24). Therefore, it is well established that plasma or urinary measurements of catecholamines offer limited diagnostic accuracy for the diagnosis or exclusion of both NBL and other catecholamine-producing tumors (25). Thus, both types of catecholamine-producing tumors may present with normal concentrations of catecholamines in plasma or urine.

Diagnosis of NBLs currently depends principally on biopsy-based histopathology. In contrast, for diagnosis of PCCs/PGLs, measurements of plasma or urinary metanephrines, the O-methylated metabolites of catecholamines, are the recommended first-line tests (26). The diagnostic superiority of metanephrines over catecholamines is explained by the fact that the first are produced from metabolism of catecholamines in large amounts within tumor cells, a process that is independent of catecholamine secretion 27. Additional measurements of plasma methoxytyramine, the O-methylated metabolite of dopamine, are important for diagnosis of patients with dopamine-producing tumors (28) especially among patients with high risk of PPGLs (29). In contrast, measurements of the urinary catecholamine metabolite VMA, often measured in conjunction with urinary homovanillic acid (HVA), are of limited diagnostic utility (Eisenhofer et al. 2022 submitted data, 30) for catecholamine-producing tumors, as particularly tumor-derived VMA is diluted by considerable amounts of VMA produced in other sources (31).

In the herein presented cohort, catecholamines and/or metanephrines were measured in urine. Previous studies in adult cohorts but also isolated reports of pediatric cases, though, have shown superior diagnostic performance of plasma over urinary metanephrines (29, 32, 33). However, blood sampling can be an inconvenient procedure for children. In particular, blood should always be drawn after overnight fasting and at least after 20 min of supine rest. In addition, blood sampling may often be a stressful condition for young children and may lead to activation of the sympathetic nervous system and therefore to increased proportions of false-positive results. Taking the above into consideration, measurements of metanephrines in urine constitute a more practical approach, compared to plasma metanephrines, with spot urine collections the preferred method in children. If this approach is decided, clinicians should be aware that urinary methoxytyramine has negligible diagnostic value, as it is mainly derived from sources largely independent of the circulating metabolite or dopamine (34). Finally, it is of paramount importance that laboratories utilize appropriate assays and have available appropriate age-adjusted reference intervals for children with further consideration of sex differences (35–38). Liquid chromatography with electrochemical detection (LC-ECD) was initially the method of choice for the diagnosis of PCC/PGL (39) but in the last decade has been largely replaced by liquid chromatography with tandem mass spectrometry (LC-MS/MS) (40). Easy-to-use immunoassays are not recommended due to underestimation of concentrations in conjunction with upper limits of reference intervals that are also too high for reliable detection of tumors (41).

IMAGING FEATURES

For best patient care, adequate visualization of the tumor and potential metastases is essential. For the diagnosis of an adrenal mass in children as well as for staging purposes, ultrasonography (US) followed by magnetic resonance imaging (MRI) and computed tomography (CT) for the evaluation of lung metastases is a standard initial imaging modality (42, 43). Since CT and functional imaging modalities are associated with radiation exposure, they

should be used in a restrictive and targeted manner particularly in children.

Indeed, all patients in our case series underwent MRI and CT (patients with ACC from a foreign country), respectively, in addition to ultrasonography. Details on imaging features were not available; however, we may suggest that imaging features were consistent with differential diagnosis of NBL as assessed by the local radiologist. One may speculate that an experienced pediatric radiologist would have established another prioritized differential diagnosis based on imaging features.

All three imaging modalities are widely and readily available, but they, in many cases by themselves, cannot determine the exact entity of the mass nor its behavior (ACA vs. ACC, PCC with malignant potential). Of note, the International Neuroblastoma Risk Group (INRG) Project proposed a new staging system designed for tumor staging before any treatment. The INRG Staging System includes image-defined risk factors (IDRFs) for localized disease (44, 45). Details on imaging diagnostic steps and features in ACT, PCC/PGL, and NBL are given in **Figure 1**.

Additional information such as the results of laboratory assessment and functional imaging is needed. On radiological imaging, most ACCs and malignant PCCs are inhomogeneous with irregular margins and irregular enhancement of the solid components after administration of i.v. contrast medium.

For planning of tumor resection, morphological imaging is important to exclude or verify local invasion or tumor extension into the inferior vena cava, as well as lymph node or other metastases (lung and liver).

Functional imaging using radiotracers may aid in the diagnosis and staging of adrenal tumors. It should be used preoperatively only in hormonally inactive tumors. The radiotracer should be chosen according to clinical settings, the results of morphological imaging, and especially the results of hormonal screening (particularly in patients with PCC/PGL); in some patients, the result of a biopsy including genetics may already be available and guide tracer selection (46).

The standard for functional imaging of NBL is I-123-meta-iodobenzylguanidine (mIBG) (11, 47). Imaging may be used to

FIGURE 1 | Imaging diagnostics **(A)** and features **(B)** of adrenal tumors in children. CT, computed tomography; FDG, fluorodeoxyglucose; mIBG, metaiodobenzylguanidine; MRI, magnetic resonance imaging; PET, positron emission tomography; SPECT, single-photon emission computed tomography; US, ultrasonography; *, CT in selected cases only due to radiation exposure.

identify metastases as well as patients suitable for I-131-mIBG radionuclide therapy. Imaging is performed by planar scintigraphy but also as single-photon emission computed tomography (SPECT) sometimes in combination with low-dose CT (SPECT/CT). I-123-mIBG targets the norepinephrine transporter which is highly expressed on both NBL and PCC/PGL due to the similar origin of these tumors; correspondingly, a differentiation between NBL and PCC/PGL is not possible using 123-mIBG. In line with this, mIBG scintigraphy was performed prior to surgery in two out of five patients subsequently diagnosed with PCC/PGL. In both patients, the lesions were mIBG positive. Hence, mIBG imaging did not add in the differential diagnosis of the lesions.

In case no uptake is observed, an F-18-fluorodeoxyglucose-positron emission tomography (F-18-FDG-PET) may be performed. F-18-FDG-PET is a useful tool for distinguishing potentially malignant lesions from benign tumors in radiologically indeterminate adrenal lesions (48). The uptake of the tracer reflects the glucose metabolic activity of the respective tissue; therefore, F-18-FDG-PET is not able to reliably differentiate between (malignant) tumor entities. Uptake can sometimes also be seen in benign lesions, such as adrenal adenomas or (benign) PCCs. Of note, I-123-mIBG imaging is superior to F-18-FDG PET/CT in the assessment of disease extent in high-risk neuroblastoma; however, F-18-FDG PET/CT has significant prognostic implications in these patients (49, 50).

F-18-L-Fluoro-L-3, 4-dihydroxyphenylalanine (F-18-FDOPA), another tracer used for PET imaging, is the precursor of catecholamines and therefore shows uptake in tumors derived from the adrenal medulla (NBL and PCC/PGL). Accordingly, a differentiation between NBL and PCC/PGL is not possible using F-18-FDOPA. However, ACT do not commonly show uptake of these radiotracers.

Somatostatin-receptor-targeted PET (SSR-PET) has widely replaced somatostatin receptor scintigraphy because of superior imaging characteristics. Usually, somatostatin receptor agonists labeled with the positron emitter Gallium-68 (Ga-68-DOTA-NOC, -TOC, -TATE) are used. Catecholamine-producing tumors most notably PGLs are known to highly express SSR (51); however, also in ACT an SSR expression may be found (52). The imaging may be used to identify patients suitable for peptide receptor radionuclide therapy (PRRT).

In patients with an established diagnosis of (malignant) PCC/PGL, F-18-FDG-PET appears to be inferior to other PET imagings, targeting the somatostatin receptor (SSR-PET) in a pediatric population with an *SDHB* variant (53) and malignant tumors; however, it may be used if SSR-PET is not available. F-18-FDOPA-PET may be used in the abovementioned entity as well and has been shown to be of value in tumors associated with *VHL*, *NF1*, and *RET* alterations (51).

SURGICAL APPROACH TO PEDIATRIC ACT AND PCC

A microscopically complete resection is of utmost importance in the treatment of both ACC and PCC/PGL; however, biopsy was performed in two out of 10 patients with ACT and two out of five patients with PCC/PGL; therefore, one patient in whom complete tumor resection was deemed impossible.

Open surgery with transperitoneal access is the standard treatment of all patients with localized and locally advanced-stage ACCs when complete resection can be achieved (54, 55). Care must be taken to avoid tumor spillage in any case. However, in our case series, tumor spillage by intraoperative tumor rupture occurred in two out of 10 patients subsequently diagnosed with ACC and ACx, respectively. Intraoperative tumor rupture is associated with an inferior prognosis and implicates intensified systemic treatment also in patients otherwise not qualifying for systemic therapy.

A multinational survey including 68 children and adolescents with adrenal masses including PCC (n = 9) and ACT (n = 1) demonstrated that the minimally invasive approach was safe in masses up to 145.6 ml with a low complication rate (56). In a single-center study, Wu and colleagues reported their experience with open vs. laparoscopic adrenalectomies for stage I/II adrenocortical carcinoma and a tumor size <10 cm in adults (57). In this study, local and peritoneal recurrence rates were 42% after laparoscopic adrenalectomy and 22% after open adrenalectomy ($p = 0.035$); however, there was no significant difference between the open and laparoscopic approaches regarding OS and EFS. The authors concluded that the open approach should be considered as treatment of choice. Even though data on the surgical approach in patients with intraoperative tumor rupture were not available, both patients in our case series presented with large tumors with approximately 300 ml tumor volume.

The resection status is a major predictor of prognosis for ACC in children and adolescents (58). A margin-free complete resection, in fact, provides the only means to achieve long-term survival. To obtain a R_0 resection of a locally advanced ACC, it can be mandatory to resect (parts of) adjacent organs such as the wall of the vena cava, liver, spleen, colon, pancreas, and/or stomach. Locoregional lymphadenectomy improves tumor staging, but the role of lymph node dissection in patients with localized disease remains unclear (3).

Laparoscopic adrenalectomy is a safe and effective procedure for PCC/PGL in children (59). In adults, no difference in the 5-year-EFS in malignant PCC between laparoscopic and open approaches was demonstrated (60), but tumor size >6 cm was associated with a decreased likelihood for minimally invasive surgery (60). A meta-analysis of open vs. laparoscopic surgery in PCC involving 626 adult patients demonstrated that laparoscopic surgery is safe and effective and causes less intraoperative hemodynamic instability (61). No data on the prognostic role of radical surgery and lymphadenectomy are available up to now for pediatric PCC and synchronous metastatic diseases.

Of note, a preoperative preparation with adrenergic blocking drugs is mandatory for patients with PCC/PGL (26). In our case series, no child with PCC/PGL underwent preoperative preparation with adrenergic blockade despite clinical suspicion of catecholamine excess in several cases. This is dangerous and

completely inappropriate. Preoperative preparation requires alpha-adrenergic blockade to control blood pressure and to prevent perioperative cardiovascular complications. Beta blockers are used if significant tachycardia occurs after alpha blockade. Preoperative medical treatment is recommended for 7 to 14 days to allow adequate time to normalize blood pressure and heart rate. Treatment should also include a high-sodium diet and fluid intake to reverse catecholamine-induced blood volume contraction preoperatively to prevent severe hypotension after tumor removal (26).

In summary, adrenal surgery should be performed only in experienced centers. Laparoscopic adrenalectomy should be considered extremely carefully and only performed in centers with a consolidated experience in laparoscopic adrenal surgery, in which principles of oncologic surgical treatment are strictly respected.

HISTOLOGICAL DIAGNOSIS

The pathological differential diagnosis of adrenal neoplasia is still largely based on morphological features requiring an experienced pediatric pathologist. This is corroborated by our data; diagnosis was established by a pediatric reference pathologist in six out of 10 patients with ACT and one out of five patients with PCC/PGL.

Differentiating benign from malignant adrenocortical tumors is very challenging on a biopsy only and may lead to misdiagnosis (48, 62). Furthermore, the biopsy comes with significant risks such as hemorrhage and the risk of tumor dissemination precluding a R_0 resection (63). The latter is a major prognostic factor in children and adolescents with ACT (58, 64).

In patients with PCC/PGL, adrenal biopsy can be life-threatening due to biopsy-related complications such as hemorrhage, capsular disruption with tumor implantation, hypertensive crisis, myocardial infarction, arrhythmia, stroke, or death (65–68).

If a biopsy is planned, rarely required in adrenal lesions, ACT and PCC/PGL must be excluded by biochemical testing prior to the biopsy to avoid potential life-threatening complications. However, two out of 10 children with ACT and two out of five children with PCC/PGL were biopsied either percutaneously or by open surgery.

The diagnosis of an ACT is based on morphology and the immunohistochemical expression of, e.g., inhibin and melan A. The differential diagnosis between ACA and ACC is challenging. The most widely used diagnostic score for tumors in the pediatric age group has been introduced by Wieneke et al. (69) and includes the following parameters: weight, size, extension into adjacent tissue and organs, vena cava invasion, venous invasion, capsular invasion, tumor necrosis, number of mitoses, and atypical mitotic figures. A score >3 suggests malignancy. More recently, a five-item microscopic score was proposed by Picard et al. (13) including capsular invasion, venous invasion, tumor necrosis, number of mitoses, and Ki-67 proliferation index.

In our case series, ACA was diagnosed in four out of 10 patients aged <1 year with small ACT, while ACCs were diagnosed in five out of 10 patients ranging from 0.2 to 15.8 years with predominantly large tumors (range, 18–480 ml). In addition, ACT was classified as ACx in one 0.4-year-old patient with a large tumor of 304 ml.

For PCC, the situation is similarly demanding (70). Several histologic features (local invasiveness, growth pattern, presence of necrosis, cellularity, spindled morphology, nuclear pleomorphism and hyperchromasia, mitotic activity, and atypical mitosis) comprise the PCC adrenal gland scaled score (71). However, there is currently no consensus on the adoption of a formal scoring system for these tumors. According to the current World Health Organization (WHO) classification, malignancy is still defined by the presence of metastases to a site where PCC/PGL tissue is not normally present, i.e., liver or bone, to avoid confusion with multiple primary tumors (72, 73). No single histological finding can predict metastatic disease.

In our cohort, diagnosis of PGL was only established by a pediatric reference pathologist in one out of five cases with metastatic disease to the lungs, while in four out of five patients diagnoses were already established by local pathologists. Worth noting is that one out of five patients with a pathogenic *SDHD* variant presented with multifocal disease, and in one out of five patients tumor invasion into the VCI was documented. Both clinical presentations cannot clearly distinguish between NBL and PCC/PGL.

NBL is one of the "small, round blue cell" neoplasms in childhood (74, 75). The histologic subtypes of the neuroblastic tumors appear to correlate with the normal differentiation patterns of the sympathetic nervous system (74–77). The typical NBL is composed of small but uniformly sized cells containing dense, hyperchromatic nuclei and scant cytoplasm. The presence of neuritic processes, or neuropil, is a pathognomonic feature of all but the most primitive NBL (75). The Homer–Wright pseudorosette, another diagnostic feature of NBL, seen in 15% to 50% of cases, is composed of neuroblasts surrounding areas of eosinophilic neuropil. Distinguishing NBL from other tumors of childhood often requires techniques beyond hematoxylin and eosin staining and light microscopy. Immunohistochemistry is a helpful adjunct to light microscopy. NBL will stain with monoclonal antibodies recognizing, e.g., synaptophysin, tyrosinhydroxylase, and PHOX2B.

DISCUSSION

The differential diagnosis of adrenal masses in children and adolescents can be challenging. In our registry, we identified 15 children and adolescents, who were initially mistaken as NBL and subsequently diagnosed with ACT or PCC/PGL.

The incidence of NBL in children and adolescents is about 30–50-fold higher compared to ACT and about 10-fold higher compared to PCC/PGL. Thus, diagnosis of NBL particularly in young children presenting with adrenal masses is more likely; however, ACT and PCC/PGL must also be considered in

childhood cases. NBLs impress as adrenal/abdominal/thoracic masses sometimes infiltrating into adjacent tissues. Signs and symptoms of catecholamine excess such as hypertension and tachycardia are rare but can occur (11). Of note, even in the absence of hypertension and signs and symptoms of a catecholamine producing tumor, a PCC/PGL cannot be ruled out (32).

Although calcifications in NBLs are frequent, lack of evidence of calcification in ultrasonography and/or magnetic resonance imaging does not exclude diagnosis of NBL. In addition, functional imaging in particular mIBG imaging cannot distinguish between NBL and PCC/PGL. Thus, most importantly, thorough preoperative workup to reliably confirm or exclude a PCC/PGL with plasma or urinary metanephrines is essential; the fact that biochemical testing was not performed in three out of five patients subsequently diagnosed with PCC/PGL reveals that this need has not been firmly embedded in clinical practice. In case a delay in treatment is clinically acceptable, genetic testing for PCC/PGL syndromes may add to diagnostic confirmation.

Usually, presenting clinical signs and symptoms in patients with ACT differ from those of patients with NBL and PCC/PGL. In asymptomatic patients, differential diagnosis is much more sophisticated. In all cases of an adrenal mass, a comprehensive hormonal analysis is strongly recommended (**Figure 2**).

The diagnostic accuracy of the workup is high if applied appropriately (including pre-analytics) and the preoperative hormone pattern may serve as a fingerprint of the tumor during periodic surveillance and follow-up. We suggest performing biochemical testing in dedicated laboratories with experience in pediatric endocrinology. In patients with apparently inconclusive results, seeking advice from a center may be helpful.

Of note, three out of 10 patients with ACT were diagnosed with Beckwith–Wiedemann syndrome and hemihypertrophy, respectively. These syndromes are associated with an increased risk to develop a wide spectrum of malignancies including among others NBL as well as ACT.

Prognosis of ACT patients with intraoperative tumor spillage is inferior compared to complete tumor resection necessitating intensified treatment (64). Thus, in children and adolescents with adrenal masses in whom definite diagnosis cannot be established prior to surgery, microscopical complete tumor resection instead of biopsy must be performed.

A final diagnosis was established by a pediatric reference pathologist in six out of 10 ACT cases and in one out of five PCC/PGL cases. Initial misdiagnosis resulted in NBL-directed multidrug chemotherapy in five patients with subsequent diagnosis of ACT and in one patient with PGL. Original pathology reports were not available. Thus, it was not possible to determine why particularly ACTs were initially misdiagnosed as NBL by local pathology assessment. Due to the sophisticated differential diagnosis of adrenal neoplasia and their rarity in children, consultation of an experienced pediatric pathologist is strongly recommended.

CONCLUSION

The differential diagnosis of adrenal neoplasias and associated extra-adrenal tumors in children and adolescents may be challenging necessitating interdisciplinary and multidisciplinary

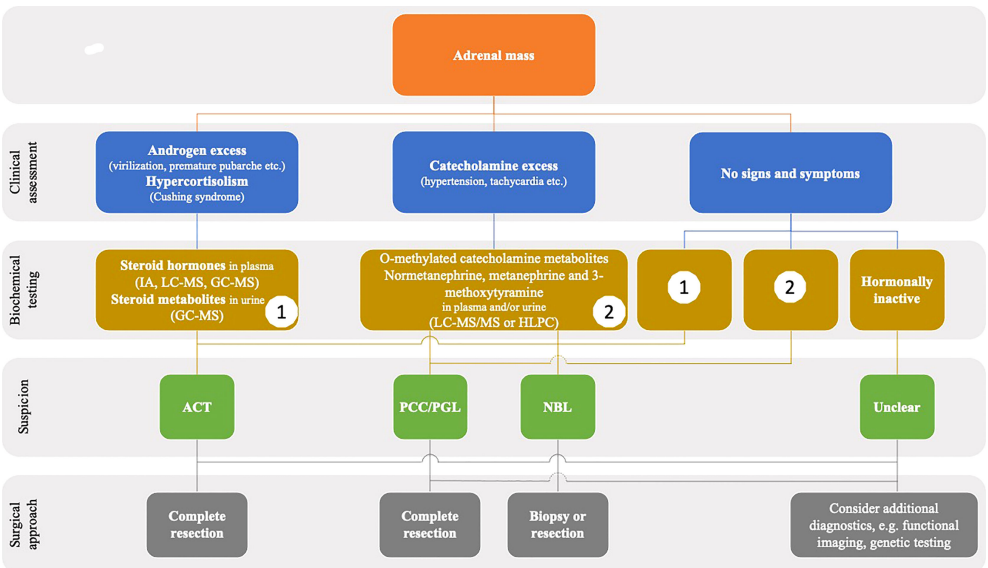

FIGURE 2 | Flowchart showing the diagnostics steps in children presenting with an adrenal mass in imaging. GC-MS, gas chromatography-mass spectrometry; IA, immunoassay; LC-MS, liquid chromatography-mass spectometry.

efforts. We suggest a step-wise assessment starting with conventional imagings, e.g., sonography and MRI, and hormonal testing. In ambiguous and/or hormonally inactive cases though comprehensive biochemical testing, preoperative functional imaging may aid in the diagnosis. Microscopical complete tumor resection by an experienced surgeon is vital to preventing poor outcome in children and adolescents with ACT and/or PCC/PGL. Finally, specimens need to be assessed by an experienced pediatric pathologist to establish diagnosis.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of Luebeck (approval number 97-125) and Otto-von-Guericke-University Magdeburg (approval number 174/12), Germany. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

All authors confirmed they have contributed to the intellectual content of this paper. MiK, conception and design, acquisition of data, analysis and interpretation of data, drafting the article; CP and MP, conception and design, drafting parts of the article; MaK, provision of study material or patients; SW, drafting parts of the article, provision of study material or patients; MH, drafting parts of the article, provision of study material or patients; CV, revising the article for intellectual content; GS, drafting parts of the article, MKr, drafting and revising parts of the article; TS, provision of study material or patients; BH, provision of study material or patients; MF, acquisition of data, provision of study material or patients; PV, acquisition of data, provision of study material or patients; AR, acquisition of data, analysis and interpretation of data, provision of study material or patients, revising the article for intellectual content. All authors contributed to the article and approved the submitted version.

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REVIEW
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Pediatric Metastatic Pheochromocytoma and Paraganglioma: Clinical Presentation and Diagnosis, Genetics, and Therapeutic Approaches

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Although pediatric pheochromocytomas and paragangliomas (PPGLs) are rare, they have important differences compared to those in adults. Unfortunately, without timely diagnosis and management, these tumors have a potentially devastating impact on pediatric patients. Pediatric PPGLs are more often extra-adrenal, multifocal/metastatic, and recurrent, likely due to these tumors being more commonly due to a genetic predisposition than in adults. This genetic risk results in disease manifestations at an earlier age giving these tumors time to advance before detection. In spite of these problematic features, advances in the molecular and biochemical characterization of PPGLs have heralded an age of increasingly personalized medicine. An understanding of the genetic basis for an individual patient's tumor provides insight into its natural history and can guide clinicians in management of this challenging disease. In pediatric PPGLs, mutations in genes related to pseudohypoxia are most commonly seen, including the von Hippel-Lindau gene (*VHL*) and succinate dehydrogenase subunit (*SDHx*) genes, with the highest risk for metastatic disease associated with variants in *SDHB* and *SDHA*. Such pathogenic variants are associated with a noradrenergic biochemical phenotype with resultant sustained catecholamine release and therefore persistent symptoms. This is in contrast to paroxysmal symptoms (e.g., episodic hypertension, palpitations, and diaphoresis/flushing) as seen in the adrenergic, or epinephrine-predominant, biochemical phenotype (due to episodic catecholamine release) that is commonly observed in adults. Additionally, PPGLs in children more often present with signs and symptoms of catecholamine excess. Therefore, children, adolescents, and young adults present differently from older adults (e.g., the prototypical presentation of palpitations, perspiration, and pounding headaches in the setting of an isolated adrenal mass). These presentations are a direct result of genetic determinants and highlight the need for

pediatricians to recognize these differences in order to expedite appropriate evaluations, including genetic testing. Identification and familiarity with causative genes inform surveillance and treatment strategies to improve outcomes in pediatric patients with PPGL.

Keywords: pediatric, metastatic, pheochromocytoma, paraganglioma, clinical presentation, diagnosis, genetics, therapeutic approach

INTRODUCTION

Pheochromocytomas and paragangliomas (PPGLs) are catecholamine-producing tumors that arise from chromaffin cells of the adrenal gland and extra-adrenal tissue, pheochromocytomas (PCC) and paragangliomas (PGL), respectively. Pediatric PPGLs have important differences compared to those in adults, a consequence of the more frequent genetic predisposition seen in the younger population (80% in pediatric patients compared to 40% in adults, see **Table 1**) (2, 4). In children with PPGL, disease is more likely to be extra-adrenal (30-60%), bilateral adrenal (12-48% of pediatric vs. 7-14% of adult patients), recurrent (13-38%), and multifocal (17-62%) (2, 4–8). The most common underlying genetic causes of pediatric PPGL are von Hippel-Lindau syndrome (VHL, 27-51%), mutations in succinate dehydrogenase subunit genes (collectively referred to as *SDHx*, 13-39% in *SDHB* and 8-10% in *SDHD*), multiple endocrine neoplasia type 2 (MEN-2, 0.6-10%), and neurofibromatosis type 1 (NF-1, 1-3%) (2, 4, 5, 7–11).

The underlying pathologic mechanisms that give rise to PPGL are heterogeneous and have been organized into clusters of genes by their effect on different intracellular pathways and gene expression profiling, including pseudohypoxia (cluster 1), kinase signaling (cluster 2), and Wnt signaling (cluster 3) (10, 12–15). The significance of this molecular taxonomy is not only descriptive but also helps to define different aspects of chromaffin cell metabolism that have direct clinical applications, such as the prototypical examples of VHL (cluster 1) and MEN-2 (cluster 2).

PCCs in patients with MEN-2 produce either only epinephrine or both norepinephrine and epinephrine (and their metabolites normetanephrine and metanephrine, respectively; collectively termed metanephrines) and have higher catecholamine levels due to increased expression of tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis (12). PCCs in VHL patients produce norepinephrine almost exclusively, related to decreased expression of the enzyme phenylethanolamine *N*-methyltransferase (PNMT) which converts norepinephrine to epinephrine (12). Furthermore, it was shown that expression of proteins involved in catecholamine secretion are differentially regulated between MEN-2 and VHL, as VHL-related PCCs show decreased expression of some secretory components, resulting in a less coordinated secretion system and therefore, continuous secretion of catecholamines in sharp contrast to paroxysmal release of catecholamines by MEN-2 PCCs (13). One study reported that, on average, norepinephrine-secreting PPGLs stored 1,760,000 picograms (pg) of norepinephrine/gram (g) tissue with 53% released each day, in contrast to epinephrine-secreting PPGLs that contained 3,801,000 pg of epinephrine/g tissue with only 5% released daily (16). Norepinephrine has a higher affinity for α_1-adrenergic receptors, and epinephrine has a higher affinity for β_1-adrenergic receptors, which broadly translates into more of an increased risk for hypertension in those with tumors that produce predominantly norepinephrine and more risk for tachycardia and arrhythmias in those tumors that produce predominantly epinephrine (17).

Taken together, these molecular and biochemical features are foundational for understanding the different clinical features in these patients, with paroxysmal hypertension occurring in the context of the adrenergic biochemical phenotype (increased epinephrine and metanephrine) of MEN-2 as compared to sustained hypertension with the noradrenergic biochemical phenotype (increased norepinephrine and normetanephrine) of VHL. Additionally, decreased expression of dopamine β-hydroxylase or tyrosine hydroxylase can result in a dopaminergic or biochemically silent (non-secreting) phenotype, respectively, which may both be seen from mutations in cluster 1 genes (18). Pediatric PPGL is more often due to pathogenic variants in cluster 1 genes (particularly *VHL*, *SDHx*, and *EPAS1*; at least 57-80% of patients with pediatric PPGL when somatic variants are included) and is thus more likely to have a non-adrenergic (noradrenergic, dopaminergic, or non-secreting) biochemical phenotype (93% of pediatric patients without increased plasma metanephrine vs. 57% in adults, see **Table 1**) (2, 4, 5, 7).

About 10-20% of PPGLs are diagnosed in pediatric patients (19), therefore, it is important for clinicians who care for pediatric patients to be aware of this treatable cause of hypertension, not only to alleviate the symptomatic burden of catecholamine excess, but also to make a timely diagnosis due to the risk of metastatic disease and consequent morbidity and mortality. Among adults under the age of 35 years with PPGL, many similarities to pediatric PPGL are seen (including hereditary, noradrenergic, and multifocal disease), and bilateral tumors were seen even more often than in children or in adults over the age of 35 years, suggesting that these tumors may have evaded clinical detection and were in fact present earlier in childhood (2), thus highlighting the need for clinicians to be aware of the possibility of PPGL in children and adolescents. Retrospective analysis from the Department of Defense Serum Repository found that in adults, biochemical evidence of elevated plasma metanephrines was seen at a median of 6.6 years (elevation above the upper reference limit [URL]) and 4.1 years (3 times above the URL) prior to diagnosis of PCC (20). Therefore, it is reasonable to suspect that pediatric PPGLs may

TABLE 1 | Data on pediatric and adult PPGLs from studies directly comparing the two populations.

	Children	Adults
Clinical Presentation (1)		
Hypertension (sustained)	93%	68%
Hypertension (paroxysmal)	7%	26%
Normotension	0%	5%
Headache	95%	90%
Sweating	90%	92%
Tachycardia/dysrhythmias	35%	72%
Weight loss	15%	72%
Location and Behavior (2, 3)		
Unilateral PCC	12-22%	23-56%
Bilateral PCC	12-20%	9-26%
Solitary PGL	34%	22%
Multifocal	24-33%	5-14%
Metastatic	50%	29%
Synchronous metastases	26%	43%
Metachronous metastases	75%	57%
Recurrent primary tumors	30%	14%
Biochemistry (2)		
Adrenergic	7%	43%
Non-adrenergic*	93%	57%
Genetics (2, 3)		
Causal Gene** (*Cluster*)	70-84%	36-44%
VHL (1B)	27-32%	10-13%
SDHB (1A)	39-44%	17-26%
SDHD (1A)	10-16%	11-21%
RET (2)	3-4%	9-47%
NF1 (2)	1%	4%

*Non-adrenergic includes noradrenergic, dopaminergic, and non-secreting biochemical phenotypes. **Includes germline and somatic etiologies.

go undetected for many years before diagnosis, putting these patients at risk not only for continued tumor growth and metastatic disease but also catastrophic complications if stored catecholamines in a clinically occult PPGL are suddenly released when provoked by surgery, induction of anesthesia, or as an adverse effect of medication.

While isolated PPGLs are often definitively treated by surgical removal, metastatic disease requires additional treatment modalities, such as chemotherapy, radiotherapy, targeted molecular therapies, or ablative therapies (21). Metastatic disease, defined by the presence of chromaffin tumor cells in tissues without chromaffin cells, is a major aspect of PPGL care and is itself largely genetically determined (1). Metastatic disease can occur in 12% of pediatric patients, in general, but may be up to 70% among *SDHB* mutation carriers, the second most commonly mutated gene in pediatric PPGL after *VHL* (19, 22, 23). The sites of involvement are most often bone, lymph nodes, liver, and lungs (though primary PGLs have been described in liver and lungs), which can result in significant impairment in quality of life and prognosis (24, 25). Metastases may be present at the time of initial diagnosis (synchronous) or later (metachronous or non-synchronous); in pediatric PPGL, metastases are less often synchronous as compared to adults, underscoring the importance of long-term surveillance for any child diagnosed with PPGL (2). In addition to those patients who harbor a mutation in *SDHB*, other established risk factors for metastatic disease include tumor size ≥ 5 cm, extra-adrenal PGL, dopaminergic phenotype (plasma 3-methoxytyramine higher than three times the URL), and a Ki-67 index > 3% (3, 26).

Finally, metastatic disease has direct implications in the approach to and management of pediatric PPGL, including imaging and treatment. Advances in functional imaging modalities with positron emission tomography/computed tomography (PET/CT) scans using different radionuclides provide insight into how patients from particular genetic backgrounds can receive an increasingly personalized approach to management of these challenging tumors. And while metastatic PPGL is incurable, knowledge of the molecular pathogenesis can direct treatments in some cases, informed by the cellular pathways disrupted by the genetic predisposition. For these reasons, the genetic background may guide management of the pediatric PPGL patient, and genetic testing is essential to providing optimal care for these patients.

CLINICAL FEATURES

PPGLs are estimated to occur at an incidence of 0.57 per 100,000 person-years, of which, up to 20% of PPGLs are diagnosed in pediatric patients, at an average age of 11 years (19, 22, 23, 27). Most pediatric patients are symptomatic (around 90%), and hypertension is the most common presentation of pediatric PPGL (64-93%), which causes pediatric hypertension in up to 1% of cases (7, 8, 22). Therefore, one should suspect PPGL in pediatric patients with sustained hypertension, with headache (39-95%), diaphoresis (90%), palpitations (53%), and signs/symptoms of mass effect or as an incidental mass (30%) (**Table 1**) (8, 11, 22).

Pediatric hypertension is defined as an auscultatory blood pressure > 130/80 mmHg for children ages 13 years and above or ≥ 95th percentile for age, sex, and height for children ages 1 to 12 years, on more than 3 occasions, according to the most recent Clinical Practice Guideline from the American Academy of Pediatrics (28). Pediatric hypertension can be further complicated by hypertensive emergency in patients with catecholamine excess, such as retinopathy, resulting in visual disturbances, and hypertensive encephalopathy, resulting in seizures and disorientation (22, 29). Hypertrophic and dilated cardiomyopathy can arise as sequelae of hypertension in pediatric patients with PPGL; among pediatric patients with dilated cardiomyopathy, surgical excision of their tumors led to resolution of hypertension and improved cardiac function (8, 30).

Tachycardia and dysrhythmias, by contrast, are seen more often in adults, which may be related to the relatively higher proportion of cluster 2 mutations in adults resulting in stimulation of cardiac β_1-adrenergic receptors by epinephrine (22). Stimulation of β-adrenergic receptors by epinephrine in cluster 2 mutations results in hepatic glycogenolysis and gluconeogenesis, which may account for the higher proportion of adults as opposed to children who present with elevated fasting glucose levels (22, 31).

In addition to the signs and symptoms noted above, other findings related to catecholamine excess may be non-specific and include pallor, orthostatic hypotension and syncope, shortness of breath, abdominal pain, nausea, vomiting, constipation, diarrhea, hyperglycemia, polyuria and polydipsia, anxiety, behavioral symptoms, worsening performance in school, and ADHD (9, 30, 32). Interestingly, among pediatric patients harboring an *SDHB* mutation, sweating was significantly associated with earlier development of metastatic disease (p = 0.0073), and for those without metastases at initial diagnosis, patients with tumor pain developed metastases at an earlier interval than those without tumor pain (p = 0.0088) (23).

Presenting signs and symptoms may also be related to mass effect of the growing tumor, and patients may present with a palpable abdominal mass on physical examination (30). Abdominal masses may present with abdominal pain and distension or back pain, and bladder masses may result in hematuria or symptoms with voiding (11, 32). Parasympathetic head and neck PGLs (HNPGLs) would not be expected to produce catecholamines, and the clinical presentation may be related to mass effect on cranial nerves and other local structures, resulting in hearing loss, tinnitus, hoarseness, dysphagia, cough, pain, or feeling of fullness in the neck (32). As in adults, PPGLs may also be found as an incidental mass on imaging studies performed for other indications (8, 11). For patients with clinical findings of or genetic predisposition to PPGL, biochemical evaluation is indicated.

BIOCHEMICAL FOUNDATIONS

The biosynthesis of catecholamines, metanephrines, and 3-methoxytyramine (3-MT, the *O*-methylated metabolite of dopamine), as derivatives of tyrosine, is shown in **Figure 1**. Plasma free metanephrines should be measured, as this highly sensitive test can help to rule out PPGL in children, as in adults (33). Eisenhofer et al. found that the optimal combination of diagnostic sensitivity (97.9%) and specificity (94.2%) was achieved by using age-nonspecific URLs for metanephrine (446 picomoles/liter [pmol/L]) and 3-methoxytyramine (107 pmol/L) with age-specific URLs for normetanephrine, increasing from age 5 years (542 pmol/L) to age 65 years (1092 pmol/L) as modeled by the equation $URL_{NMN} = (2.07 \times 10^{-3} \times age^3) + 545$ (pmol/L) (**Table 2**) (34). Although plasma fractionated metanephrines are the test of choice in children as in adults, urinary free metanephrines may be considered without much loss of sensitivity (97.9% for plasma free metanephrines and 93.4% for urinary free metanephrines), which may be preferable to avoid venipuncture in some children (35).

Dopamine-producing PPGLs show elevations of 3-MT; thus, plasma 3-MT should be used to increase the diagnostic sensitivity in PGLs, especially for HNPGLs (22.1% to 50%) using a cut-off of 0.1 nanomoles/liter (nmol/L) (36). Urinary dopamine reflects renal metabolism and is not helpful to identify dopaminergic PPGL (36). Although testing for 3-MT is of more limited clinical availability than that of metanephrines, it is helpful to assess risk of metastatic disease, as plasma elevations above 0.2 nmol/L had a 57% sensitivity and 85% specificity for metastatic disease (37).

Chromogranin A is a biomarker of neuroendocrine tumors, including PPGL, and has been shown to increase the sensitivity of diagnosis when used in conjunction with metanephrines, especially in patients with *SDHB*-related PCC or sympathetic PGL, though its performance was not as good in patients with HNPGL (38). As its production is independent of the catecholamine metabolism pathway, it is a helpful adjunct to detect non-secreting tumors.

Care must be taken to reduce false-positive biochemical laboratory results. This can be achieved by avoiding circumstances that provoke an adrenergic response at the time of collection. For this reason, the patient should be placed in a supine position with venipuncture performed 20-30 minutes before biochemical labs are obtained – as both non-supine position and venipuncture may lead to catecholamine release (32). Medications that can artifactually increase plasma or urinary fractionated metanephrines include acetaminophen, α-methyldopa, tricyclic antidepressants, monoamine oxidase inhibitors, sympathomimetics, catecholamine reuptake inhibitors, mesalamine/sulfasalazine, phenoxybenzamine, levodopa, anesthetics, neuromuscular blockers, antiemetics, linezolid, peptide hormones, steroids, cocaine, and opioids (17, 39). Glucocorticoids potentiate catecholamine biosynthetic enzymes and should be avoided in patients with PPGL due to risk of causing PCC crisis (40). Therefore, when able, these medications should be held before biochemical labs are obtained.

Cellular consequences of cluster 1 mutations converge on hypoxia-inducible factor 2α (HIF-2α), a transcription factor that dimerizes with HIF-1β and mediates downstream effects such as blocking the glucocorticoid-induced expression of PNMT (41). Gain-of-function mutations in *EPAS1* (encoding for HIF-2α) contribute to aberrant neuroendocrine-to-mesenchymal transition which gives rise to PPGLs and contributes to their metastatic behavior (41). Biochemically, this translates to the observation that PNMT and other biosynthetic enzymes are down-regulated in patients with cluster 1 mutations, such that the biochemical phenotype is either noradrenergic, dopaminergic, or non-secreting, but not expected to be adrenergic.

Finally, the metabolic role of succinate dehydrogenase in the tricarboxylic acid (TCA) cycle and electron transport chain is related to diagnostic imaging and may impact treatment. Mutations in TCA cycle genes (cluster 1A) result in decreased function of these metabolic enzymes and increase dependency on glycolysis for energy production (15). Radiologically, this correlates with higher uptake of ^{18}F-fluorodeoxyglucose (^{18}F-FDG) on PET imaging for tumors of a cluster 1 genetic background when compared with cluster 2 (42). In addition to its role in the conversion of succinate to fumarate in the TCA cycle, succinate dehydrogenase also plays a crucial role as complex II in the mitochondrial electron transport chain, using reducing equivalents of $FADH_2$ to ultimately generate ATP (43, 44). Defects in SDH subunit genes (i.e., *SDHx*) may increase the cell's dependence on NADH oxidation through complex I, resulting

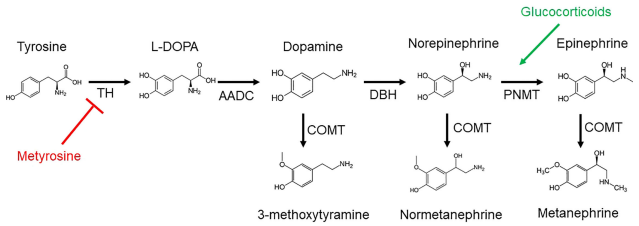

FIGURE 1 | Catecholamines and metanephrines are derived from tyrosine by the enzyme tyrosine hydroxylase (TH), resulting in L-3,4-dihydroxyphenylalanine (L-DOPA). Aromatic L-amino acid decarboxylase (AACD) generates dopamine from L-DOPA. Dopamine β-hydroxylase (DBH) acts on dopamine to produce norepinephrine, followed by the enzyme phenylethanolamine *N*-methyltransferase (PNMT) to yield epinephrine. Norepinephrine and epinephrine are *O*-methylated by catechol-*O*-methyltransferase (COMT) to produce normetanephrine and metanephrine, respectively. The corresponding *O*-methylated metabolite of dopamine is 3-methyoxytyramine (3-MT). Metyrosine inhibits (red) TH. Glucocorticoids stimulate (green) PNMT.

in increased availability of NAD^+ to be used by poly (ADP-ribose) polymerase (PARP) in base excision repair of DNA (43). Therefore, *SDHx* mutations increase chemoresistance to genotoxic agents by stimulating DNA repair mechanisms via PARP. For these reasons, PARP inhibition may deprive these tumors of this protective mechanism and play a role in personalized treatment tailored by genetics (43).

GENETICS

Overview

Pediatric PPGLs are often found to have a genetic cause, which is most commonly due to a germline mutation in one of the following susceptibility genes: *VHL*, *SDHx*, *RET*, and *NF1*. Genes related to pseudohypoxia in cluster 1 are subdivided into cluster 1A with TCA-cycle genes, including the *SDHx* genes, and cluster 1B related to hypoxia signaling, most significantly *VHL* and *EPAS1*. The other two important genetic causes of pediatric PPGL, *RET* and *NF1*, are in cluster 2, related to kinase signaling (15, 26).

Genetic syndromes predisposing to PPGL are all inherited in an autosomal dominant manner, but with the caveat that pathogenic variants in *SDHD* (as well as *SDHAF2* and *MAX*) increase PPGL susceptibility when paternally inherited (18). However, identifying a maternally inherited *SDHD* variant has implications for screening extended family members and subsequent generations. While a family history of PPGL is often seen in pediatric patients with PPGL, the absence of a family history should not disincline clinicians from referring for genetic counseling and testing, as one must consider the possibility of *de novo* mutations. All patients with PPGL should be referred for genetic counseling and evaluation (1).

Succinate Dehydrogenase Subunit Defects

Mutations in all four subunits of succinate dehydrogenase (subunits A, B, C, and D) and assembly factor gene *SDHAF2* have been described in PPGL. In addition to sequence variants and copy number changes contributing to the genetic pathogenesis of *SDHx* variants, disease resulting from epigenetic alteration of *SDHC* by increased promoter methylation (i.e., epimutation) resulting in decreased expression have also been described, especially in PGLs and gastrointestinal stromal tumors (GISTs) (45, 46). Accumulation of succinate results in impaired prolyl hydroxylation of HIF-2α, resulting in the pseudohypoxic expression pattern seen for these cluster 1A genes, as well as other epigenetic effects on gene expression (47). In addition to PPGLs, other tumors, including renal cell carcinoma (RCC), GIST, pituitary adenoma, pulmonary chondroma (as part of Carney triad, which also includes PGL and GIST), and papillary thyroid cancer, as well as thyroid nodules have been reported in patients with *SDHx* mutations or *SDHC* epimutation; estimated penetrance of RCC in *SDHx* carriers is 2-3% (mostly *SDHB*), but 85% of GISTs diagnosed in childhood are related to *SDHx* mutations (4, 7, 48). No additional imaging is recommended to screen for these tumors in asymptomatic *SDHx* carriers (48). While genotype-phenotype correlations have been explored in *SDHx* genes, particularly for *SDHD* and *SDHB*, variable expressivity is seen, such that even among family members with the same variant, the sites and extent of disease and presence of metastases may vary (48).

Mutations in *SDHB* (on chromosome 1p36.13) are the most well-established genetic risk factor for metastatic PPGL. In a study from the National Institutes of Health that assessed 125 patients with metastatic PPGL, 32 presented before 20 years old, of which 23 (71.9%) were found to have a germline mutation in

TABLE 2 | Age-specific upper reference limits for normetanephrine.

	Pediatric				Adult	
Age (years)	5	12	19	35	50	65
Normetanephrine	542	549	559	634	804	1092

Units expressed as pmol/L. Values between 5 and 65 years of age were interpolated based on the equation in reference (31).

SDHB (49). Although the penetrance of PPGL in *SDHB* was initially thought to be higher, a Dutch study using data ascertained from clinical genetics centers (as opposed to PPGL tertiary care centers) found that the penetrance was closer to 21% by age 50 and 42% by age 70 (50). The risk of metastatic disease for *SDHB* carriers with PPGL is reported to be about 34-71%; in a study of 64 pediatric PPGL patients with *SDHB* mutations reported by Jochmanova et al., 70% developed metastases at a median age of 16 years (those patients being diagnosed at a median age of 12 years). Among those with metastases, only 19% had synchronous metastases, with the highest risk of metastatic disease within the first 2 years after diagnosis and again between 12-18 years after diagnosis (23), highlighting the critical role of lifelong surveillance and monitoring in these patients. Recurrent disease was seen in 20% of these patients, at a median age of 16 years (median time from diagnosis to recurrence was 2 years); recurrence was more often as sympathetic PGL (sPGL) than PCC (85% compared to 38%, respectively). Tumor size also played an important role in the timing of metastases as patients with tumors ≤ 5 cm developed metastatic disease at a median interval of 7 years compared to 2 years in those with tumors > 5 cm (23). All patients who died (12.5%) had metastatic disease, surviving for a median duration of 7.5 years after diagnosis of metastatic disease; no differences in the time to death were seen by comparison among the 5 most common mutations (one missense, two premature nonsense mutations, and two exonic deletions) (23). A genetic analysis of two European datasets (Germany and Great Britain) comparing different types of mutations in *SDHB* carriers with PPGL found that truncating mutations in *SDHB* seemed to confer a higher risk of PPGL than missense variants (62% vs. 38%), which remained statistically significant after removing prominent founder variants from one of the datasets; malignancy was also more common in those with truncating variants (62% vs. 38%), but no significant difference was seen for HNPGL between these different *SDHB* variants (47% for truncating vs. 53% for missense variants). No difference in age of diagnosis was seen between truncating and missense *SDHB* variants (46).

Mutations in *SDHD* (on chromosome 11q23.1) have a higher penetrance than those in *SDHB* (> 80%), and the autosomal dominant inheritance pattern is modified by maternal imprinting such that the disease is usually inherited from the paternal allele (46). Cases involving confirmed maternal inheritance are rare and involve additional genetic events, such as post-zygotic loss of chromosome 11 material as demonstrated in one study of 20 such patients with only one who was confirmed to have PPGL (35 years woman with PCC) (51). *SDHD* mutations are commonly seen in HNPGLs, but thoracic PGLs and PCCs are also seen (4, 18). Metastatic risk in *SDHD* is reported to be around 15-29% (26). In a study of 32 pediatric PPGL patients who developed metastatic disease, the three patients with a primary HNPGL were all identified to have a *SDHD* mutation (49), however in three other studies of pediatric PPGL patients, 17 of 177 patients, 5 of 88 patients, and 2 of 25 patients were found to have *SDHD* mutations, and only 2 of 17 later developed metastatic disease (4, 5, 7).

Pathogenic variants in *SDHC* (on chromosome 1q23.3) and *SDHA* (on chromosome 5p15.33) contributing to PPGL are much less common, with estimates of lifetime penetrance for PPGL at 8.3% and 1.7%, respectively (52). While metastatic disease risk is estimated to be low for *SDHC* mutations, metastatic risk in *SDHA* is 30-66% (26). Bausch et al. reported that among 177 pediatric PPGL patients, there was one patient who carried pathogenic variants in each of these genes: a 12 years male with head and neck PGL (*SDHC*) who did not develop metastases and a 15 years male with PCC (*SDHA*) who died within 13 years of follow-up (4). Interestingly, SDHC and SDHD share structural (anchoring the SDH complex to the inner mitochondrial membrane) and bioenergetic roles (the SDHC-SDHD dimer transfers electrons from iron-sulfur clusters of SDHB to ubiquinone in the electron transport chain) as well as both commonly presenting as head and neck PGLs (18, 44).

International consensus guidelines were published for PPGL screening in asymptomatic *SDHx* carriers (*SDHA*, *SDHB*, *SDHC*, and paternally-inherited *SDHD* variants), which should start in childhood at age 6-10 years for *SDHB* carriers and otherwise at 10-15 years and include clinical evaluation with symptom assessment, physical examination, and measurement of blood pressure; biochemical testing with plasma or urine metanephrines; and magnetic resonance imaging (MRI) studies of the head and neck as well as of the thorax, abdomen, and pelvis (48). If the initial evaluation is negative, follow-up should include annual clinical evaluation, biochemical testing every 2 years, and MRI every 2-3 years. Functional imaging by PET/CT was included for initial screening in adults, but no recommendation was made for screening by functional imaging in children (48).

Von Hippel-Lindau Syndrome

Due to pathogenic variants in *VHL* (on chromosome 3p25.3), VHL syndrome is due to loss of function of this tumor suppressor gene that encodes for an E3 ubiquitin ligase that targets HIF-2α for degradation (53). *VHL* is a cluster 1B gene and is associated with a noradrenergic biochemical phenotype. PPGL develops in about 10-25% of VHL patients, typically presenting as PCC, though sympathetic and parasympathetic PGLs can also be seen, and the risk of metastatic disease is 5-8% (26, 54). Other tumors seen include angiomas and hemangioblastomas of the retina and central nervous system, renal and pancreatic cysts, RCC, endolymphatic sac tumor, pancreatic neuroendocrine tumor, and papillary cystadenoma of the epididymis or broad ligament (54). Genotype-phenotype correlations have been reported in VHL: type 2 VHL, associated with higher risk of PCC, is usually due to missense variants, whereas type 1 VHL, with a lower risk for PCC, includes truncating variants and exon deletions as well as missense mutations (53, 54). In about 20% of cases, patients will have a *de novo* mutation; interestingly, a study of Spanish PPGL patients found a much higher *de novo* rate of 60% overall and 50% for the pediatric group, though the authors attributed this to sample size (29, 54). VHL is the most common genetic cause of pediatric PPGL and tends to present the earliest at an average age of 11-12 years (4, 5, 7, 8, 22), in contrast to those syndromes

arising from pathogenic variants in cluster 2 – specifically, MEN-2 (14-20 years) and NF1 (16-17 years); PCC may be the first manifestation of VHL disease (4, 5, 7, 8, 22). While screening recommendations for VHL may vary, PCC has been identified in a girl as young as 2 years, so measurement of blood pressure at each medical appointment and annual plasma free metanephrines or 24-hour urine fractionated metanephrines starting as early as 2 years but no later than 5 years has been proposed (54). Other aspects of screening for VHL-associated tumors, including retinal examination, audiology evaluation, and MRI studies are not discussed here but should be included as part of the comprehensive care for these patients.

EPAS1 Gain-of-Function Syndrome

One notable exception to the general rule of germline susceptibility is Pacak-Zhuang syndrome (PZS), characterized by polycythemia, PPGL, and duodenal somatostatinoma (55, 56). PZS is usually due to post-zygotic somatic mutations (rarely associated with germline inheritance) in the *EPAS1* gene (on chromosome 2p21), encoding for the transcription factor HIF-2α (55, 56). This syndrome was first described in two female patients with congenital polycythemia and onset of PGL during adolescence (55, 56). In PZS, gain-of-function mutation results in impaired prolyl hydroxylation of HIF-2α, increasing its stability by interfering with ubiquitination by the VHL protein (pVHL). Transcriptional effects include expression of erythropoietin, resulting in polycythemia, and vascular endothelial growth factor (VEGF), contributing to a pro-angiogenic phenotype, and resulting in decreased expression of PNMT, characteristic of cluster 1 mutations (41, 55). This increased stability also affects differentiation of neural crest progenitor cells that may promote metastatic behavior such as facilitating the neuroendocrine-to-mesenchymal transition (41). Pamporaki et al. reported that 4 of 92 pediatric patients (4.3%) compared to 5 of 519 adult patients (1.0%) with PPGL were found to have a somatic mutation in *EPAS1* from tumor tissue, emphasizing how frequently cluster 1 mutations, even post-zygotic mutations, are seen in the pediatric population (2). In another cohort, Redlich et al. identified 1 of 88 (1.6%) pediatric PPGL patients with an *EPAS1* somatic mutation and clinical features of PZS (including abdominal PGL) but without evidence of metastatic disease (7). A study of 7 patients with PZS showed that PGLs were multiple and recurrent in all patients but only 2 of 7 (29%) had metastatic disease (57). These patients highlight the benefit of somatic – in addition to germline – genetic testing when patient tumor tissue is available, as it may provide additional clues to the underlying etiology and raise awareness of associated clinical features and risk of multiplicity, recurrence, and malignancy.

Neurofibromatosis Type 1

Mutations in the *NF1* gene (encoding for neurofibromin), located on chromosome 17q11.2, are responsible for neurofibromatosis type 1. Though both are in cluster 2, in contrast to the *RET* gene, *NF1* is a tumor suppressor that negatively regulates the RAS-MAPK signaling pathway. Partly due to historical challenges in sequencing related to the size of this gene, clinical diagnostic criteria were developed at the National Institutes of Health and include the presence of café-au-lait macules, axillary or inguinal freckling, neurofibromas, optic glioma, Lisch nodules, distinctive bone lesions, and an affected first-degree relative (58). While an often-cited retrospective analysis showed that PCCs were seen in 0.1 to 5.7% of patients with NF-1, 3.3 to 13.0% of NF-1 patients were found to have PCC on autopsy (59). A prospective study of adults with NF-1 identified PCC in 7.7% of patients, suggesting that these tumors are often found incidentally in asymptomatic patients (60). Unilateral adrenal disease is most common (78-84%), with bilateral disease seen in only 9.6 to 16.6% of patients with PCC, and metastatic disease is seen in up to about 11.5% of patients, though these estimates are derived mostly from adult NF-1 patients. Extra-adrenal locations include the organ of Zuckerkandl, abdominal sympathetic chain, and bladder (59–61). Adrenal findings in NF-1 include benign adrenal nodules, adrenal hyperplasia, and PCCs, which may be found histologically as composite tumors (with ganglioneuroma, ganglioneuroblastoma, neuroblastoma, and mixed neuroendocrine-neural tumor); GIST, renal oncocytoma, and carcinoid tumors have also been reported in NF-1 (59, 60). By contrast, pediatric PPGL in NF-1 is far less commonly seen, with reports indicating that these patients comprise only 1-3% of pediatric PPGL cohorts and present as teenagers (15-17 years), though 3 of 8 patients developed metastatic disease (one of which had microscopic residual tumor on histopathological analysis) (2, 4, 7). An important consideration is that up to 50% of the cases of NF–1 arise *de novo*, another reason why the absence of a family history *per se* should not preclude NF–1 from the differential diagnosis in the presence of suggestive clinical findings (59).

Multiple Endocrine Neoplasia Type 2

MEN-2 is due to pathogenic variants in the *RET* gene (on chromosome 10q11.21), a cluster 2 proto-oncogene encoding for a receptor tyrosine kinase that plays an important role in the development of neural crest cells (62). MEN-2A is associated with the combination of medullary thyroid cancer (MTC, > 90%), hyperparathyroidism (15-30%), and PCC (57%) and accounts for up to 95% of MEN-2 (including familial MTC [FMTC], a variant of MEN-2A), whereas MEN-2B constitutes about 5% of MEN-2 and is characterized by early and aggressive MTC, PCC (50%), mucosal neuromas, and a marfanoid habitus (63, 64). As a proto-oncogene, disease arises due to gain-of-function mutations in *RET*, often due to missense mutations at cysteine residues, such as at codons 609, 618, and 620 in exon 10 or 634 in exon 11, which result in MEN-2A (63, 64). In contrast, about 95% of individuals with MEN-2B have the p.M918T mutation in exon 16, and about 75% of patients have disease resulting from a *de novo* mutation (63, 64). Genotype-phenotype correlations are particularly important in MEN-2, and the 2015 Revision of the Guidelines for Management of Medullary Thyroid Cancer by the American Thyroid Association has classified *RET* variants into three risk categories on the basis of risk for MTC, which for the "high" and "highest" risk categories also correspond to those mutations for the greatest risk of PCC:

"highest" (only the p.M918T mutation), "high" (p.A883F and p.C634 mutations), and "moderate" (other mutations) (63). With mutations of the cysteine residue at codon 634, the risk of PCC, hyperparathyroidism, and cutaneous lichen amyloidosis is higher, and specifically for the p.C634R mutation, there is a higher risk of metastatic MTC at diagnosis (64). For *RET* mutations at codons 918, 883, and 634, screening for PCC should begin at age 11 years, but for those moderate risk mutations, screening may begin at age 16 years (63). PCCs in MEN-2 are often multicentric and bilateral, developing in the setting of diffuse nodular adrenal medullary hyperplasia (63). Metastatic risk of PCC is < 5% (26). Among 6 studies of pediatric PPGL patients with MEN-2 due to a *RET* mutation, ten patients were identified (representing 0.6-3.3% of each cohort except for 13.0% in one study), all with PCC Age at PCC diagnosis ranged from 14-20 years; eight patients had bilateral or recurrent disease, and no patients were reported to have metastatic disease (2, 4, 5, 7, 8, 29). Though not discussed here, other aspects of MEN-2, including hyperparathyroidism and MTC, require clinical consideration and management (63).

IMAGING

Anatomic imaging is essential to the diagnosis for any patient being evaluated for PPGL and is typically performed by CT or MRI. In pediatric patients, high signal-intensity T2-weighted MRI is preferable over CT to reduce radiation exposure and may be better suited to identify extra-adrenal tumors, invasion into the spinal canal, and involvement of major vessels (2, 9, 11, 65). Children may not tolerate MRI as well as adults and may require sedation, so clinicians should be mindful regarding the choice of sedative so as not to cause precipitous catecholamine release; medications such as opioids (except for fentanyl) can cause histamine release, which may stimulate catecholamine secretion (66).

Anatomical imaging is used for tumor localization and detection of metastases after positive biochemical testing but also for screening, as non-functional tumors may be negative on biochemical evaluation, such as in patients with *SDHx* mutations or those with parasympathetic PGLs arising in the head and neck (48). For follow-up imaging in the setting of metastases, CT and MRI can be complementary, as CT is better for visualizing lung lesions, and MRI is better for liver lesions (26).

Functional imaging provides insight into the molecular features of PPGLs by use of different radiopharmaceuticals, which is especially informative in metastatic disease. *"Theranostics"* refers to this unification of *thera*peutic and diag*nostic* utility with the same radiopharmaceutical platform, for example using ^{123}I-labeled metaiodobenzylguanidine (MIBG) scintigraphy to identify PPGLs which can then be treated by ^{131}I-MIBG (67). MIBG is an analog of norepinephrine and enters tumor cells via the norepinephrine transporter (42, 68). Medications such as over-the-counter cough and cold treatments, calcium channel blockers, labetalol, and tricyclic antidepressants can interfere with MIBG uptake and should be avoided before treatment. Special preparation is also required to reduce accumulation of radioiodine in the thyroid (68). While highly specific, MIBG may not be as sensitive as other functional imaging modalities, particularly in hereditary PPGL, and especially in those with *SDHx* mutations. Additionally, sensitivity is higher for PCC than PGL (88% vs 67%, respectively), and in metastatic PPGL, per-lesion sensitivity is < 60% (42). Despite these limitations in the pediatric population with metastatic PPGL, MIBG may still have a useful theranostic role in patients where ^{123}I-MIBG avidity is observed in all lesions.

The other relevant application of the theranostic paradigm is for tumors expressing somatostatin receptors – especially type 2 (SSTR2) – that bind to somatostatin analogs (SSAs) linked to a chelator (DOTA) for the radionuclide. Typically, ^{68}Ga- or ^{64}Cu-DOTA-SSA is used for imaging, and ^{90}Y- and ^{177}Lu-DOTA-SSAs deliver the radiation dose to the sites of tumors, with the latter referred to as peptide receptor radionuclide therapy (PRRT) (42, 69). ^{68}Ga-DOTATATE is commonly used since DOTATATE binds preferentially to SSTR2 over other somatostatin receptors, as opposed to DOTATOC or DOTANOC (42). ^{68}Ga-DOTATATE is particularly useful for cluster 1A mutations (especially *SDHx*), HNPGLs, metastatic PPGL, and pediatric PPGL (42). Among nine pediatric patients with *SDHx* mutations, both ^{18}F-FDG and ^{68}Ga-DOTATATE detected lesions in all of the patients but ^{68}Ga-DOTATATE had superior sensitivity on a per-lesion basis (94% compared to 79% on ^{18}F-FDG) and was also more sensitive than anatomic imaging by CT or MRI with contrast enhancement (74%). ^{68}Ga-DOTATATE was especially superior to CT/MRI for mediastinal lesion detection and superior to ^{18}F-FDG for detection of adrenal and liver lesions, though ^{18}F-FDG and CT/MRI outperformed ^{68}Ga-DOTATATE for detection of other abdominal lesions (**Figure 2**) (70). These data offer a compelling case for performing ^{68}Ga-DOTATATE PET/CT or ^{68}Ga-DOTATATE PET/MRI with contrast enhancement to detect such abdominal lesions. Currently both functional and anatomic imaging are required for staging and for assessing treatment response in pediatric metastatic PPGL patients and, therefore, simultaneous PET/MRI may be considered in this cohort due to decreased radiation exposure, fewer instances requiring sedation or general anesthesia, fewer appointments, and simultaneous imaging with two advanced diagnostic imaging techniques (whole-body PET and MRI) (71). Recommended imaging modalities for different types of PPGL based on genetic and clinical features are summarized in **Table 3**. A 2015 meta-analysis by Han et al. pooled patients of unknown genetic background, finding a significantly superior detection rate of ^{68}Ga-DOTA-conjugated somatostatin receptor-targeting peptide (^{68}Ga-DOTA-SST) PET (93%) as compared to other functional imaging modalities (72). Therefore, in cases where a genetic etiology is not known, ^{68}Ga-DOTATATE PET may be performed.

^{18}F-fluorodihydroxyphenylalanine (^{18}F-FDOPA) enters cells via the large neutral amino acid transporter (LAT-1) and enters into the catecholamine synthesis pathway (42). Preparation for

^{18}F-FDOPA PET/CT involves fasting and administration of carbidopa to block decarboxylation of DOPA to dopamine, improving uptake in target tissue. There are no known interfering medications (42). ^{18}F-FDOPA PET/CT is particularly helpful for imaging PPGLs in clusters 1B (VHL and PZS) and 2 (MEN-2 and NF-1) and has an advantage over MIBG in that normal adrenal tissue has lower uptake, increasing the sensitivity to detect nonmetastatic PCC (94% in patients of known genetic background and up to 100% in patients with apparently sporadic nonmetastatic PCC); indeed, the detection of metastatic PPGL may also vary on the basis of the genetic background with greater sensitivity in non-*SDHx* PPGL (42, 73).

^{18}F-FDG is a radiolabeled form of glucose and is taken up by glucose transporters (particularly GLUT-1) and enters into the glycolytic pathway (42). Preparation for ^{18}F-FDG involves fasting, controlling hyperglycemia, and avoidance of ambient cold temperature to prevent artifacts from glucose metabolism in thermogenic brown fat (42, 74). The sensitivity of ^{18}F-FDG in detection of metastatic PPGL is superior to MIBG (usually > 80%), particularly in *SDHx* patients, with sensitivity in metastatic disease for *SDHx* patients ranging from 83-92% compared to 62% for non-*SDHx* (42). Furthermore, while there are no studies of direct comparisons, GIST appears to be better appreciated on ^{18}F-FDG compared to ^{68}Ga-DOTATATE, and therefore, some *SDHx* patients would benefit from ^{18}F-FDG in addition to ^{68}Ga-DOTATATE PET/CT scan when GIST is suspected (75, 76).

MANAGEMENT

After biochemical studies and tumor localization by imaging, definitive treatment of PPGL should be addressed. In addition to surgery of the primary tumor, when appropriate, patients with metastatic disease require other treatment modalities to address disease at sites of metastasis, such as chemotherapy, radiotherapy, ablative therapy, or other targeted therapies, such as somatostatin analogs or small molecule inhibitors (21). Clinical trials for pediatric patients with advanced/metastatic

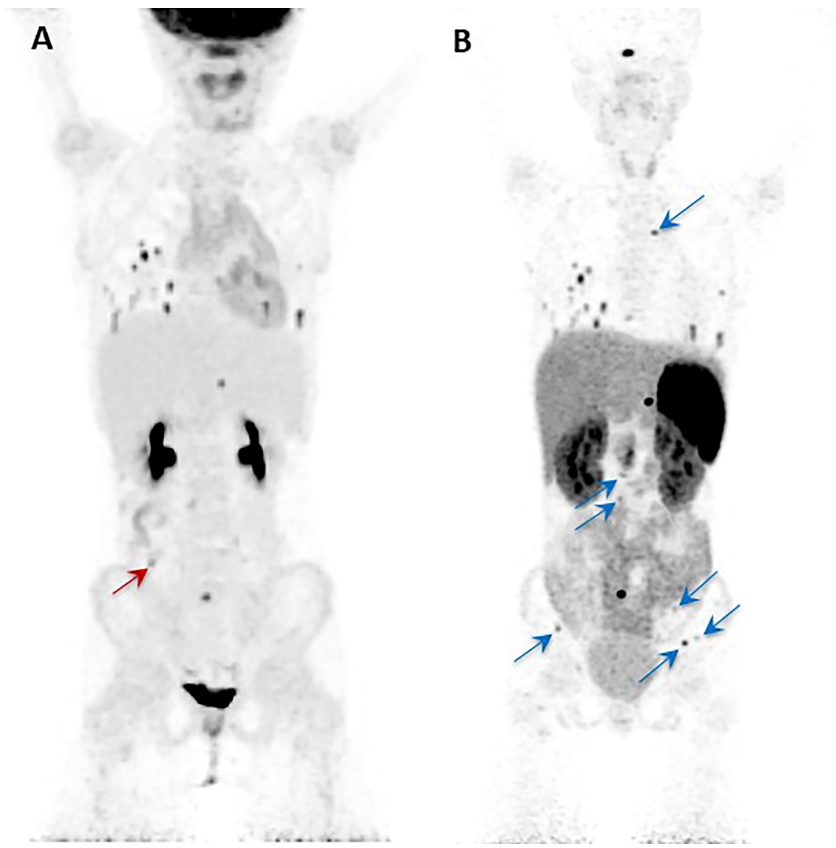

FIGURE 2 | Anterior maximal intensity projection (MIP) images of the ^{18}F-FDG PET/CT **(A)** and ^{68}Ga-DOTATATE PET/CT **(B)** studies of a 10-year-old *SDHB* positive girl. She was diagnosed initially with metastatic disease at the age of 8 years. Her right paraaortic, retroperitoneal primary paraganglioma was surgically resected. On presentation to our institution, the progression of her disease was demonstrated by metastatic lesions in bone, lungs, and abdomen as shown in the images **(A, B)**. The single red arrow on image **(A)** indicates the one lesion (abutting bowel) localized by ^{18}F-FDG PET/CT that is not visualized by the ^{68}Ga-DOTATATE PET/CT. Similarly, all the additional lesions (transverse process of T4 spine, L2-L5 vertebral bodies, left ilium, and left and right iliac wings) localized by the ^{68}Ga-DOTATATE PET/CT (blue arrows) are not visualized by ^{18}F-FDG PET/CT **(B)**. This figure was adapted from the figure that was initially published as Figure 2 by Jha et al. (70).

TABLE 3 | Functional imaging recommendations for pediatric PPGL.

PPGL Type	Recommended Imaging Modality	Alternative
Cluster 1A Metastatic	^{68}Ga-DOTATATE PET/CT	^{18}F-FDG PET/CT
Cluster 1B Cluster 2 Sporadic PCC	^{18}F-DOPA PET/CT	^{123}I-MIBG scintigraphy
HNPGL	^{68}Ga-DOTATATE PET/CT	^{18}F-DOPA PET/CT

Recommended imaging modalities for different PPGL types. Note that ^{123}I-MIBG scintigraphy should also be considered for metastatic disease if ^{131}I-MIBG therapy would be performed.

PPGL that are recruiting, active, or approved are listed in **Table 4**.

The first medication clinicians should consider in pediatric PPGL is an α_1-adrenergic antagonist, such as the non-competitive long-acting phenoxybenzamine or competitive short-acting drugs, typically doxazosin. Alpha-adrenergic antagonists help to reduce the symptoms associated with catecholamine excess, improve hypertension, and prepare patients for therapeutic interventions, therefore α-adrenergic antagonists should be started in all PPGL patients (1, 26). Blood pressure and heart rate should be monitored, and β-adrenergic blockade should be considered in patients who have persistent tachycardia but only after starting α-adrenergic blockade for at least 2 to 3 days to avoid the effect of unopposed α-adrenergic stimulation (16). To prevent complications related to catecholamine release during treatment, patients should start α-adrenergic blockade 7 to 14 days before surgery or before non-surgical treatments, such as chemotherapy or radiotherapy, as these may all result in catastrophic surges of stored catecholamines (26). Competitive inhibition of tyrosine hydroxylase by α-methyl-L-tyrosine (metyrosine, or Demser®) results in decreased synthesis of all downstream catecholamines and can be helpful for patients with highly elevated levels of catecholamines to improve hemodynamic stability before and after surgery but must be used cautiously as it is contraindicated in patients with severe depression and suicidal ideation (17, 26). Calcium channel blockers, especially amlodipine, can also be helpful in controlling hypertension in pediatric patients with PPGL (77).

Surgical interventions should be considered even for patients with metastatic disease, to reduce the tumor burden contributing to catecholamine excess and to improve radiopharmaceutical uptake in remaining metastatic lesions (26). Genetic testing preoperatively can be helpful to inform the surgical approach, as cortical-sparing adrenalectomy is preferred in younger patients to reduce the risk of adrenal insufficiency (and requirement for exogenous steroids) and does not seem to increase the risk of recurrence for patients with mutations in *VHL*, *RET*, or *NF1*, but in patients with higher risk for metastatic disease, such as those with *SDHB* mutations, more extensive resection may be needed (21).

The approach to the patient with metastatic disease depends on the extent of disease and the rate of progression. For patients with very extensive disease or rapid progression, chemotherapy should be tried first, with first-line therapy consisting of cyclophosphamide, vincristine, and dacarbazine (CVD); temozolomide as monotherapy may also be considered as an alternative or a second-line agent in these cases (78). The impact of SDH deficiency on chemoresistance, as previously mentioned, suggests that PARP inhibitors such as olaparib may be useful in combination with temozolomide, and a clinical trial is currently recruiting to evaluate this combination in adults (NCT04394858). Hypoxia-inducible factors increase expression of growth factors such as VEGF, platelet-derived growth factor (PDGF), and transforming growth factor α (TGFα), which bind to their respective receptor tyrosine kinases (53). Therefore, additional therapies to consider include tyrosine kinase inhibitors with anti-VEGF activity, such as sunitinib (53). As a last resort, additional approaches to consider include immunotherapy with checkpoint inhibitors (e.g., pembrolizumab or combination nivolumab-ipilimumab) in patients with cluster 1 mutations (consequent to impaired immune recognition related to pseudohypoxia) or mTORC1 inhibitors (e.g., everolimus) in patients with cluster 2 mutations (mTORC1 regulates cellular activity downstream of kinase signaling pathways), but additional studies are needed in children with metastatic PPGL (78).

For patients with less rapid progression or smaller tumor burden, systemic radiotherapy can be particularly useful as part of a personalized approach when lesions have avidity on imaging scans for the corresponding diagnostic radionuclides. For patients with DOTA-SSA-avid lesions, as is often seen with cluster 1 mutations, PRRT with ^{177}Lu-DOTA-SSA ("hot SSA") or SSAs that do not carry radiopharmaceuticals (referred to as "cold SSAs") may also be helpful, especially for *SDHx* patients with DOTATATE-avid lesions, and additional clinical trials are evaluating these in children (**Table 4**). Conventional or high-specific activity (HSA) ^{131}I-MIBG (referred to as Ultratrace™, or Azedra®) may be particularly useful for patients with cluster 2 mutations who are more likely to have adrenal disease but requires positive findings on diagnostic ^{123}I-MIBG scan before use (69). When considering systemic radiotherapy, clinicians should ideally perform both ^{123}I-MIBG and ^{68}Ga-DOTATATE scans to determine which radiopharmaceutical best shows all the metastatic lesions in order to guide which radiotherapy should be used to treat the patient (79).

Locoregional approaches may be helpful for certain aspects of metastatic disease. External radiation therapy can be useful for head and neck primary disease due to a more favorable risk profile as compared to surgical intervention and for rapidly-growing metastatic tumors, such as in bone, to provide symptomatic relief (1, 26, 78). Treatment of a primary HNPGL by external beam radiation therapy, therefore, may be helpful for those with *SDHD* mutations. Bone metastases are also treated

TABLE 4 | Clinical trials for metastatic PPGL therapies in pediatric patients that are recruiting, active, or approved.

Intervention	Class	NCT Number	Title
Belzutifan	HIF-2α inhibitor	NCT04924075	Belzutifan/MK-6482 for the Treatment of Advanced Pheochromocytoma/Paraganglioma (PPGL) or Pancreatic Neuroendocrine Tumor (pNET) (MK-6482-015)
DFF332	HIF-2α inhibitor	NCT04895748	DFF332 as a Single Agent and in Combination with Everolimus & Immuno-Oncology Agents in Advanced/ Relapsed Renal Cancer & Other Malignancies
^{177}Lu-oxodotreotide/ DOTATATE	PRRT	NCT04711135	Study to Evaluate Safety and Dosimetry of Lutathera in Adolescent Patients With GEP-NETs and PPGLs
^{177}Lu-Octreotate	PRRT	NCT02743741	Lu-DOTATATE Treatment in Patients with ^{68}Ga-DOTATATE Somatostatin Receptor Positive Neuroendocrine Tumors
^{177}Lu-DOTATATE	PRRT	NCT02236910	An Open Label Registry Study of Lutetium-177 (DOTA0, TYR3) Octreotate (Lu-DOTA-TATE) Treatment in Patients with Somatostatin Receptor Positive Tumors
^{177}Lu-DOTATATE	PRRT	NCT01876771	A Trial to Assess the Safety and Effectiveness of Lutetium-177 Octreotate Therapy in Neuroendocrine Tumours
^{90}Y-DOTA tyr3-Octreotide	PRRT	NCT00049023	Radiolabeled Octreotide in Treating Children with Advanced or Refractory Solid Tumors
^{131}I-MIBG	MIBG	NCT03015844	A Compassionate Use/Expanded Access Protocol Using ^{131}I-MIBG Therapy for Patients with Refractory Neuroblastoma and Metastatic Pheochromocytoma
^{131}I-MIBG	MIBG	NCT01850888	MIBG for Refractory Neuroblastoma and Pheochromocytoma
^{131}I-MIBG	MIBG	NCT01590680	Expanded Access Protocol Using ^{131}I-MIBG
^{131}I-MIBG	MIBG	NCT01413503	A Phase II Study of ^{131}I- Metaiodobenzylguanidine (MIBG) for Treatment of Metastatic or Unresectable Pheochromocytoma and Related Tumors
^{131}I-MIBG	MIBG	NCT01377532	Compassionate Use of ^{131}I-MIBG for Patients with Malignant Pheochromocytoma
^{131}I-MIBG	MIBG	NCT00107289	Iodine I-131 Metaiodobenzylguanidine in Treating Patients with Recurrent, Progressive, or Refractory Neuroblastoma or Malignant Pheochromocytoma or Paraganglioma
Ultratrace ^{131}I-MIBG	MIBG	NCT02961491	Expanded Access Program of Ultratrace Iobenguane I-131 for Malignant Relapsed/Refractory Pheochromocytoma/Paraganglioma
Tipifarnib	Farnesyltransferase inhibitor	NCT04284774	Tipifarnib for the Treatment of Advanced Solid Tumors, Lymphoma, or Histiocytic Disorders with *HRAS* Gene Alterations, a Pediatric MATCH Treatment Trial

with bisphosphonates or denosumab (3, 26). Interventional procedures such as radiofrequency ablation, cryoablation, or ethanol injection can be used in cases for treatment of a single metastatic lesion or oligo-metastases (3, 26). Furthermore, patients with inoperable primary tumors can be considered for potential treatment with a cold-SSA if lesions are ^{68}Ga-DOTATATE-avid, as reported in an adult *SDHB* patient with pterygopalatine fossa PGL, who was successfully controlled for 36 months with octreotide (80).

Belzutifan is one of the newer targeted therapies that specifically inhibits HIF-2α and has been approved to treat VHL-associated tumors (81). Given the central role that HIF-2α plays in hypoxia signaling, this therapy could be incredibly impactful for patients with cluster 1 mutations. A recent case report demonstrated the efficacy of belzutifan in treating multiple PGLs in an adolescent patient with PZS, with improvement in biochemical (normetanephrine and chromogranin A) and hematologic (erythropoietin and hemoglobin) markers and reduced tumor size on imaging. Belzutifan was well-tolerated in this patient with minimal side effects, most notably, anemia that did not require transfusion (81).

While many studies examining prognosis, disease progression, and outcomes in PPGL are focused on adults or include both adults and children, it seems that the most important prognostic factor for disease-free survival (DFS) is extent of surgical resection of the primary tumor (for pediatric PPGL: 45.6% for complete resection vs. 24.1% for incomplete resection, $p < 0.001$) (7, 21, 30). Redlich et al. described other significant differences in 10-year DFS in pediatric patients with PPGL, including the presence of metastatic disease (29.6% vs. 43.5% in those without metastatic disease, $p = 0.014$), and PGL (36.6% vs. 47.8% in PCC, $p = 0.039$), whereas the presence of an *SDHB* mutation was associated with lower DFS but did not reach statistical significance (45.1% in non-*SDHB* vs. 24.4% in *SDHB*, $p = 0.063$) (7). Outcomes in pediatric patients with *SDHB* mutations have been well-described by Jochmanova et al., and specific outcomes are also discussed above (23).

PERSPECTIVES AND FUTURE DIRECTIONS

This review highlights important features to consider in pediatric patients with PPGL, with an emphasis on metastatic disease. Pediatric PPGL is overwhelmingly due to an underlying genetic predisposition, especially from cluster 1 genes associated with pseudohypoxia. The central role of hypoxia signaling by HIF-2α in these patients results in a pro-metastatic phenotype and disruption of normal development of chromaffin cell precursors that can result in increased susceptibility to PPGL in multiple tissues. Indeed, the biochemical and secretory features could be described as more primitive or underdeveloped in pediatric patients with cluster 1 mutations predisposing them to PPGL. All pediatric PPGL patients require lifelong surveillance and monitoring, even those with cluster 2 mutations who may not present with PPGL until their teenage

years. To the extent possible, these rare and challenging patients should be managed at centers of expertise with multidisciplinary teams that can address the different aspects of care.

Rather than viewing the pediatric patient with a genetic mutation as having an immutable risk factor for PPGL, the knowledge of this risk can guide clinicians to develop an appropriate screening and management plan with their patients and families, especially since the penetrance of PPGL is incomplete for all of the susceptibility genes (26). Genetic counseling can play an important supportive role in addition to contributing valuable information about risk of disease. Reducing anxiety by dispelling misconceptions about genetic risks as well as emphasizing preventive strategies and forming a screening plan to help reduce anxiety and empower patients and families is a key part of the approach to genetic counseling as shown in a study of patients with *SDHx* mutations. Out of 164 patients, only 2 (1.2%) had increased anxiety in response to genetic testing results, and only 1 (0.6%) did not proceed with any preventive measures nor any screening for her positive children (82).

Though not as prevalent a cause of metastatic disease as cluster 1 mutations, additional studies of targeted therapies for patients with cluster 2 mutations are needed in the pediatric population as our increased understanding of cellular pathways has generated specific therapeutic targets. Mweempwa et al. demonstrated efficacy of a selective RET inhibitor (selpercatinib) in metastatic PCC due to an activating gene fusion of *RET-SEPTIN9* in an adult with recurrent PCC with biopsy-confirmed metastatic disease to the lungs and liver with negative urinary metanephrines but highly elevated chromogranin A (33,710 μg/L). After 12 weeks of therapy with selpercatinib, chromogranin A had decreased to 598 μg/L, and pulmonary and hepatic tumors demonstrated reduction in size on CT (46% in the sum of diameters of lesions), raising the possibility that this targeted therapy may also be useful for pediatric patients with activating *RET* mutations; however additional studies are needed (83). Similarly, targeted therapy directed by the genetic background may also inform the approach to patients with NF-1. Selumetinib selectively inhibits MEK in the RAS-MAPK pathway, and in children with inoperable plexiform neurofibromas, treatment with selumetinib resulted in a reduction in tumor volume (median reduction of 27.9%) and progression-free survival of 84% (compared to 15% in natural history controls) at 3 years from treatment initiation. MEK inhibitors have also shown responses in other *NF1*-related tumors (e.g., optic pathway glioma), suggesting that PPGLs driven by *NF1* mutations might also be considered for this targeted therapy (84).

PPGLs have been described as the tumors that are the most highly genetically determined of any human tumor, and in children, around 80% have a germline predisposition. When accounting for those with additional somatic changes (e.g., *EPAS1* gain-of-function mutations), an even higher proportion is realized. Germline genetic testing to determine an underlying cause of PPGL is mandatory, especially in children with metastatic disease, but somatic tumor genetic testing has clear benefits as well and may identify new genes and targetable mechanisms that can personalize the care of these vulnerable patients.

AUTHOR CONTRIBUTIONS

MK drafted and revised the initial manuscript, figures, and tables. MN aided in conceptualizing and contributing to the manuscript. He also revised the manuscript. AJ aided in conceptualizing, contributing, and revising the manuscript. KP conceived, conceptualized, critically reviewed the manuscript for intellectual content, and aided in revision of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Biochemical Diagnosis of Catecholamine-Producing Tumors of Childhood: Neuroblastoma, Pheochromocytoma and Paraganglioma

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Catecholamine-producing tumors of childhood include most notably neuroblastoma, but also pheochromocytoma and paraganglioma (PPGL). Diagnosis of the former depends largely on biopsy-dependent histopathology, but this is contraindicated in PPGL where diagnosis depends crucially on biochemical tests of catecholamine excess. Such tests retain some importance in neuroblastoma though continue to largely rely on measurements of homovanillic acid (HVA) and vanillylmandelic acid (VMA), which are no longer recommended for PPGL. For PPGL, urinary or plasma metanephrines are the recommended most accurate tests. Addition of methoxytyramine to the plasma panel is particularly useful to identify dopamine-producing tumors and combined with normetanephrine also shows superior diagnostic performance over HVA and VMA for neuroblastoma. While use of metanephrines and methoxytyramine for diagnosis of PPGL in adults is established, there are numerous pitfalls for use of these tests in children. The establishment of pediatric reference intervals is particularly difficult and complicated by dynamic changes in metabolites during childhood, especially in infants for both plasma and urinary measurements, and extending to adolescence for urinary measurements. Interpretation of test results is further complicated in children by difficulties in following recommended preanalytical precautions. Due to this, the slow growing nature of PPGL and neglected consideration of the tumors in childhood the true pediatric prevalence of PPGL is likely underappreciated. Earlier identification of disease, as facilitated by surveillance programs, may uncover the true prevalence and improve therapeutic outcomes of childhood PPGL. For neuroblastoma there remain considerable obstacles in moving from entrenched to more accurate tests of catecholamine excess.

Keywords: pediatric, pheochromocytoma, paraganglioma, neuroblastoma, catecholamines, metanephrines, methoxytyramine, homovanillic acid

INTRODUCTION

Catecholamine-producing tumors include neuroblastoma, as well as pheochromocytoma and paraganglioma (PPGL), all derived from cells of the neural crest. Neuroblastoma occurs almost exclusively in childhood and originate from immature embryonic neuroblast cells that undergo transformation to form tumors at intra-adrenal and extra-adrenal locations. PPGL similarly occur at respective intra-adrenal and extra-adrenal locations, but originate from chromaffin cells or their chromoblast precursors, and are usually detected in adulthood though may be overlooked in childhood.

It is now apparent that neuroblasts, sympathoblasts, chromoblasts and mature chromaffin cells originate from neural crest derived Schwann cell precursors by way of different transitions and manifest by variable stages of differentiation (1, 2). These emerging concepts about development from different neural crest derivatives are fundamental to a complete understanding of the utility of catecholamine-related biomarkers for diagnosis of neuroblastoma and PPGL. In particular, differences in transition of neural-crest derived tumor precursors appear to be recapitulated in the considerable heterogeneity of presentations of both neuroblastoma and PPGL, including the nature of catecholamine production.

While neuroblastomas are characterized by poorly developed catecholamine biosynthetic and secretory pathways, there is nevertheless variation in this that relates to differences in catecholamine-associated features important for biochemical testing and disease aggressiveness (3–6). PPGL on the other hand show more mature catecholamine biosynthetic and secretory pathways compared to neuroblastoma, though even within those two intra- and extra-adrenal groups of tumors there is considerable heterogeneity in differentiation that impacts the nature of catecholamine secretory and metabolic products employed for biochemical diagnosis (7).

Of relevance to this article, PPGL detected in childhood and young adulthood tend to be poorly differentiated and often occur secondary to a particular group of mutated genes (i.e., so called cluster 1 group mutations) that predispose to more often extra-adrenal paraganglioma and multifocal adrenal and extra-adrenal tumors than the tumors that originate from cluster 2 group mutations (7–9). As detailed later, such differences have relevance to biochemical testing strategies and interpretation of biochemical test results in children with suspected chromaffin cell tumors.

It is also important to appreciate that PPGL are typically slow growing with a volume doubling time of 5-7 years (10, 11). From this it can be expected that it would take at least 15 years for a tumor to enlarge from 1.5 to 3 cm in diameter (**Figure 1**). Furthermore, in the early stages when tumors are less than 1.5 cm, they are unlikely to produce enough catecholamines for detection by standard biochemical tests let alone to evoke the signs and symptoms that may alert clinicians to the possibility of the tumor. Even when tumors do attain a size and level of catecholamine secretion sufficient to evoke signs and symptoms, there is usually considerable delay in recognizing the significance of this (12). Given that the median diameter of PPGL detected on the basis of signs and symptoms is 4 cm (13), it can be appreciated that for any PPGL first diagnosed in adults under an age of 30 years, the tumor is likely to have originated in childhood. The prevalence of childhood PPGL commonly cited in the literature is 10-20% (14–16); however, with the considerations outlined above, this likely represents an underestimate, particularly for patients with cluster 1 type gene mutations who have a median age of tumor diagnosis of 29 to 32 years (17).

As in adulthood the most important factor for an early diagnosis of PPGL in children and adolescents is attentiveness to the clinical clues of these tumors by pediatric caregivers. For familial cases involving offspring or siblings identified with germline mutations of tumor susceptibility genes an earlier diagnosis can be facilitated by enrolment into routine surveillance programs beginning as early as 5 years of age depending on the mutated gene (18–20).

MODES OF CLINICAL SUSPICION

The initial mode of clinical suspicion of a PPGL is usually based on signs and symptoms of catecholamine excess. Signs and symptoms in children as in adults include hypertension, particularly paroxysmal hypertension, as well as palpitations, excessive sweatiness, headache, visual disturbances, pallor, anxiety, tremor, panic/anxiety, constipation, and nausea and/or vomiting (14, 21, 22). Among these signs and symptoms, any of a few may be present and mostly in episodes. Weight loss associated with a lowered body mass index and high heart rate are also useful to consider, in fact, more so than hypertension, which is relatively common in adults and of limited discriminatory value (23). High blood pressure in children and adolescents is, however, uncommon and in combination with any other signs and symptoms should always be considered as a potential indicator of a catecholamine-producing tumor. Nighttime sweatiness, polyuria and disturbances of vision or mental status have been reported in pediatric cases of PPGL (15), and might warrant particular attention.

As in adults, some children with PPGLs may be normotensive and asymptomatic (24), particularly when tumors are found as part of surveillance programs involving family members with a known mutation of a tumor susceptibility gene (25). Children with incidentally discovered adrenal or extra-adrenal masses based on imaging studies for reasons other than a suspected PPGL can also be normotensive and asymptomatic and have a catecholamine-producing tumor (26, 27).

For neuroblastoma, which have a more limited hereditary background compared to PPGL and do not usually secrete catecholamines in amounts sufficient to cause signs and symptoms, the mode of initial clinical suspicion is different from that for chromaffin cell tumors. Since routine screening for the tumors is now out of favor, neuroblastoma are usually suspected based on findings of a palpable abdominal mass or as a mass found incidentally during ultrasonography (28), including on occasion during prenatal ultrasound (29). Masses in the neck

Abbreviations: PPGL, pheochromocytoma and paraganglioma; LC-MS/MS, liquid chromatography with tandem mass spectrometry; VMA, vanillylmandelic acid; HVA, homovanillic acid; PNMT, phenylethanolamine N-methyltransferase; COMT, catechol-O-methyltransferase; MAO, monoamine oxidase; DHPG, 3,4-dihydroxyphenylglycol; MHPG, 3-methoxy-4-hydroxyphenylglycol; MAX, myc-associated factor X; VHL, von-Hippel Lindau; SDHB, succinate dehydrogenase subunit B; RET, rearranged during transfection proto-oncogene; NF1, neurofibromatosis type 1.

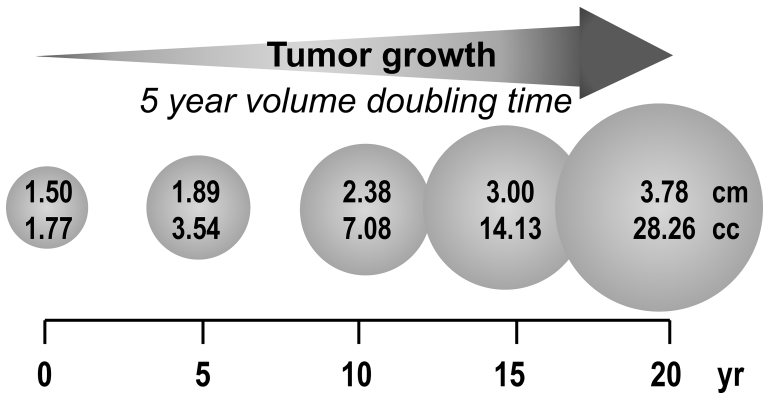

FIGURE 1 | Estimated duration (years) for a 1.5 cm PPGL to reach 3 to 4 cm based on reported minimum tumor volume doubling time of 5 years.

or thoracic regions may be discovered on the basis of Horner's syndrome. Children with neuroblastoma may also present with fever, weight loss, bone or joint pain, or other symptoms that may evoke discovery during imaging studies. Hematological abnormalities from bone marrow involvement can also lead to discovery of these tumors and eventual diagnosis achieved by histopathology often after percutaneous needle biopsy.

CATECHOLAMINE SYNTHESIS, METABOLISM AND SECRETION

Both PPGL and neuroblastoma are characterized by synthesis and metabolism of catecholamines within tumor cells. For any appreciation of the use of catecholamine-related biomarkers for diagnosis of these tumors it is useful to understand the pathways of catecholamine biosynthesis, storage, metabolism and secretion (30).

Catecholamine biosynthesis starts with conversion of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) by the rate limiting enzyme, tyrosine hydroxylase. DOPA is then converted to dopamine by aromatic-L-amino acid decarboxylase, an enzyme with a wide tissue distribution and broad substrate specificity. Dopamine is then transported by vesicular monoamine transporters into vesicular storage granules, where it is further converted to norepinephrine by dopamine β-hydroxylase, an enzyme with a unique presence in vesicular storage granules. Presence of phenylethanolamine N- methyltransferase (PNMT) in adrenal chromaffin cells leads to further conversion of norepinephrine to epinephrine (**Figure 2**); however, since PNMT is a cytosolic enzyme, this step depends on leakage of norepinephrine from vesicular storage granules into the cell cytoplasm. Epinephrine is then translocated back into storage granules from where it can be actively secreted as a circulating hormone.

Importantly and contrary to usual textbook depictions, vesicular stores of catecholamines do not exist in a static state until exocytotic secretion (31). Rather, vesicular stores of catecholamines exist in a highly dynamic equilibrium with the surrounding cytoplasm, with passive outward leakage into the cytoplasm counterbalanced by inward active transport under the control of vesicular monoamine transporters. The processes of active exocytotic secretion versus passive leakage of catecholamines from vesicular stores are entirely different and contribute differently to the metabolism of catecholamines produced at different sites including within tumor cells (32). These differences, however, are rarely appreciated despite the importance of this understanding for clinical applications of catecholamine-related biomarkers for diagnosis of catecholamine-producing tumors (30).

Exocytotic secretion of catecholamines involves the emptying of contents of vesicular catecholamine stores into the surrounding extracellular space. The process for hormonal secretion from the adrenal medulla involves closer proximity to the bloodstream than for norepinephrine secreted by sympathetic neurons, which acts locally rather than systemically. Close to 90% of norepinephrine secreted by sympathetic nerves is removed back into nerves by neuronal uptake so that only a small proportion escapes into the bloodstream or is metabolized at extra-neuronal locations before entry into the bloodstream (31). Some of the norepinephrine recaptured by sympathetic nerves is deaminated intraneuronally to 3,4-dihydroxyphenylglycol (DHPG) by monoamine oxidase (MAO), but most is returned to vesicular stores *via* vesicular monoamine transporters. By far the most DHPG is produced not after neuronal reuptake but rather after vesicular leakage of norepinephrine into the cytoplasm. DHPG is thereby the main initial metabolite produced from norepinephrine (**Figure 2**). DHPG is further metabolized at extraneuronal sites by catechol-O-methyltransferase (COMT) to 3-methoxy-4-hydroxyphenylglycol (MHPG). Thereafter most MHPG is metabolized in the liver by alcohol dehydrogenase to vanillylmandelic acid (VMA), the main urinary metabolic end-product of norepinephrine metabolism (33).

The processes of metabolism for catecholamines synthesized and secreted by adrenal chromaffin cells are somewhat similar to those for catecholamine-producing tumors, but entirely different from those for the norepinephrine produced in sympathetic nerves (31). First and foremost, while sympathetic nerves express only MAO, chromaffin cells also express COMT and thereby produce O-methylated metabolites (31). These include

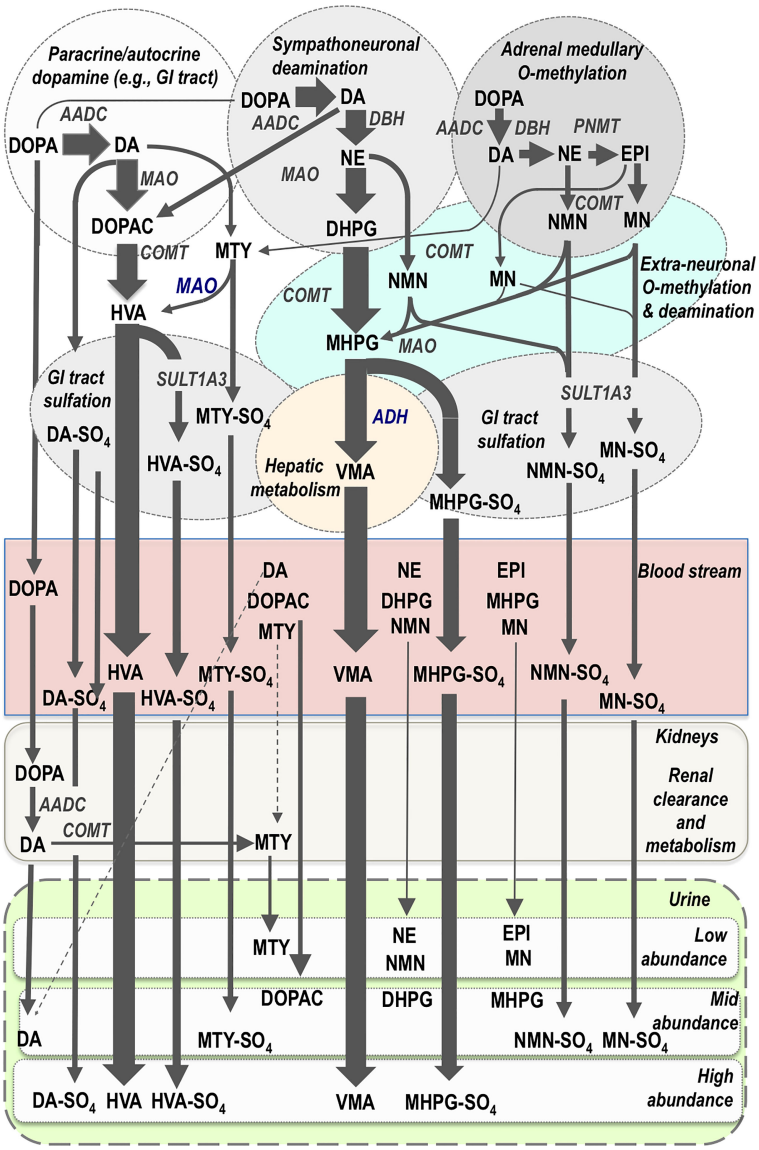

FIGURE 2 | Pathways of catecholamine metabolism according to initial biosynthesis of dopamine (DA) in paracrine and autocrine systems, of DA and norepinephrine (NE) in sympathoneuronal systems and of DA, NE and epinephrine (EPI) in chromaffin cells of the adrenal medulla. Thereafter the main metabolic pathways are shown according different compartments of metabolism and according to entry into the bloodstream and thereafter clearance and metabolism by the kidneys for elimination in the urine. Flux through metabolic pathways is reflected by arrow size and final abundance of metabolites in the blood stream and urine. The normetanephrine (NMN), metanephrine (MN) and methoxytyramine (MTY) in the bloodstream are produced in low abundance, primarily from adrenal chromaffin cells, and thereby provide better diagnostic signals for PPGLs than metabolites produced in higher abundance and largely from sources other than adrenal chromaffin cells. Almost all catecholamines and their metabolites undergo some degree of sulfate conjugation in humans, but only the main sulfate-conjugated metabolites are displayed. Note also the high abundance of dopamine metabolites, despite their minor source from sympatho-adrenal medullary systems. AADC, aromatic amino acid decarboxylase; DBH, dopamine-beta-hydroxylase; PNMT, phenylethanolamine-N-methyltransferase; MAO, monoamine oxidase; COMT, catechol-O-methyltransferase; SULT1A3; sulfotransferase isoenzyme 1A3; ADH, alcohol dehydrogenase; DOPA, 3,4-dihydroxyphenylalanine; DOPAC, 3,4-dihydroxyphenylacetic acid; DHPG, 3,4-dihydroxyphenylglycol; MHPG, 3-methoxy-4-hydroxyphenylglycol; HVA, homovanillic acid; VMA, vanillylmandelic acid; MTY-SO_4, methoxytyramine-sulfate; NMN-SO_4, normetanephrine-sulfate; MN-SO_4, metanephrine-sulfate; HVA-SO_4, homovanillic acid-sulfate; MHPG-SO_4, 3-methoxy-4-hydroxyphenylglycol-sulfate; DA-SO_4, dopamine-sulfate.

metanephrine from epinephrine, normetanephrine from norepinephrine and methoxytyramine from dopamine (**Figure 2**). The same former two metabolites can also be produced at extraneuronal locations from the catecholamines secreted into the bloodstream from the adrenals or from the norepinephrine secreted by sympathetic nerves. For circulating metanephrine, over 90% is derived from epinephrine metabolized within adrenal chromaffin cells rather than from epinephrine secreted from the same chromaffin cells (34). For circulating normetanephrine, about 25% is produced within

adrenal chromaffin cells and 75% from extraneuronal metabolism of norepinephrine secreted by sympathetic nerves.

The processes for metabolism of dopamine are somewhat different in that substantial amounts of this catecholamine are not produced in the sympatho-adrenal system, but rather in diffuse paracrine systems of the gastrointestinal tract, kidneys and other tissues (35, 36) (**Figure 2**). In the kidneys, dopamine is produced after renal uptake and local metabolism of circulating DOPA (37). Thus, more than 90% of urinary dopamine is formed from circulating DOPA rather than circulating dopamine (38). Urinary methoxytyramine appears to be derived by similar processes that may also include renal O-methylation of the dopamine produced from circulating DOPA (39). Finally, the end-product of dopamine metabolism, homovanillic acid (HVA), is derived from the combined actions of COMT and MAO; unlike VMA, the production of HVA does not require any additional actions of hepatic alcohol dehydrogenase so that HVA has distinctly different sources from VMA (35).

Apart from final metabolism of catecholamines and catecholamine metabolites to HVA and VMA, all compounds also undergo varying degrees of sulfate conjugation (**Figure 2**). This process occurs mainly in gastrointestinal tissues, the site of expression of the required sulfotransferase isoenzyme, SULT1A3 (40). The sulfated metabolic end-products are primarily removed by the kidneys and excreted in urine. This is particularly important for dopamine and its metabolites, but also provides an important metabolic pathway for O-methylated catecholamine metabolites. Thus, the normetanephrine, metanephrine and methoxytyramine commonly measured in urine after acid hydrolysis mainly reflect sulfate conjugates that have partly different sources from the more rapidly cleared and thus much lower concentrations of circulating free metabolites (41).

TUMORAL CATECHOLAMINE METABOLISM

With the considerations outlined above concerning the subcellular, cellular and organ wide compartmentalized disposition of catecholamines it can be better appreciated why the O-methylated metabolites of catecholamines offer the best biomarkers of catecholamine-producing tumors, as also displayed according to the simplifications of **Figure 3**. For cluster 1 norepinephrine-producing noradrenergic PPGL typical of childhood (**Figure 3**, panel A), the tumor-derived signal for free normetanephrine commonly shows a stronger and larger proportional increase above normal plasma concentrations than for circulating norepinephrine, 90% of which is derived from sympathetic nerves (34). That large proportion serves to dilute the diagnostic signal from tumors considerably more than for circulating normetanephrine. Moreover, the tumoral production of normetanephrine is continuous, whereas the exocytotic secretion of norepinephrine by tumors can be intermittent or minimal unless provoked. The signal produced by VMA is also largely diluted by the considerable amounts of this metabolite originally derived from the DHPG produced in sympathetic nerves, which lack COMT. Similarly, the normetanephrine sulfate produced in gastrointestinal tissues from locally secreted normetanephrine dilutes the signal for this metabolite, thereby explaining lower diagnostic signal for urinary deconjugated normetanephrine than for free normetanephrine (42).

Similar considerations to those outlined above also clarify why measurements of plasma free normetanephrine provide a stronger diagnostic signal than urinary VMA for children with neuroblastoma (4). However, unlike PPGL, in neuroblastoma it is the dopamine metabolites and not the norepinephrine metabolites that provide the more consistently increased biomarkers of excess catecholamine production. The considerations for catecholamine metabolism in neuroblastoma are also somewhat different than in PPGL where COMT is expressed in larger abundance than MAO, the expression of which is also lower than in adrenal medullary chromaffin cells (43, 44). Neuroblastomas express not only COMT (45), but also MAO in high abundance (46), and thus produce significant amounts of both deaminated and O-methylated catecholamine metabolites (**Figure 3**, panel B). Nevertheless, since HVA is normally produced in substantial amounts by an array of different pathways, this production acts to dilute the diagnostic signal for this metabolite when produced by neuroblastoma. In contrast, free methoxytyramine circulates at very low plasma concentrations so the diagnostic signal for this metabolite is stronger than for urinary HVA (4). For some tumors plasma concentrations of DOPA and 3-O-methyldopa provide the only signal (4, 47), presumably due to their poorly differentiated nature, including minimal expression of aromatic-L-amino acid decarboxylase, the enzyme that converts DOPA to dopamine.

BIOCHEMICAL TESTS OF CATECHOLAMINE EXCESS

For neuroblastoma the value of measuring catecholamine metabolites as biomarkers, rather than the catecholamines themselves, was established in the 1970s from the observations of LaBrosse and colleagues (48). As shown by these investigators, neuroblastomas display a relative lack of the catecholamine storage vesicles characteristic of mature chromaffin cells and their PPGL derivatives. Thus, these tumors usually do not present with hypertension or increased plasma or urinary catecholamines, so that biochemical tests have traditionally depended on measurements of catecholamine metabolites, in particular HVA and VMA.

Intra-tumoral metabolism of catecholamines in PPGLs was also described in the 1960s by Crout and Sjoerdsma (49). Nevertheless, because PPGLs are characterized by hypertension and symptoms of catecholamine excess, those early findings were largely ignored and diagnosis continued to focus on measurements of catecholamines. It was not until after the

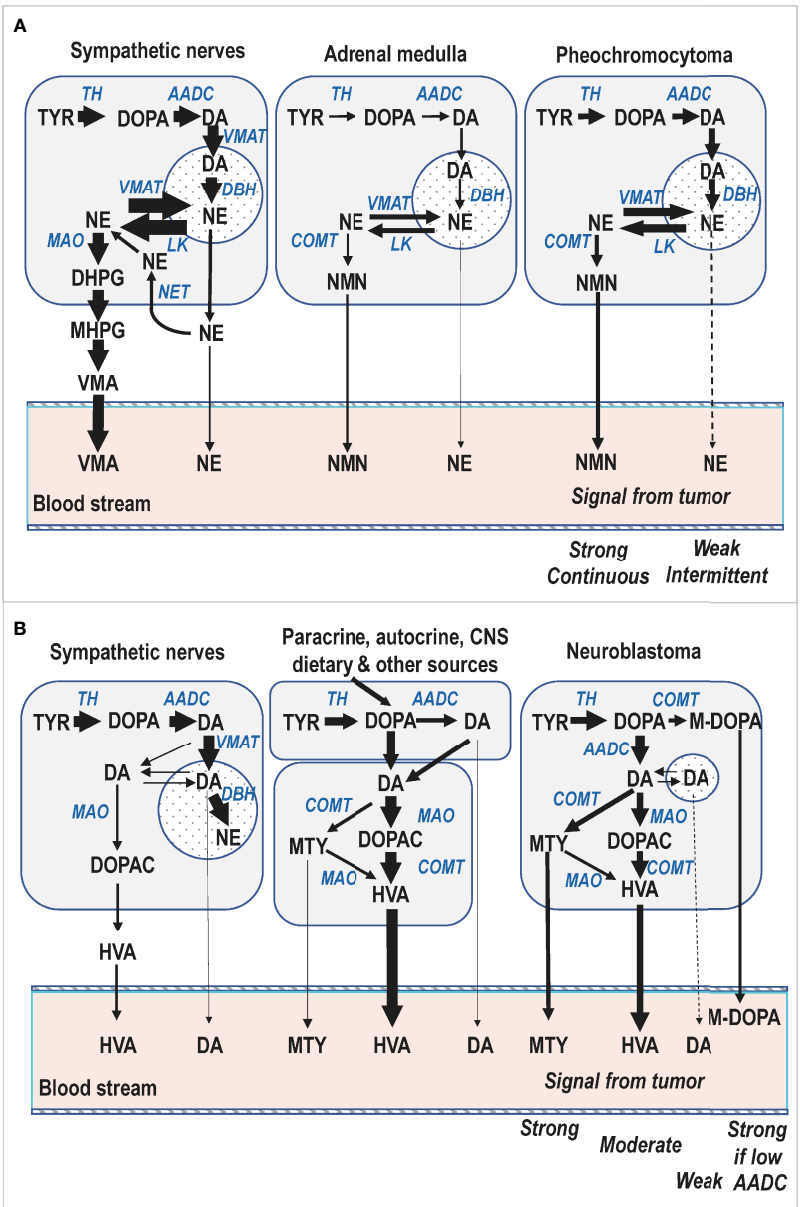

FIGURE 3 | Simplified diagrammatic representations of catecholamine biosynthetic and metabolic pathways in a pediatric patient with a cluster 1 norepinephrine-producing PPGL **(A)** and another with a dopamine-producing neuroblastoma **(B)**. For both panels the storage vesicles where dopamine is converted to norepinephrine and from where catecholamines are secreted by exocytosis are depicted by the circular compartments. For neuroblastoma the smaller size of that compartment serves to illustrate lower numbers of vesicular storage granules and associated minimal catecholamine secretory activity of these tumors. AADC, aromatic amino acid decarboxylase; DBH, dopamine-beta-hydroxylase; MAO, monoamine oxidase; COMT, catechol-O-methyltransferase; VMAT, vesicular monoamine transporter; LK, leakage of catecholamines from vesicles; NET, cell membrane norepinephrine transporter; DOPA, 3,4-dihydroxyphenylalanine; M-DOPA, 3-O-methyl-dopa; DA, dopamine; NE, norepinephrine; DOPAC, 3,4-dihydroxyphenylacetic acid; DHPG, 3,4-dihydroxyphenylglycol; MHPG, 3-methoxy-4-hydroxyphenylglycol; HVA, homovanillic acid; VMA, vanillylmandelic acid; MTY, methoxytyramine; NMN, normetanephrine.

turn of the 21st century that emphasis moved from catecholamines to their O-methylated metabolites, the metanephrines (50). Shift in emphasis from catecholamines to metanephrines for diagnosis of PPGLs followed the understanding outlined earlier about how catecholamines are synthesized, stored and metabolized independently of secretion and by different pathways. Consequently, recommendations today mandate measurements of plasma free or urinary fractionated metanephrines as initial screening tests for PPGLs (51). Among these tests, plasma measurements offer superior diagnostic accuracy than urinary measurements (42). For the latter it should be appreciated that these may involve either the free metabolites or the more commonly used and much higher concentrations of sulfate-conjugated metabolites measured

TABLE 1 | Diagnostic performance of biochemical biomarkers for pediatric PPGL.

Study reference	Sample matrix	Biomarker	Diagnostic sensitivity	Diagnostic specificity
Sarathi et al., *Endocr Prac* 2012;18:694-9 (52)	Plasma	NMN+MN	100% (17/17)	85.7% (67/78)
Weise et al., *J Clin Endocrinol Metab* 2002;87:1955-60 (25)	Plasma	NMN+MN	100% (12/12)	94% (31/33)
		NE+EPI	92% (11/12)	91% (30/33)
	Urine	NMN+MN	100% (5/5)	95% (21/22)
		NE+EPI	100% (10/10)	83% (25/30)
Barontini et al., Ann NY Acad Sci 2006;1073:30-7 (98)	Urine	NMN	95% (55/58)*	No data
		MN	68% (39/58)*	No data
		NE	98% (57/58)*	No data
		EPI	45% (26/58)*	No data
		VMA	93% (54/58)*	No data
Perel et al., *Pediatr Hemat Oncol* 1997;14:413-22 (99)	Urine	MN	91.7% (11/12)*	No data
		VMA	95.7% (22/23)*	No data

No information provided on reference intervals.

together with the free metabolites after an acid hydrolysis deconjugation step. The measurements of urinary free metabolites offer some diagnostic advantages over the combined free and deconjugated metabolites, though the value of urinary free methoxytyramine for identification of dopamine producing tumors is limited (42). Most likely this reflects origins of urinary free methoxytyramine from renal uptake, clearance and metabolism of circulating DOPA (**Figure 2**).

The vast majority of studies that have examined the diagnostic performance of biochemical tests for patients with PPGL have been in adults, with only a few isolated reports in children some of which employed outdated measurements of urinary VMA or spectrophotometric measurements of total metanephrines rather than fractionated normetanephrine and metanephrine (**Table 1**). Nevertheless, there have been two reports that documented high diagnostic accuracy of plasma free metanephrines for childhood cases of PPGL (25, 52).

The two catecholamine metabolites that continue to provide the mainstay for biochemical testing of neuroblastoma are HVA and VMA, usually measured in urine. Since the HVA and VMA derived from neuroblastoma or PPGL are diluted by considerable amounts of the same metabolites produced from other sources, these metabolites are relatively poor diagnostic markers for catecholamine-producing tumors. For adult pheochromocytoma, diagnostic sensitivity of VMA reaches only to 46-77% compared to 97-99% for plasma free metanephrines at similar specificities (53). In a prospective trial involving 1.5 million neonates only 73% of all infants detected at follow-up with neuroblastoma had elevated urinary excretion of HVA or VMA at screening (54). Moreover, many that were detected by screening were those that spontaneously regressed while those that were missed were usually aggressive. Consequently, screening programs involving urinary HVA and VMA have been abandoned. Today, diagnosis of neuroblastoma depends primarily on biopsy-dependent histopathology, which combined with genomic biomarkers (e.g., *MYCN* amplification) can provide information for staging and therapeutic intervention (55).

Reflecting historical precedence, almost all reports that have examined the utility of tests of catecholamine excess to identify children with neuroblastoma have included measurements of urinary HVA and VMA (**Table 2**). Nevertheless, there have been recent reports that have examined utility of plasma free or urinary measurements of normetanephrine and methoxytyramine. Thus, similar to PPGL, there is now evidence that measurements of plasma normetanephrine and methoxytyramine provide excellent biomarkers for identification of patients with neuroblastoma (4, 56, 57), including one study that showed the expected superiority over urinary HVA and VMA (4). Introduction of new biochemical tests for neuroblastoma is, nevertheless, largely made futile by reliance on biopsy-dependent histopathological diagnosis and measurements of urinary HVA and VMA for assessing tumoral catecholamine production.

Urinary rather than plasma methoxytyramine has also recently been advanced as an alternative to urinary HVA for identification of neuroblastoma (58). This metabolite, similar to urinary dopamine, appears to be largely derived from DOPA and is thus not a particularly good biomarker of tumoral dopamine metabolism (42). Nevertheless, these measurements appear to have prognostic utility (5). Since plasma DOPA also shows prognostic utility in neuroblastoma (47, 59), it is possible that this may underly the prognostic utility of urinary methoxytyramine. With prognostic rather than diagnostic utility in mind, there may be a rationale to advance from current antiquated reliance on urinary HVA and VMA to new and improved methods for biochemical testing of neuroblastoma.

REFERENCE INTERVALS

The need to establish reference intervals for biochemical tests of catecholamine excess represents another impediment to moving from outdated methods of diagnosis to new and improved biochemical tests to detect catecholamine-producing tumors. For adults it is relatively simple to obtain blood or urine specimens to establish reference intervals and further test utility of those reference intervals in patient populations. The practical and ethical barriers to procure blood or urine specimens for establishing reference intervals are more complex to negotiate for pediatric than adult populations (60). Consequently, advances in place for adults can be considerably delayed to implement for children.

As with many biomarkers, those involving tests of catecholamine excess can show variable differences between adults and children and within the pediatric population differences according to developmental age and sex. For plasma free normetanephrine and

TABLE 2 | Diagnostic performance of biochemical biomarkers for neuroblastoma.

Study Reference	Sample matrix	Biomarker	Diagnostic sensitivity	Diagnostic specificity
Peitzsch et al., *Pediatr Blood Cancer* 2019;e28081 (60)	Plasma	NMN	72.9% (70/96)[1]	97.6% (40/41)[1]
		MTY	96.8% (93/96)[1]	97.6% (40/41)[1]
		NMN+MTY	97.9% (94/96)[1]	95.1% (39/41)[1]
	Urine	VMA	73.2% (60/82)	84.8% (28/33)
		HVA	75.6% (68/90)	87.9% (29/33)
		VMA+HVA	82.2% (74/90)	84.8% (28/33)
Barco et al., *Clin Biochem* 2019;66:57-62 (57)	Plasma	NMN	80.4% (43/54)[2]	100%[2]
		MTY	88.2% (48/54)[2]	95.8%[2]
		NMN+MTY	92% (50/54)[2]	92%[2]
	Urine	VMA	79% (30/38)[2]	No data
		HVA	90% (34/38)[2]	No data
Franscini et al., *Pediatr Blood Cancer* 2015;62:587-K2793 (56)	Plasma	NMN	100% (10/10)	No data
		MTY	100% (10/10)	No data
Hwang et al., *Molecules* 2021;26:3470 (100)	Urine	VMA	75% (15/20)[3]	No data
		HVA	85% (17/20)[3]	No data
		VMA+HVA	90% (18/20)[3]	No data
Verly et al., *Eur J Cancer* 2017;72:235-43	Urine	NMN	89% (268/301)[4]	No data
		MTY	69% (208/301)[4]	No data
		NMN+MTY	92% (277/301)[4]	No data
		VMA	66% (199/301)[4]	No data
		HVA	82% (247/301)[4]	No data
		VMA+HVA	84% (253/301)[4]	No data
Barco et al., *Clin Biochem* 2014;47:848-52 (57)	Urine	VMA	81.6% (146/179)[2]	93.8%[2]
		HVA	80.5% (136/169)[2]	92.3%[2]
		VMA+HVA	81.9% (130/159)[2]	91.2%[2]
Strenger et al., *Pediatr Blood Cancer* 2007;48:504-9 (101)	Urine	VMA	80.7% (92/114)[5]	No data
		HVA	71.9% (82/114)[5]	No data
		DA	61.3% (70/114)[5]	No data
		VMA+HVA	88.6% (101/114)[5]	No data
		VMA+HVA+DA	91.2 (104/114)[5]	No data
Monsaingeon et al., *Eur J Pediatr* 2003;162:397-402 (102)	Urine	NMN	96.6% (28/29)[2]	97%[2]
		VMA	96.6% (28/29)[2]	96%[2]
		HVA	93.1% (27/29)[2]	99%[2]
Schilling et al., *NEJM* 2002;346:1047-53 (54)	Urine	VMA +HVA	73% (109/149)	99.8% (1,470,864/1,472,469)
Candito et al., *Med Pediatr Oncol* 1992;20:215-20 (103)	Urine	NMN	72% (13/18)	No data
		MTY	89% (16/18)	No data
		VMA	78% (14/18)	No data
		HVA	67% (12/18)	No data
Tuchman et al., *Pediatrics* 1987;79:203-5 (104)	Urine	VMA	72.5% (29/40)[6]	No data
		HVA	90% (36/40)[6]	No data
LaBrosse et al., *Cancer Res* 1980;40:1995-2001 (105)	Urine	VMA	71% (151/213)[7]	No data
		HVA	75% (159/213)[7]	No data

1) Reference intervals from Peitzsch et al., Clin Chim Acta 2019;494:100 (60).
2) performance characteristics derived from ROC analysis.
3) Reference intervals from Rifai, N. Tietz Textbook of Clin Chem Mol Diag, 6th ed.; Elsevier: Louis, MO, USA, 2018 (106).
4) Reference intervals from Davidson et al., Ann Clin Biochem 2011;48(Pt 4):358 (63).
5) Reference intervals from Kerbl et al., Eur J Cancer 1996;32A:2298 (107).
6) Reference intervals from Tuchman et al., Pediatrics 1985;75:324 (108).
7) Reference intervals from Gitlow et al., J Lab. Clin Med 1978;72:612 (109), Voorhess, Pediatrics 1967;39:252 (110).

methoxytyramine there are highly dynamic changes in early childhood with markedly higher plasma concentrations in neonates that drop rapidly within the first year (**Figure 4**). Thereafter, concentrations level off, though for normetanephrine slowly climb later in adolescence and continue to increase throughout adulthood. In contrast, plasma concentrations of metanephrine increase during early infancy, are higher in young children than adults and remain higher in males than females (25). The reciprocal changes of methoxytyramine and normetanephrine compared to metanephrine in early childhood are suggested to reflect apoptosis of neural crest-derived cells of the paraganglia from which the former metabolites derive, changes that contrast with development of the adrenal chromaffin cells responsible for almost all circulating metanephrine (60). For use of O-methylated catecholamine metabolites as biomarkers of neuroblastoma it is essential to employ age-specific reference intervals, which can be achieved from polynomial curve fitting (56, 60). For children over 5 years of age, plasma concentrations of methoxytyramine and normetanephrine remain relatively constant throughout childhood and all that is required are reference intervals for that broader age range. For metanephrine, concentrations are higher in boys than girls and particularly in younger children, when slightly higher cut-offs may be preferable compared to adolescents and adults.

Since diagnosis of catecholamine-producing tumors of childhood have historically involved measurements in urine, reference intervals for this sample matrix are those that have received the most attention (61–65). However, such measurements are not without associated problems for establishing reference intervals. Since 24-hr collections of urine from both infants and older children are unreliable and difficult, spot urines are the method commonly employed with dilutional differences corrected using creatinine (61). This, however, has the problem of introducing another variable as a denominator (**Figure 5**). Urinary outputs of creatinine vary according to diet, exercise and most importantly muscle mass (66, 67). Consequently, urinary outputs of creatinine are higher in males than females and are positively correlated to body mass or body surface area and increase substantially from early infancy throughout childhood (67–69). There are also body size related increases in urinary outputs of catecholamines and their metabolites, but these are not as substantial as those for creatinine. Thus, although 24-hr urinary outputs of catecholamines and metabolites increase throughout childhood, when expressed as a ratio to urinary outputs of creatinine there are marked decreases throughout childhood and particularly in infancy (61–65).

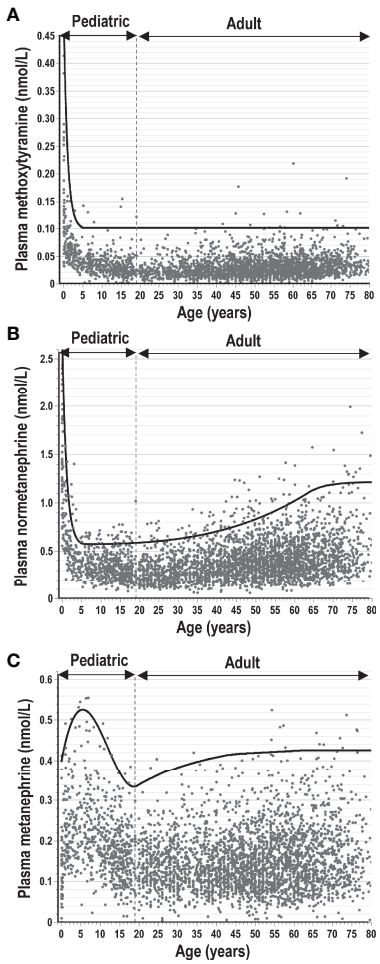

FIGURE 4 | Plasma concentrations of methoxytyramine **(A)**, normetanephrine **(B)** and metanephrine **(C)** from infancy and early childhood through adolescence to later ages in adults. Concentrations are derived from measurements in 3706 individuals (including 707 children from 0-18 years of age) without catecholamine producing tumors according to previously published data (4, 25, 42, 60). The solid lines indicate upper limits of reference intervals, which for adults and children over 5 years of age were optimized to provide optimum diagnostic sensitivity for normetanephrine at optimal specificity and lower proportions of false-positive results (<1%) for metanephrine and methoxytyramine.

Due to the dynamic changes in urinary excretion of both creatinine and catecholamine metabolites, reference intervals must be established for different age groups (61–65). Thus, for example, as reported by Davidson et al. (63), upper limits for urinary excretion of normetanephrine as a ratio to creatinine vary considerably from 0.529 nmol/mol for infants below one year to 0.123 nmol/mol for children 8 to 10 years of age and 0.086 nmol/mol for those between 14 to 19 years of age. As reported by Cole et al. (61), use of creatinine to correct for dilutional influences on measured concentrations of VMA and HVA in spot urine samples suffers from several limitations that impact reliability of measurements as a laboratory test for neuroblastoma. These investigators proposed further adjustments for sex and body weight.

For all reference intervals due consideration must also be given to the methods of measurement. Early spectrophotometric methods as well as more recent immunoassay methods can be inaccurate and suffer from interferences so that reference intervals developed by these methods can be unreliable (70). Even for techniques employing gas or liquid chromatographic separation there can be differences in measurements between laboratories so that reference intervals should be validated by each laboratory and not simply involve those reported in the literature. Mass spectrometric methods offer opportunities for both improved analytical specificity and accuracy. By participation in interlaboratory proficiency programs or comparison studies there is now the possibility for both harmonized measurements and reference intervals (71).

PREANALYTICAL CONSIDERATIONS

Apart from appropriately established reference intervals and accurate measurement methods, consideration of preanalytics and other patient specific variables is crucial to interpretation of analytical test results (72), particularly those that fall close to either side of upper limits of reference intervals. For both plasma and urinary measurements, it is important that samples are procured with patients under conditions of minimal physiological or emotional distress (**Figure 5**). In adults it is well established that emergency situations and acute illness can result in plasma concentrations or urinary outputs of metanephrines that can be indistinguishable from those in patients with catecholamine-producing tumors (73). The same can also be expected for children, so that ideally testing for a catecholamine-producing tumor should be undertaken after recovery from a hypertensive emergency or severe illness.

Exercise or other forms of physical activity should be avoided or minimized before blood collections or preceding and during urine collections. First morning urine collections are associated with lower urinary outputs of catecholamines and metabolites due to the preceding nighttime rest and have been proposed as a means to minimize false-positive results (74). However, as yet this has not been verified for diagnosis of catecholamine-producing tumors in childhood.

For measurements of plasma metanephrines it is important that blood samples are taken after at least 20 minutes of comfortable supine rest, but for young children this can be difficult and requires close supervision (25). Similarly, although it is standard practice to acquire blood samples by direct venipuncture, this is established in adults to result in higher plasma concentrations and rates of false-positive results than sampling using a previously inserted intravenous cannula (75). For children, the emotional distress of venipuncture can be expected to be more troublesome than for adults. With preanalytical precautions in place high diagnostic sensitivity is preserved and false-positive results are minimal according to upper limits of reference intervals appropriately determined for children (25).

For younger children, stress-free blood sampling can be highly problematic, which represents a limitation of plasma compared to the urinary measurements typically used when testing children for suspected neuroblastoma. The volume of blood required can also be a limiting factor for neonates. However, new advances in sample preparation procedures before mass spectrometry is now allowing accurate and precise measurements of plasma methoxytyramine and other O-methylated metabolites in as little as 50 µl of plasma (76). With such advances it might even be possible to obtain blood by heel prick, which might provide an advantage of a blood versus a urine test.

Dietary influences and medications can also be important to consider. Certain foods can increase plasma concentrations of methoxytyramine as well as urinary outputs of catecholamine metabolites (77). For blood sampling such influences can be avoided by an overnight fast; however, for infants and urine collections, dietary restrictions may be preferable or more practical (60). Drugs that significantly impact monoamine systems, such as norepinephrine uptake blockers, can be troublesome in adults but are rarely administered to children. Although some antihypertensives and other medications can result in analytical interferences for some methods of analysis, such drugs are not usually troublesome with modern mass spectrometric methods (78).

BIOCHEMICAL TEST INTERPRETATION

Apart from assessment of whether test results are positive or negative, the pattern and nature of increases of the three O-methylated catecholamine metabolites can provide other information about a catecholamine producing tumor that cannot be so easily gleaned from measurements of the catecholamines themselves or other metabolites such as HVA and VMA. Extents and patterns of

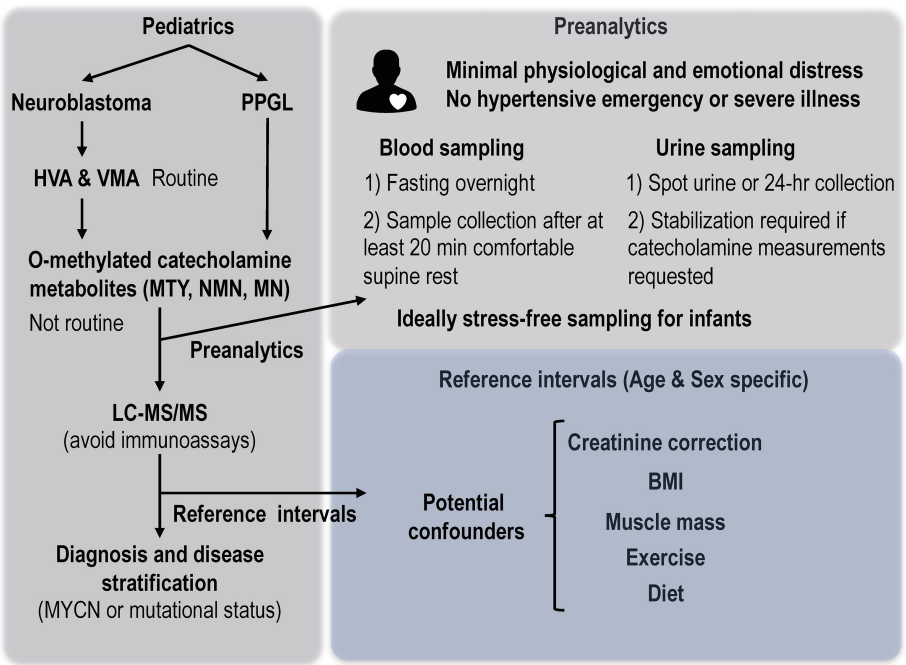

FIGURE 5 | Considerations of preanalytics and reference intervals for interpretation of biochemical tests of catecholamine excess in children. MTY, methoxytyramine; NMN, normetanephrine; MN, metanephrine; BMI, body mass index.

increases above reference intervals not only allow assessments of relative probabilities of a tumor, but also other information such as location, size, disease aggressiveness and type of disease-causing mutations (79, 80). For neuroblastoma the information is a little more limited than for PPGL, but from increases in methoxytyramine can be used to assess likelihood of *MYCN* amplification (4, 5), which has prognostic significance.

For PPGL, increases in plasma methoxytyramine and metanephrine can be used to assess likelihood of extra- versus intra-adrenal tumor locations (80), the former more common in pediatric than adult patients. Increases in methoxytyramine relative to normetanephrine can also be used to assess risk of metastatic disease (79), which as in neuroblastoma may also relate to the developmental aspects of the tumors and associated degree of differentiation. Related to this, relative increases of the three metabolites can be used to assess probabilities of underlying mutations in groups of genes (80), these differing in prevalence among children and young adults compared to older adults with the tumors (7, 8).

Magnitudes of increases above normal of all combined O-methylated metabolites correlate with disease burden and can thereby be used to predict tumor size (80). The generally slow growing nature of PPGLs is also reflected by time-dependent increases in plasma concentrations of the O-methylated metabolites, which can be useful in follow-up to better confirm a PPGL. This can be particularly useful in surveillance programs, such as in children with identified mutations of the von-Hippel Lindau (*VHL*) gene whereby relative changes over time can signal development of a PPGL better than a single point measurement (25).

DIFFERENTIAL DIAGNOSIS

Neuroblastoma is more common in children than PPGL and since clinical presentations can overlap, there can be difficulties in the differential diagnosis of the two tumor entities (22). This difficulty has been highlighted in this issue by a case series of five children with PPGL who were initially diagnosed mistakenly with neuroblastoma, including some who underwent biopsy and all of whom were inadequately prepared before surgical intervention (81). Though commonly employed for neuroblastoma, percutaneous biopsy of a PPGL is potentially dangerous and is therefore contraindicated for those tumors (82, 83). Furthermore, because all patients, including children, with PPGL should be prepared for surgery using adrenergic blocking drugs (22, 84), it is imperative that there is no mistaking a PPGL for a neuroblastoma before scheduling fine needle biopsy or surgical intervention. Adding to the difficulties in differential diagnosis, cases have also been reported of composite neuroblastoma and pheochromocytoma (85–87). Furthermore, some gene mutations, such as those impacting the *myc-associated factor X* (*MAX*) gene, can predispose to both childhood neuroblastoma and pheochromocytoma (88, 89).

For PPGL at least, identification of disease depends crucially on biochemical tests of catecholamine excess; thus, if there is any doubt about a presumed neuroblastoma, the same tests should be considered in those cases. In the case series presented in this issue, involving five patients with PPGL and a mistaken initial diagnosis of neuroblastoma, only two underwent any form of testing for catecholamine excess (81). The problem remains, however, whether such tests can be used to distinguish neuroblastoma from PPGL.

Since both PPGL and neuroblastoma show overlapping patterns of increases in plasma dopamine and norepinephrine metabolites, as well as in some cases the precursor catecholamines themselves, these measurements alone are unlikely to offer a solution for differential diagnosis in all pediatric cases of these tumors. Plasma concentrations of 3-O-methydopa are negligibly impacted in patients with PPGL (personal observations), but show increases above cut-offs in about 75% of patients with neuroblastoma (4). Such increases may therefore be useful for distinguishing neuroblastoma from PPGL, but cannot solve the problem for more fully differentiated cases of neuroblastoma. As outlined earlier, neuroblastoma differ from PPGL in their relative expression of the primary catecholamine-metabolizing enzymes, MAO and COMT, with the former deaminating enzyme more highly expressed in neuroblastoma than PPGL. Thus, it is possible that, in addition to measurements of 3-O-methyldopa, different patterns of deaminated and O-methylated metabolites might be useful for differential diagnosis. This, however, remains to be determined.

Technologies such as those involving circulating tumor cells or DNA and protein biomarkers offer potential molecular diagnostic strategies for therapeutic stratification and monitoring in neuroblastoma (90, 91). Such approaches might also be also useful for differential diagnosis. Nevertheless, it will take considerable time to develop and establish liquid biopsies for these purposes, let alone prospectively determine any clinical utility, particularly in pediatric solid tumors (92).

Differences in imaging features between neuroblastoma and PPGL, such as presence of calcifications in the former, could also offer clues to help distinguish the two types of tumors (93). However, calcifications can occasionally occur in PPGL (94). Imaging characteristics of encasement, invasion and infiltration are relatively common in neuroblastoma (95), but far less so in PPGL and might thereby offer other clues to differentiate the two tumor types. Nevertheless, as indicated in the case series covered in this issue, there can be exceptions. As yet any use of functional imaging for differential diagnosis appears undocumented. Therefore, without histopathological confirmation, differential diagnosis of neuroblastoma and PPGL must continue to rely on a combination of considerations, including presentation and clinical features of the patient, imaging characteristics as well as whatever laboratory tests may be available.

BIOCHEMICAL TESTING IN DISEASE SURVEILLANCE PROGRAMS

While surveillance programs for neuroblastoma have all but disappeared, those for PPGLs are becoming increasingly important due to recognition of the high hereditability of the tumors. For families with identified germline mutations in tumor susceptibility genes, screening is recommended to start in childhood as early as 5 years for some gene mutations, such as for *succinate dehydrogenase subunit B* (*SDHB*) and *VHL*, that lead to activation of pseudohypoxia pathways (18, 19). A slightly later start at about 10

years is currently recommended for mutations of other pseudohypoxia genes and later still for mutations of genes impacting kinase signaling pathways, such as those encoding the rearranged during transfection proto-oncogene (*RET*) and the neurofibromatosis type 1 (*NF1*) gene (96, 97).

Since mutations of genes such as *VHL* and *SDHB* that result in activation of pseudohypoxia pathways lead to PPGLs that do not produce epinephrine, the focus of biochemical testing in children as in adults with these mutations should be on normetanephrine. However, for mutations of succinate dehydrogenase subunit genes, the resulting tumors often also produce methoxytyramine and sometimes only methoxytyramine (80). Therefore, for these children biochemical testing should include measurements of methoxytyramine, which for assessing tumoral dopamine production should involve measurements in plasma rather than urine (42). On the other hand, for children with mutations of *RET*, *NF1* or other genes that primarily impact kinase signaling pathways, interpretation of biochemical tests must include measurements of metanephrine since the associated tumors express PNMT and invariably produce epinephrine (80).

For all surveillance involving children, reference intervals established for pediatric populations are essential. With plasma measurements, use of reference intervals established for adults is likely to lead to false-negative results when applied to children over 5 years of age. This is particularly important for normetanephrine, which shows higher plasma concentrations in adults than in children (25).

FUTURE PERSPECTIVE

While biochemical tests of catecholamine excess in neuroblastoma are of limited importance for diagnosis compared to biopsy based histopathological diagnosis and molecular analyses, there are situations where biopsy is either not possible or may be contra-indicated. For such situations biochemical tests of catecholamine excess remain useful, but for the most part continue to rely on measurements of VMA and HVA, tests that have long been abandoned for PPGL. There are now better tests of catecholamine excess than urinary HVA and VMA, but it will likely take considerable time, if ever, before these will enter the clinical mainstream for neuroblastoma. For PPGL such tests have been widely available for the past two decades, but still need to be better integrated into pediatric care. The need for appropriate transition of pediatric to adult care for childhood cases of PPGL is another challenge, and particularly important in light of the high rate of disease recurrence and/or metastasis for tumors that originate in childhood. PPGL, however, remain rare tumors and patient management requires a depth of knowledge not within reach for most pediatricians who might first encounter affected children. Appropriate management of all patients with PPGL, but especially those in childhood, requires a multidisciplinary approach. This need is now recognized by patient-led organizations that are establishing procedures for review and accreditation of centers with appropriate resources and expertise. Through these and other efforts it is possible that earlier diagnosis of PPGL might be achieved and the true prevalence of childhood PPGL clarified. On the other hand, biochemical tests of catecholamine excess in children with neuroblastoma are likely to follow the trend of the past six decades and continue to remain sub-optimally based on historical precedence rather than more ideally on contemporary understanding.

AUTHOR CONTRIBUTIONS

GE drafted the first version of manuscript, while MP, NB and AH contributed to the editing and revision of the manuscript. All authors provided conceptual input and MP additionally assisted with compilation of data from the literature.

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Adrenalectomies in children and adolescents in Germany – a diagnose related groups based analysis from 2009-2017

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Background: Adrenalectomies are rare procedures especially in childhood. So far, no large cohort study on this topic has been published with data on to age distribution, operative procedures, hospital volume and operative outcome.

Methods: This is a retrospective analysis of anonymized nationwide hospital billing data (DRG data, 2009-2017). All adrenal surgeries (defined by OPS codes) of patients between the age 0 and 21 years in Germany were included.

Results: A total of 523 patient records were identified. The mean age was 8.6 ± 7.7 years and 262 patients were female (50.1%). The majority of patients were between 0 and 5 years old (52% overall), while 11.1% were between 6 and 11 and 38.8% older than 12 years. The most common diagnoses were malignant neoplasms of the adrenal gland (56%, mostly neuroblastoma) with the majority being younger than 5 years. Benign neoplasms in the adrenal gland (D350) account for 29% of all cases with the majority of affected patients being 12 years or older. 15% were not defined regarding tumor behavior. Overall complication rate was 27% with a clear higher complication rate in resection for malignant neoplasia of the adrenal gland. Bleeding occurrence and transfusions are the main complications, followed by the necessary of relaparotomy. There was an uneven patient distribution between hospital tertiles (low volume, medium and high volume tertile). While 164 patients received surgery in 85 different "low volume" hospitals (0.2 cases per hospital per year), 205 patients received surgery in 8 different "high volume" hospitals (2.8 cases per hospital per year; p<0.001). Patients in high volume centers were significant younger, had more extended resections and more often malignant neoplasia. In multivariable analysis younger age, extended resections and open

procedures were independent predictors for occurrence of postoperative complications.

Conclusion: Overall complication rate of adrenalectomies in the pediatric population in Germany is low, demonstrating good therapeutic quality. Our analysis revealed a very uneven distribution of patient volume among hospitals.

KEYWORDS

pediatric, neuroblastoma – diagnosis, therapy, adrenocortical adenocarcinoma, outcome, volume, adrenalectomia

Introduction

Adrenal neoplasia are overall rare, especially in childhood. They can arise from the adrenal cortex or the adrenal medulla with different histopathologic origin, distinct differences in dignity (benign/malignant), age distribution, prognosis and hormone production. In addition, in both localizations, several genetic underpinnings have been identified (1–4).

Patients present with a wide range of symptoms, from clinical signs of an unspecific abdominal swelling or recognized symptoms related to hormone overproduction to an incidental finding of an adrenal mass in radiography.

Neoplasms of the adrenal medulla are mostly neuroblastoma followed by pheochromocytoma and other neuroendocrine tumors, that may be found incidentally due to a lack of symptoms but typically present with symptoms such as sweating, flushing, weight loss etc. due to catecholamine excess. In the vast majority of cases, surgical treatment is necessary (1). Since neuroblastoma account for approx. 7% of childhood tumors and have their median age around the age of 2 (5), the majority of infant adrenal tumors can be assigned to neuroblastoma.

Among childhood neoplasms arising from the adrenal cortex, only around 20% are benign adenomas (ACA) with an excellent prognosis, whereas the majority are adrenocortical carcinoma (ACC) with malignant behavior. Pediatric ACC are predominantly hormone secreting and surgical complete resection is the only curative treatment option. Distinction between benign and malign tumors remains challenging (1, 4). There is no known specific age distribution of the tumor entities.

Regardless of the genetic background, age, hormone production and malignant behavior, adrenalectomy is a cornerstone within the multidisciplinary treatment algorithms. However, substantial differences exist between minimally invasive adrenalectomy for small, mostly benign adrenal tumors, and open extended resections for large malignant tumors infiltrating surrounding tissue. Dependent on the surgical approach, there are significant differences regarding postoperative morbidity as well as mortality rates.

For several decades, it has become increasingly obvious that individual surgeon volume and annual hospital case load clearly correlate with patients' postoperative outcome (6, 7). This has especially been proven for complex cancer surgery in adults (8, 9). Recent data from nationwide analysis also demonstrate a volume – outcome relationship for adrenalectomies of adults (10, 11). To date, it is unclear whether there is also a correlation between hospital volume and patient outcome for adrenal surgery in pediatric patients. Furthermore, no systematic analysis of complication rates and surgical approaches in pediatric patient undergoing adrenal surgery exists.

In this study, we used nationwide billing data to describe a cohort of patients below 21 years undergoing adrenalectomy. Primary outcome was occurrence of postoperative complications by patients' age, hospital volume as well as main diagnosis leading to adrenalectomy. Secondary outcome was, among others, was age distribution of malignant and non-malignant adrenal neoplasia within this cohort.

Methods

This is a register based, retrospective nationwide cohort-study on the basis of anonymized diagnose related groups (DRG) billing data provided by the "Statistische Bundesamt" (Federal Statistical Office in Germany) of all adrenal gland resections among children and adolescents performed between 2009 and 2017 in Germany. Hospital reimbursement in Germany is regulated by the DRG-system. Hospitals report the patients diagnoses (primary diagnose as well as potential postoperative complications) their performed medical procedures as well as discharge data to the incurrence. This data are also reported to the Federal Statistical Office.

Identification of patients for this study was done using OPS codes including "adrenal…" or "adrenal gland…". The resulting

ICD codes (Supplementary Table 1) led to the inclusion of ICD codes (Supplementary Table 2) after application of an in-detail bias analysis (ICD codes which were most likely the main diagnosis and led to an accompanying adrenal gland resection were excluded due to possible bias, Figure 1). In each individual case, the OPS codes (Supplementary Table 3) were hierarchized and the most radical procedure was defined as the main intervention (for procedural hierarchy see Supplementary Table 4). Primary nephral diagnoses (like C64, renal cell carcinoma) were not included due to high bias risk.

Relevant complication DRG coding was conducted on the basis of clinical relevance.

Data acquisition was conducted in close contact with the Research Center of the Federal Statistical Office *via* remote analysis (FDZ of the Federal Statistical Offices and the federal states; Data source: Diagnosis-Related Group Statistics (2009-17)) and in accordance with their guidelines for handling highly sensitive patient-record data. Each patient record was assigned an age, gender, an anonymized institute identifier, procedural codes (OPS), main and secondary diagnoses (ICD-10-GM), length of stay and reason for admission and discharge. Duplicates were identified and, in case of occurrence, one data set was chosen randomly to minimize bias. We only analyzed complete data records. ICD-codes for complications were identified among patients' secondary diagnoses.

All patient records of patients older than 21 years of age were excluded. Before this was done, the same identification criteria were applied to all DRG data and, as a result, all adrenalectomies were primarily identified, regardless of age. This is how this work can present "total number of adrenalectomies performed at the same hospital, including grown-ups" (see in Results section).

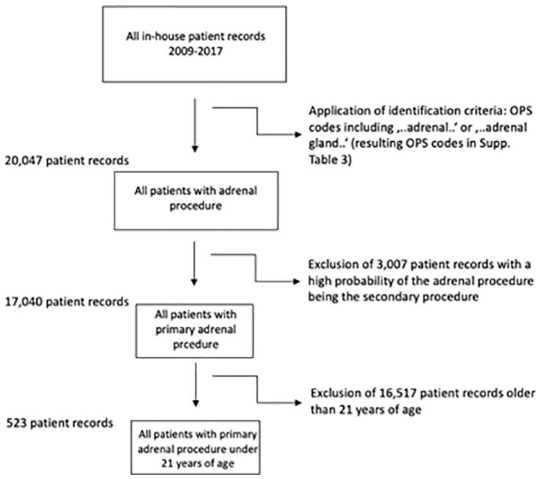

FIGURE 1
Inclusion criteria and flow-chart. Resulting ICD codes after application of OPS Codes in Supplementary Table 1, final ICD codes after exclusion of most likely secondary procedures in Supplementary Table 2.

Patient records were into three volume tertiles categories of approximately equal size based on their pooled number of adrenal gland resections between 2009 and 2017 (12, 13). For this the entire cohort is divided in three equally sized groups according to the annual hospital volume of pediatric adrenal resections.

Odds ratios (OR) were calculated as risk assessment between the primary dependent variable "hospital complication" and the primary independent variable "dignity" as well as secondary independent variables. A multivariable logistic regression model was used to analyze the relation between hospital tertile and hospital mortality while taking into account possible confounding variables. In order to account for different comorbidity structures, we used the comorbidity score first introduced by Stausberg et al. (14) whose validity has been affirmed in the German variant of the ICD-system. Eligible confounding variables including comorbidity, age and gender were controlled for. Trends were assessed using non parametric trends (15). We excluded the presence of significant multicollinearities among confounding variables. Using the Mantel-Haenszel method, relevant effect modification was checked for.

For the multivariable logistic regression model, the relation between hospital tertile and in-house mortality was determined while accounting for possible confounders and the clustered data structure treating the constant hospital identifier as random effect. Likelihood tests were used to assess the regression model accuracy. Refitting the models for different quadrature points and comparing the values of the estimators helped check the accuracy of the random effects estimators. A resulting maximum difference of $\leq 10^{-4}$ between the distinct quadrature points was accepted.

Graphs were generated using Prism (Version 9). Stata (Version 16; StataCorp LP, USA) was used for all statistical analysis. P-values of ≤ 0.05 were considered significant.

Results

A total of 20.047 patients with an adrenal surgical procedure code were (OPS 5-071 for partial and 5-072 for adrenalectomy, among others, details in Supplementary Figure 3) reported to the German Federal Statistical Office between 2009 and 2017, of whom 17.040 had a primary adrenal surgery due to a pathology within the adrenal gland (see Figure 1). Of these, 523 patients had an age below 21 years. In this cohort, the median age was 8.6 (± 7.7) years, 262 patients (50.1%) were female and roughly 56% had a malignant neoplasia of the adrenal gland. In total, 109 hospitals performed at least one adrenal surgery in the observation period, the mean length of stay was 11.9 (± 11.7) days, and 174 resection procedures (33.3%) were performed using a minimally invasive (laparoscopic/retroperitoneoscopic) approach.

The most frequent main diagnosis was benign neoplasia of the adrenal gland (n= 152, 29.1%; D350) followed by malignant neoplasia of the adrenal medulla (n=134; 25.6%; C741),

malignant neoplasia of the adrenal gland (not otherwise specified) (n=114, 21.8%; C749) and malignant neoplasia of the adrenal cortex (n=43, 8.2%; C740).

When analyzing the patient cohort according to the patient's age (see also Table 1), there were significant differences. With higher age, a clear shift towards female patients (p<0.001), a shorter length of stay (p<0.001) and a higher percentage of minimally invasive procedures (p<0.001) were observed. Also, the rate of malignant adrenal tumors decreased with increasing age (p<0.001).

As we observed significant differences within the distribution of malignant and non-malignant neoplasia, we divided the cohort accordingly. Patients with a malignant neoplasia were in average 10 years younger (mean 4.2 ± 5.3years; p<0.001)), were more often male (60.9%; p<0.001), had a higher comorbidity score (p<0.001) and a longer length of stay (p<0.001) (details are given in Table 2).

As surgery is one cornerstone within the multimodal treatment of patients with adrenal tumors, we analyzed the occurrence of postoperative complications. Overall, more than

TABLE 1 Patients ≤21 years of age: patient characteristics by age groups.

z No. of patients between the age of … years	0-1	2-5	6-11	12-17	18-21	P-value ‡
n	128 (24.5)	134 (25.6)	58 (11.1)	89 (17.0)	114 (21.8)	
no. of hospitals over the years 33.4 (25-38)	43	38	29	48	70	<0.001
length of stay (days)* 11.9 ±11.7	13.2 (16.8)	13.6 (10.3)	12.0 (8.7)	10.4 (8.1)	9.6 (9.1)	<0.001 n.p.
age in years* 8.6 ±7.7	0.4 (0.5)	3.0 (1.0)	8.5 (1.8)	15.1 (1.6)	19.6 (1.1)	<0.001 n.p.
comorbidity score #$_1$ *	101.9 (3.2)	102.7 (4.0)	101.9 (3.8)	99.8 (3.0)	98.7 (3.1)	<0.001 n.p.
minimally invasive procedure n (%)	25 (19.5)	12 (9.0)	13 (22.4)	46 (51.7)	78 (68.4)	<0.001
no. of females 262 (50.1)	54 (42.2)	51 (38.1)	30 (51.7)	49 (55.1)	78 (68.4)	<0.001
total no. of adrenalectomies (including adult patients) performed in same hospital	225.8 (206.0)	285.8 (214.3)	245.1 (222.1)	243.6 (287.5)	221.6 (284.7)	0.005
Exact diagnosis						<0.001
C740 (malignant, adrenal cortex) 43 (8.2)	14 (10.9)	13 (9.7)	5 (8.6)	6 (6.7)	5 (4.4)	
C741 (malignant, adrenal medulla) 134 (25.6)	52 (40.6)	52 (38.8)	16 (27.6)	9 (10.1)	5 (4.4)	
C749 (malignant, adrenal gland, n.o.s.) 114 (21.8)	42 (32.8)	56 (41.8)	n.s. (<10)	n.s. (<10)	3 (2.6)	
D350 (benign, adrenal gland) 152 (29.1)	11 (8.6)	7 (5.2)	19 (32.8)	50 (56.2)	65 (57.0)	
D441 (unknown dignity, adrenal gland) 41 (7.8)	n.s. (<10)	6 (4.5)	5 (8.6)	11 (12.4)	11 (9.7)	
other #$_2$ (n.s.) 39 (7.5)	n.s. (<3)	0	n.s. (<7)	n.s. (<10)	25 (21.9)	

Left pillar: total numbers and (%). *values are mean (± s.d.); ‡ Chi^2 test for difference between subgroups. n.p. for non-parametric tests. Details in Supplementary N.s. not stated due to German data law legislation (at least one group is n<3). #$_1$ Comorbidity score introduced by Stausberg et al. (14) and validated in the German ICD-10 system. #$_2$ 'other' combines other resulting main diagnoses (of which each single one was n<20, all ICD codes of the main diagnoses in this study in Supplementary Table 2) after application of OPS codes as identification criteria (Supplementary Table 3).

TABLE 2 Patients ≤21 years of age: patient characteristics by dignity.

Tumor dignity	malignant	benign	P-value ‡
n	294 (56.2)	229 (43.8)	
no. of hospitals over the years (n)	59	93	
length of stay (days) *	13.9 (13.7)	9.3 (7.6)	<0.001 n.p.
age in years *	4.2 (5.3)	14.4 (6.4)	<0.001 n.p.
comorbidity score *	102.4 (3.9)	99.3 (2.8)	<0.001 n.p.
Minimally invasive procedure n (%)	35 (11.9)	139 (60.7)	<0.001
no. of females	115 (39.1)	147 (64.2)	<0.001
average total no. of adrenal resections (including adult patients #$_1$) performed in same hospital	229.6 (273.8)	257.7 (217.6)	
Age groups (years)			<0.001
0-1 (128, 24.5%)	108 (36.7)	20 (8.7)	
2-5 (134, 25.6%)	121 (41.2)	13 (5.7)	
6-11 (58, 11.1%)	32 (10.9)	26 (11.4)	
12-17 (89, 17.0%)	17 (5.8)	72 (31.4)	
18-21 (114, 21.8%)	16 (5.4)	98 (42.8)	

*values are mean (± s.d.); ‡ Chi^2 test for difference between subgroups. n.p. for non-parametric tests. #$_1$ Adult patients (age >21) were not part of the study cohort but total adrenal gland resection volume per hospital was determined.

one quarter had at least one complication, which is mainly directly associated with the surgical procedure like postoperative bleeding. To note, patients with a malignant tumor had a significant risk to suffer from any postoperative complication; especially the need for blood transfusions (approx.40%; p<0.001) was significantly more common than in non-malignant tumors.

To analyze the impact of hospital volume on the postoperative outcome, we divided the entire cohort in three equally sized groups of low, medium and high-volume centers according to the hospital volume of pediatric adrenal resections over the entire study period (details are listed in Table 3). This categorization revealed 85 hospitals operated on 31.4% (n=164) of all patients defined as "low volume". Another 16 hospitals operating an 29,5% (n=154) patients defining as "medium volume" and 8 hospitals operating 39.3% (n=205) of all patients defined as "high volume" This means that, on average, each low volume hospital performed two pediatric adrenal gland resections over the course of 9 years. In addition, the overall caseload of adrenal resections regarding all age groups (including adult patients) in the eight high volume centers was significantly higher than in the low volume centers. Patients in high volume centers were significantly younger (p<0.001), were more likely to have a malignant neoplasia (p<0.001) and underwent more extended (p<0.001) and open resections (p=0.015). For example, while less than 10% of patients underwent an extended resection in a low volume center, approximately one third of patients underwent an extended resection in a high volume center. In line with this

TABLE 3 Patients ≤21 years of age: patient characteristics by hospital tertiles.

No. of patients who had surgery in a hospital with a patient volume in the…	low volume tertile	medium volume tertile	high volume tertile	P-value ‡
No. of patients (523)	164 (31.4)	154 (29.5)	205 (39.2)	
No. of hospitals in total (33.4 annually on average between 2009 and 2017)	85	16	8	
average total no. of adrenal resections (including adult patients #$_1$) performed in same hospital	66.1 (44.9)	182.7 (91.3)	435.9 (281.4)	<0.001 n.p.
Length of stay (11.9 ±11.7)*	10.9 ± 11.6	11.6 ± 9.1	13.0 ± 13.3	0.057 n.p.
Age (8.6 ±7.7)*	11.3 ± 8.0	8.6 ± 7.7	6.5 ± 6.8	<0.001 n.p.
Age groups (years)				<0.001
0-1 (128, 24.5%)	34 (20.7)	38 (24.7)	56 (27.3)	
2-5 (134, 25.6%)	22 (13.4)	42 (27.3)	70 (34.2)	
6-11 (58, 11.1%)	17 (10.4)	12 (7.8)	29 (14.2)	
12-17 (89, 17.0%)	32 (19.5)	31 (20.1)	26 (12.7)	
18-21 (114, 21.8%)	59 (36.0)	31 (20.1)	24 (11.7)	
No. of females (262, 50.1%)	96 (58.5)	74 (48.1)	92 (44.9)	0.028
Comorbidity score (101.0, ±3.8)*	100.1 ± 3.0	100.7 ± 3.2	102.1 ± 4.4	<0.001 n.p.
Exact diagnosis				<0.001
C740 (malignant, adrenal cortex)	13 (7.9)	12 (7.8)	18 (8.8)	
C741 (malignant, adrenal medulla)	29 (17.7)	30 (19.5)	75 (36.6)	
C749 (malignant, adrenal gland, n.o.s.)	25 (15.2)	36 (23.4)	53 (25.9)	
D350 (benign, adrenal gland)	63 (38.4)	52 (33.8)	37 (18.1)	
D441 (unknown dignity, adrenal gland)	13 (7.9	15 (9.7)	13 (6.3)	
other (n.s.)	21 (12.8)	9 (5.8)	9 (4.4)	
Main procedure				0.019
Adrenalectomy (352, 67.3%)	121 (73.8)	100 (64.9)	131 (63.9)	
Partial adrenalectomy (46, 8.8%)	12 (7.3)	8 (5.2)	26 (12.7)	
other (125, 23.9%)	31 (18.9)	46 (29.9)	48 (23.4)	
Main procedure performed was.				0.015
. minimally invasive (174, 33.3%)	69 (42.1)	46 (29.9)	59 (28.8)	
. open (349, 66.7%)	95 (57.9)	108 (70.1)	146 (71.2)	
Overall resection…				<0.001
of adrenal gland only (413, 79.0%)	149 (90.9)	123 (79.9)	141 (68.8)	
with an accompanying resection of other organ(s) (110, 21.0%)	15 (9.1)	31 (20.1)	64 (31.2)	
Overall complication(s) (141, 27.0)	27 (16.5)	32 (20.8)	82 (40.0)	<0.001

Left pillar: total numbers in (). *values are mean (± s.d.); ‡ Chi^2 test for difference between subgroups. n.p. for non-parametric tests. Details in Supplementary Table 5. #$_1$ Adult patients (age >21) were not part of the study cohort but total adrenal gland resection volume per hospital was determined.

differences, more complications occurred in the high volume centers.

When analyzing the occurrence of complication, in line with the unequal distribution of age and malignant disease between the hospital volume we observed more frequent overall as well as surgery associated complications within the high-volume centers (Table 5).

In a crude analysis, age category, dignity, operative procedure, including minimally invasive and extended resections, and hospital volume were significantly associated with occurrence of postoperative complications (see Table 4, 5). They were therefore considered as potential confounders and included in a regression analysis. In a multivariable regression analysis, accounting for patient clustering within institutions and the effect of confounding variables, a highly significant decrease was found in postoperative complication rate following adrenal resection for patient age (p=0.046 and open procedure (p=0.001), while there was no difference regarding the annual caseload (see Table 6).

Discussion

This is the first nationwide analysis for adrenal resections within patients below 21 years. We demonstrate that there are two distinct groups of patients. First, patients with malignant neoplasia who are predominant male and younger. Second, patients with benign adrenal tumors who are more likely female and 6 years or older. The other important observation is, that there are 109 hospitals overall in Germany performing adrenalectomy in patients age blow 21.

The described cohort as well as the age distribution of benign and malignant adrenal tumors are in line with data from several

TABLE 4 Patients ≤21 years of age: complications.

No. of patients	malignant	benign	P-value
n	294 (56.2 of total)	229 (43.8 of total)	
Total of patients with complication(s) 168 (32.1)	138 (46.9)	30 (13.1)	<0.001
…. of which n had surgical complication(s) 154 (29.5)	132 (44.9)	22 (9.6)	<0.001
Bleeding occurrence 100 (19.1)	87 (29.6)	13 (5.7)	<0.001
Transfusion occurrence 129 (24.7)	116 (39.5)	13 (5.7)	<0.001
Relaparotomy occurrence 20 (3.8)	14 (4.8)	6 (2.6)	0.205
…. or other complication(s) 51 (9.8)	39 (13.3)	12 (5.2)	0.002
Endocrine insufficiency (4, 2.8)	n.s.	n.s.	0.207
Artificial respiration > 48h occurrence 19 (3.6)	n.s.	n.s.	0.003
Peritonitis/sepsis occurrence 21 (4.0)	17 (5.8)	4 (1.8)	0.020
Pneumonia occurrence 5 (1.0)	n.s.	n.s.	0.864
Acute Kidney Injury occurrence 11 (2.1)	n.s.	n.s.	0.084
Clostridium difficile occurrence 12 (2.3)	n.s.	n.s.	0.012

(Percentages of column). P values stem from Chi^2 test for difference between subgroups. N.s. not stated due to German data law legislation (at least one group is n<3).

TABLE 5 Patients ≤21 years of age: complications by patient volume.

No. of patients	low volume tertile	medium volume tertile	high volume tertile	P-value
n (%)	164 (31.4)	154 (29.5)	205 (39.2)	
Total of patients with complication(s) 141 (27.0)	27 (16.5)	32 (20.8)	82 (40.0)	<0.001
…. of which n had surgical complication(s) 127 (24.3)	23 (14.0)	26 (16.9)	78 (38.1)	<0.001
Bleeding occurrence 100 (19.1)	17 (10.4)	19 (12.3)	64 (31.2)	<0.001
Transfusion occurrence 129 (24.7)	28 (17.1)	31 (20.1)	70 (34.2)	<0.001
Relaparotomy occurrence 20 (3.8)	n.s.	n.s.	12 (5.9)	0.070
…. or other complication(s) 48 (9.2)	11 (6.7)	10 (6.5)	27 (13.2)	0.040
Endocrine insufficiency (4, 2.8)	n.s.	n.s.	n.s.	0.905
Artificial respiration > 48h occurrence 19 (3.6)	n.s.	n.s.	15 (7.3)	0.001
Peritonitis/sepsis occurrence 21 (4.0)	4 (2.4)	3 (2.0)	14 (6.8)	0.031
Pneumonia occurrence 5 (1.0)	n.s.	n.s.	n.s.	
Acute Kidney Injury occurrence 11 (2.1)	n.s.	n.s.	7 (3.4)	0.244
Clostridium difficile occurrence 12 (2.3)	4 (2.4)	4 (2.6)	4 (2.0)	0.911

(Percentages of column). P values stem from Chi^2 test for difference between subgroups. N.s. not stated due to German data law legislation (at least one group is n<3).

TABLE 6 Crude Odds Ratios and multiple regression model.

	Unadjusted OR for in-hospital complication		Multivariable logistic regression model for in-house complication	
	Odds Ratio [95% CI]	P-value	Odds Ratio [95% CI]	P-value
Age (years)				
0-1	1		1	
2-5	1.36 [0.83-2.23]	0.229	1.01 [0.57-1.79]	0.970
6-11	0.55 [0.27-1.10]	0.093	0.37 [0.16-0.86]	0.021
12-17	0.29 [0.15-0.59]	0.001	0.42 [0.18-0.98]	0.046
18-21	0.18 [0.09-0.38]	<0.001	0.40 [0.16-0.98]	0.046
Sex				
female	1		1	
male	1.21 [0.83-1.79]	0.315	0.88 [0.54-1.42]	0.588
Dignity				
benign or unknown	1		1	
malignant	3.99 [2.55-6.23]	<0.001	0.62 (0.31-1.22)	0.167
Procedure				
Adrenalectomy	1		1	
Partial adrenalectomy or other	0.48 [0.21-1.11]	0.085	0.97 [0.75-1.26]	0.825
Surgery was…				
minimally invasive	1		1	
open	6.78 [3.77-12.21]	<0.001	3.38 [1.70-6.71]	0.001
Overall resection…				
with an accompanying resection of other organ(s)	1		1	
of adrenal gland only	0.17 [0.11-0.27]	<0.001	0.30 [0.18-0.52]	<0.001
Comorbidity score (incremental)	1.33 [1.25-1.43]	<0.001	1.21 [1.12-1.30]	<0.001
Hospital Tertile				
low volume	1		1	
medium volume	1.38 [0.79-2.43]	0.259	0.82 [0.43-1.58]	0.559
high volume	3.52 [2.14-5.79]	<0.001	1.78 [0.98-3.22]	0.058

ORs are unadjusted and adjusted and are listed solely for primary dependent variable in-house complication. Overall rho = $3.81xe^{-6}$ (Confidence Interval $6.05xe^{-22}$ - 1).

large registries, also demonstrating an unequal distribution of age and dignity of adrenal neoplasia (1, 4, 16).

This uneven age distribution may be due to the fact that pediatric malignant neoplasms of the adrenal gland appear to originate in the fetal zone, and as expected for neoplasms of developing tissue, the median age at diagnosis is very young. This developmental pathway is already well known for neuroblastoma, an embryonal tumor of the autonomic nervous system, but embryonal pathogenesis of adrenocortical carcinoma in early childhood has been discussed by several authors as well. Furthermore, malignant childhood adrenal tumors are often associated with genetic alterations and tumor predisposition syndromes, such as Li-Fraumeni syndrome, leading to early onset of neoplasms (1, 4, 16, 17). Fortunately, the total perioperative mortality rate was below 1.5%. As was expected, patients with malignant neoplasia and younger patients were less likely to undergo minimally invasive resection, and this group also had a significant higher complication rate. These results are in line with billing data of the adult cohort. The aggressive growth of malignant tumors may lead to higher perioperative complications, as, for example, the increased risk of bleeding occurrence, being secondary to the tendency of malignant neoplasms to infiltrate blood vessels and nearby organs (11). Nevertheless, when analyzing billing data for occurrence of complications, some bias has to be taken into account. The available billing data reflect procedures and complications within one hospital stay, but do not identify the exact causality for potential complications. This is the case, for example, when the need of a blood transfusion is a complication of the operative procedure or when patients were initially diagnosed with anemia, for example, due to previous (chemo-) therapy, received a transfusion, and underwent adrenalectomy during the same hospital stay. This should be particularly noted when interpreting the significant higher transfusion rate within patients with malignant neoplasia. Also the high rate of documented "complications" within high volume centers could be explained by the unequal distribution of patients. For example patients within high volume centers are more likely to undergo

extended resections due to maligned disease, which per se will bias this point. This is supported by the fact, that when accounting for confounders within the multivariate analyze the Odds ratio for occurrence of complication does not differ according to hospital volume. In light of

Surprisingly, we discovered a highly unequal distribution of hospital case load, which is even more surprising considering adrenal resections are overall rare procedures. In the "low volume" tertile, there was roughly one case below 21 years within five years of the study, whereas high volume centers performed approximately 3 adrenal resections per year. In light of actual recommendations of ENSAT and other international experts, an annual case load of more than 20 adrenalectomies per year is recommended (1, 18–20). It is therefore incomprehensive why some of the patients in the study cohort have not been transferred to a high-volume center. Several studies of adult patients have demonstrated that, especially for adrenal resections, there is a significant volume-outcome correlation. From an economic point of view, high volume goes hand in hand with relevant cost savings (10, 11).

In light of the overall low mortality rate, the "small" sample size and the highly diverse cohort, we did not identify a significant volume – outcome relationship. Nevertheless, it has to be discussed if patients will benefit from a centralization of rare and complex surgical procedures within few, highly competent centers. First, these hospitals should be experienced with adrenal gland tumors as well as be equipped with highly specialized adrenal surgical units, including for pediatric patients.

The main strength of this study is the sample size of more than 500 patients with adrenal resections below 21 years of age, the completeness of data and the adjustment for confounding factors as age, malignant processes and co-morbidity.

Major limitations of this analysis are missing data of individual surgeon volume and individual surgeons' expertise and surgeons specialty (pediatric/endocrine/general) on the postoperative outcome. Furthermore, no information was available on tumor stage, tumor size, exact histopathological phenotype, hormone production, extension of resection and long-term survival of patients. Another limitation is the lack of readmission data, as the statistics include only individual cases per hospital and readmission is not accounted for. In addition, as stated above, the exact causality for potential complications such as blood transfusions could not be derived from the DRG-data set. Furthermore, the exact diagnosis remained unclear in some patients (especially C749 and D441).

In conclusion, we demonstrated that, overall, surgery for adrenal mass in children and adolescents is a save procedure. There are two groups of patients undergoing an adrenal resection with distinct profiles, younger patients with an underlying malignant disease and older patients who are less likely to suffer from maligned disease. Since tumors of the adrenal gland are overall rare and the dignity of the adrenal mass often remains unclear until definitive histopathological examination, our data suggest a treatment of these patients in few, highly specialized centers.

Data availability statement

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

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

All authors revised the manuscript and finally approved the manuscript. KU, data acquisition and statistical analysis. VW and AW, study design. MR, AW, and VW, writing the manuscript. MR, JR, TM, CG, MF, and AW, critical discussion and data interpretation. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

The handling editor CP declared a past co-authorship with the author MF.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.914449/full#supplementary-material

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Adrenal hyperplasias in childhood: An update

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Pediatric adrenocortical hyperplasias are rare; they usually present with Cushing syndrome (CS); of them, isolated micronodular adrenal disease and its variant, primary pigmented adrenocortical disease are the most commonly encountered. Most cases are due to defects in the cyclic AMP/protein kinase A (cAMP/PKA) pathway, although a few cases remain without an identified genetic defect. Another cause of adrenal hyperplasia in childhood is congenital adrenal hyperplasia, a group of autosomal recessive disorders that affect steroidogenic enzymes in the adrenal cortex. Clinical presentation varies and depends on the extent of the underlying enzymatic defect. The most common form is due to 21-hydroxylase deficiency; it accounts for more than 90% of the cases. In this article, we discuss the genetic etiology of adrenal hyperplasias in childhood.

KEYWORDS

adrenal cortex, Cushing syndrome, adrenal hyperplasia, congenital adrenal hyperplasia, childhood tumors

Introduction

Adrenocortical tumors (ACTs) are rare in childhood and adolescence (1). The majority of them (approximately 90%) are functional and thus they can present with hirsutism, acne, rapid clitoral or penile enlargement, sexual hair growth as well as increased height velocity (2). Usually, single tumors are benign unilateral adenomas but they can be malignant in rare cases and physicians should be suspicious of that as an early diagnosis impacts overall survival. Less often, patients can present with benign multinodular hyperplastic lesions including primary pigmented nodular adrenocortical disease (PPNAD), non-pigmented isolated micronodular adrenal disease (iMAD), and corticotropin (ACTH)-independent macronodular adrenal hyperplasia (AIMH) (3). These diseases usually present with cortisol excess or Cushing syndrome (CS). On the other hand, deficits of the steroidogenic enzymes of the adrenal cortex, also cause adrenal

hyperplasia but they are usually associated with decreased cortisol synthesis. These diseases are known collectively as "congenital adrenal hyperplasia" or CAH, are all autosomal recessive and have a variable clinical presentation. We review in this report both types of adrenal hyperplasias and any updates on their molecular genetics.

Adrenal gland: Embryology and early development

The human adrenal gland arises from two different embryological tissues: the cortex develops from the intermediate mesoderm of the urogenital ridge, while the medulla develops from the neural crest-derived chromaffin cells (4). The earliest recognizable form of the adrenal gland is called 'adrenal primordium' and appears at 28-30 days post conception (dpc); it is marked by the expression of steroidogenic factor-1 (SF1; also known as Ad4BP or NR5A1) which is essential for the adrenal development and steroidogenesis (5, 6). By the 7^{th}-8^{th} week of fetal development, the developing adrenal cortex delineates two distinct components, the inner fetal zone (FZ) -that comprises 80-90% of the cortical volume- and the outer definitive zone (DZ) (5, 7). By the 9^{th} week of gestation, the developing adrenal gland becomes completely encapsulated (8). The FZ cells express cytochrome P450 17α (CYP17A1), an enzyme that has both 17 hydroxylase and 17,20 lyase activity and converts pregnenolone to dehydroepiandrosterone (DHEA). At this stage, DHEA is the main product produced at ZF; later, it undergoes conversion to estrogens by the placenta for the maintenance of normal pregnancy.

By the end of the second trimester, the transitional zone (between FZ and DZ) expresses *HSD3B2*, and thus cortisol synthesis is initiated in the fetus (9). By late gestation, the DZ has begun differentiating into the zona glomerulosa (ZG) and zona fasciculata (ZF). Soon after birth, the FZ undergoes regression due to increased apoptotic activity, resulting in decrease in the weight of the adrenal glands by approximately 50% (2, 10). Zona reticularis (ZR) begins to form later in childhood; around the age of 6-8 years in females and 7-9 years in males. This process is known as adrenarche and is characterized by the production of adrenal androgens (11). Medulla is not recognized as a distinct structure with the exception of small clusters of chromaffin cells scattered throughout the cortex, until after birth (12).

Hypothalamic-pituitary-adrenal axis: Glucocorticoid secretion

Glucocorticoid secretion is regulated by the HPA axis. The most important components of the HPA axis include: 1) the corticotropin-releasing hormone (CRH)- whose actions are mediated by two receptors, CRHR1 and CRHR2- which is released, along with vasopressin, from the paraventricular nucleus of the hypothalamus and it is carried to the anterior pituitary where it stimulates 2) adrenocorticotropic hormone (ACTH) secreted from the anterior lobe of the pituitary gland, and 3) cortisol that is produced by the adrenal cortex in response to the binding of ACTH to the melanocortin type-2 receptor (MC2-R) in the ZF. The HPA axis is subject to the negative feedback cycle from the glucocorticoids; circulating cortisol inhibits primarily CRH and secondarily ACTH (13).

Renin-angiotensin-aldosterone system: Mineralocorticoid secretion

RAAS is a vital regulator of systemic vascular resistance and blood pressure, by increasing vascular tone, water and sodium reabsorption. It has three main components that include renin, angiotensin II and aldosterone. Renin, a protease synthesized in the juxtaglomerular cells of the kidney, cleaves angiotensinogen -synthesized in the liver- to the biologically inactive angiotensin I (AngI). Following that, AngI is converted to AngII by the angiotensin converting enzyme (ACE), which is found primarily in the vascular endothelium of the lungs and kidneys. AngII acts by activating AngII type 1 (AT1) receptors in the adrenal cortex, kidneys and arterioles, resulting in aldosterone release, vasoconstriction, and blood pressure increase. Release of AngII -the major stimuli of RAAS activation- is triggered by intravascular volume depletion and hyperkalemia.

Adrenal androgen secretion

The ZR in the adrenal glands produces the precursor steroids dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS) which are later converted, in the peripheral tissues, to potent androgens and estrogens (14, 15). Their secretion is partially regulated by ACTH; however, in multiple instances, the exact mechanism has not been elucidated yet.

Adrenal hyperplasias

Benign adrenocortical tumors producing cortisol.

Etiology

The vast majority of cortisol-producing adrenal hyperplasias and tumors harbor defects in the cAMP/PKA signaling pathway (16). Under normal circumstances, in the adrenocortical cells, ACTH binds to MC2R (a G protein-coupled receptor) that causes an increase of the cAMP levels and thus activation of

the PKA. PKA phosphorylates various transcription factors including cAMP response element binding protein (CREB), steroidogenic factor 1 (SF-1) and activating transcription factor 1 (ATF-1). Following that, these transcription factors bind to the cAMP response element (CRE) in the nucleus and regulate the expression of genes important in steroidogenesis (17–20).

Primary bilateral adrenal hyperplasias

This group of disorders can be categorized into two groups based on the size of the associated nodules: macronodular hyperplasias (BMAH, nodule size ≥1cm), which more commonly affect older adults and micronodular hyperplasias (MiBAH, nodule size <1cm) that occur more frequently in children and young adults. The latter includes primary pigmented adrenocortical nodular disease (PPNAD) and isolated micronodular adrenal disease (iMAD) (21). Two additional characteristics used for the classification of BAHs include the pigment that is present and whether there is atrophy or hyperplasia of the surrounding cortex tissue (22). The pigment most of the times is lipofuscin and macroscopically is seen as light to dark brown or, rarely, black (22).

Primary pigmented adrenocortical nodular disease

PPNAD in children and adolescents has been linked to periodic or atypical CS (23–25). In PPNAD the adrenal glands appear to have multiple cortisol-secreting pigmented nodules usually <4-6mm in diameter. PPNAD can present as sporadic or familial; most commonly it presents as familial PPNAD as part of Carney complex (CNC, OMIM#160980). CNC, that was first described in 1985 (26), is a tumor predisposition syndrome that presents with multiple endocrine tumors in addition to lentigines and myxomas (21, 27). In 2000, germline inactivating variants in the *PRKAR1A* gene which encodes for the regulatory type 1α-subunit of PKA, mapped on the 17q22-24 chromosomal region, was identified as the causative gene in the majority of CNC cases (28–30). These variants lead to increased activity of the PKA. More genetic alterations in the cAMP/PKA signaling pathway have been identified over the last two decades in patients with MiBAH (31–33).

Cushing syndrome

Cushing syndrome (CS) can be exogenous (or 'iatrogenic') when glucocorticoids are used in high doses for a prolonged period of time or endogenous due to overproduction of cortisol either by an ACTH-producing pituitary tumor (also known as Cushing disease -CD-) or by an ACTH-independent ACT. Endogenous CS has an incidence of 0.7-2.4 per million people per year (34); in children BAHs as etiologies of CS are much more common than in adults (23).

Clinical presentation

In most cases, CS onset is slow and gradual (35). The hallmark of CS in childhood is increasing weight with growth velocity deceleration. Other common presenting symptoms include headaches, facial plethora, hypertension, amenorrhea, hypogonadism and hirsutism (Table 1) (35–39). Virilization is not often present except for cases that the tumor produces androgens. Additional manifestations from skin such as abdominal striae, acne and bruising may also occur often.

Diagnosis

Diagnostic evaluation should be prompted in patients with high suspicion (Figure 1) (41, 42). Medical history and clinical evaluation, including growth charts in children, are vital in making the initial diagnosis. According to the Endocrine Society guidelines on the diagnostic workup of CS, after excluding exogenous glucocorticoid exposure, cortisol levels should be measured (43). Increased cortisol levels are confirmed with 24-hour urinary free cortisol (UFC) test, late-night salivary cortisol and/or a low-dose dexamethasone suppression test (1mg overnight or 2mg/day over 48 hours). The diagnostic accuracy is not 100% in any those tests and thus, usually multiple tests may be needed in order to establish the diagnosis. In some cases, falsely high UFC (known as pseudo-CS) may be obtained due to severe obesity, depression, pregnancy, alcoholism, chronic exercise, anorexia, anxiety, malnutrition or excessive water intake (>5L/day) (22). Once the presence of endogenous CS has been confirmed, ACTH is

TABLE 1 Phenotypic manifestations of pediatric Cushing syndrome.

Organ system	Manifestations
Cardiovascular	Hypertension, coagulopathy
Gonadal	Amenorrhea, virilization, gynecomastia
Skin	Acne, acanthosis nigricans, easy bruising, hirsutism, fine hair, striae
CNS	Anxiety, mood swings, depression, fatigue
Other	Facial plethora, supra-temporal and supra-clavicular fat pads, bone fractures, impaired glucose tolerance, nephrolithiasis

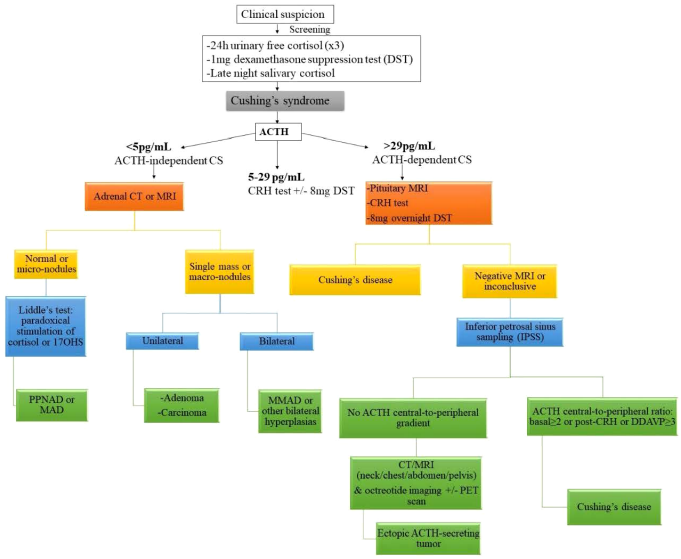

FIGURE 1
Diagnostic algorithm for suspected Cushing syndrome. ACTH adrenocorticotropic hormone, CS Cushing syndrome, CT computed tomography, DDAVP desmopressin, MAD micronodular adrenal disease, MRI magnetic resonance imaging, PET positron emission tomography, 17OHS 17-hydroxysteroid. Reprinted from Constantine A. Stratakis. Cushing Syndrome in Pediatrics. Endocrinology and Metabolism Clinics of North America. Volume 41, Issue 4, December 2012, Pages 793-803, with permission from Elsevier.

used to differentiate between ACTH-dependent and ACTH-independent CS. If ACTH levels are <5pg/mL, then ACTH-independent disease is suggested, while ACTH levels ≥29pg/ml have approximately 70% sensitivity in identifying ACTH-dependent disease in children (35, 43). Following that, high dose dexamethasone suppression test (Liddle test) is used to differentiate between CS due to adrenal causes or CD from ectopic ACTH secretion (44).

When bilateral adrenocortical hyperplasia is suspected then low-dose dexamethasone test (30ug/kg/dose every 6 hours for 8 doses) followed by high-dose dexamethasone test (120 ug/kg/dose every 6 hours for 8 doses) is performed. However, the diagnosis of CS due to PPNAD can be challenging as often has a cyclical or atypical presentation (e.g. normal or near normal 24-hour UFC). What is very useful though is that these patients appear to have a counterintuitive increase in the production of cortisol in response to high-dose dexamethasone through a PKA-mediated mechanism; this results from the overexpression of glucocorticoids receptors on the adrenocortical cells (45). For distinction from pituitary tumors causing CS, diagnostic imaging may be required, including pituitary magnetic resonance imaging (MRI) (46).

Isolated micronodular adrenocortical disease

iMAD, like PPNAD, can cause CS usually of mild to moderate severity and it can be cyclical (24, 47). It usually occurs earlier than PPNAD and presents during infancy and childhood (2).

Underlying genetic defects include inactivating variants in phosphodiesterases 11A (*PDE11A*) and 8B (*PDE8*) (48–50). *PDE11A* is located on chromosome 2q31.2, encoding a phosphodiesterase that degrades both cAMP and cGMP. It is expressed in several endocrine tissues, including the adrenal cortex (51). Thus far, five inactivating variants in *PDE11A* have been identified, three in patients with iMAD and two in patients with PPNAD (31, 32). Out of these variants, three lead to introduction of premature termination codons, whereas the other two disrupt the catalytic domain of PDE11A. Thus, all variants resulted in compromised cAMP degradation, supporting the notion that aberrantly hyperactive cAMP -dependent signaling has a causative role in the development of adrenocortical tumors. Notably, patients harboring such inactivating variants also had decreased *PDE11A4* expression (52).

Out of the cAMP-specific PDEs, the one with the highest expression in the adrenal gland is PDE8B (53), and missense variants in *PDE8B* have been associated with adrenocortical tumors; the mutant forms of PDE8B have compromised ability to degrade cAMP, resulting in abnormally elevated PKA activity. Specifically, a study of 84 patients with adrenal tumors but no identified variants in *PRKAR1A*, *PDE11A* or *GNAS*, discovered a p.H391A substitution in PDE8B (54). In addition, a p.H305P substitution was found in a two year old girl with CS and iMAD (32). The variant was transmitted to the patient from her father. However, the father's phenotype was

characterized as normal/very mild, echoing findings in female patients who inherited PDE11A variants from unaffected fathers.

ACTH-independent macronodular adrenal hyperplasia

AIMH/BMAH presenting with CS is more common in older adults and are usually sporadic (47); however, a few familial cases that have been reported appear to be in children (22). It develops progressively over the years with subclinical hypercortisolism (47, 55). In addition, BMAH can rarely occur as part of McCune-Albright syndrome (MAS) where it appears in the infantile period (<6 months old); in a few cases CS spontaneously resolved (24, 56). MAS is due to somatic activating variants in the *GNAS* gene, that encodes the α-subunit of the Gs protein, causing constant non-ACTH-dependent adrenal cortex stimulation (57).

Congenital adrenal hyperplasias

CAHs is a group of disorders of defective steroidogenesis inherited in an autosomal recessive manner (58). Inactivating variants in genes encoding enzymes that participate in the cortisol synthesis pathway result in a broad spectrum of disease severity; impaired synthesis of cortisol leads to persistently high levels of ACTH *via* negative feedback, overstimulation of the adrenal cortex and eventually adrenal hyperplasia and oversecretion of the precursors of the enzymatic defect. When the enzymatic defects are complete or almost complete overt enzymatic insufficiency ensues and those defects are known as 'classic' CAH. The 'non classic' CAH (NCCAH; also termed 'late onset') tends to be milder as the enzymatic defects are partial and patients retain some cortisol and aldosterone production. In most cases, patients with NCCAH harbor either two non-classic alleles or one non-classic and one classic, meaning that when two individuals with NCCAH have kids their risk of having a child with classic CAH is approximately 1.5-2.5% (59, 60).

The prevalence of the classic forms is 1:16,000 while for the non-classic forms is approximately 1:2,000 in the Caucasian population in the United States with a higher frequency in Hispanics, Eskimos, people of Mediterranean or Middle-Eastern descent and Ashkenazi Jews (61–65).

21-hydroxylase deficiency

21OHD (OMIM#201910, *CYP21A2*) accounts for approximately 95% of CAH cases (61, 66, 67). The enzyme 21OH catalyzes the conversion of 17-hydroxyprogesterone to 11-deoxycortisol in the ZF and progesterone to 11-deoxycorticosterone (DOC) in ZG. *CYP21A2* is located on chromosome 6p21.3, within the human leukocyte antigen (HLA) locus. About 30kb apart, the same locus harbors a non-functional highly homologous pseudogene (*CYP21A1P*). Because of that, recombination frequently occurs in the region; these recombination events are responsible for most of the *CYP21A2* inactivating variants in patients.

21OHD presents with a wide spectrum of phenotypes. Increased ACTH secretion due to inadequate production of cortisol leads to accumulation of 17-hydroxyprogesterone and progesterone. As a result, female infants with classic 21OH deficiency are exposed to excess androgens *in utero*; at birth, they present with virilization of the genitalia, including rugated or partially fused labia majora, genital hyperpigmentation, enlarged clitoris and a vagina that opens into a common urogenital sinus -like in males- while the ovaries, fallopian tubes and the uterus are normal (61). Males have minimal findings, including genital hyperpigmentation and macrogenitosomia or no physical findings of the disease (61, 68).

Linear growth is also affected as patients are at risk for centrally mediated precocious puberty due to prolonged exposure to high levels of androgens. Data from 18 centers demonstrated that their adult height is on average 1.4SD below the mean compared to the general population (69). Early diagnosis and treatment compliance are vital as both overtreatment and undertreatment increase the risk for short stature, either due to excess glucocorticoid-induced inhibition of the growth axis or due to premature epiphyseal closure (70, 71).

Approximately 75% of classic CAHs represent life-threatening salt-wasting forms and they are a result of large gene conversions, nonsense or frameshift mutations or complete deletions leading to complete absence of 21OH activity. Production of mineralocorticoids and glucocorticoids is ceased resulting in life-threatening adrenal crises in the first two weeks of life (72). When CAH is suspected, then aldosterone, serum electrolytes and plasma renin should be measured. The expected abnormalities are low aldosterone levels, hyperkalemia and elevated renin; however, because renin levels are age-specific appropriate references should be used (73). On the other hand, the NCCAH forms are more often associated with missense variants and they retain some enzymatic activity and thus are able to maintain a relatively normal amount of aldosterone and cortisol at the expense of mild to moderate excess of sex hormone precursors salt balance (72); they present with premature pubarche during childhood; however cases have been described as early as six months old (74). Females most commonly present during adolescence with hirsutism, acne and menstrual abnormalities or infertility, findings similar to polycystic ovarian syndrome (PCOS) while males often remain undiagnosed until they undergo pre-conception genetic screening or after having an affected offspring (74).

CAH-tenascin-X syndrome

Some of the deletions responsible for CAH also lead to Ehlers-Danlos syndrome (hypermobility type). This occurs when the deletion encompasses both *CYP21A2* and *TNXB* (which encodes tenascin-X; a protein of the extracellular matrix). In such cases, the resulting contiguous gene deletion syndrome is called CAH-X syndrome. Overall, CAH-X is estimated to have a prevalence of 9% among CAH patients.

More specifically, there exist 3 subtypes of CAH-X: a) CAH-X CH-1, associated with deletion of exon 35 of TNXB; b) CAH-X CH-2, caused by the c.12174 C>G (p.Cys4058Trp) variant in *TNXB*; and c) CAH-X CH-3, which is caused by a cluster of 3 variants. The mechanism of two latter subtypes involves dominant negative effects.

CAH due to other, less common defects in steroidogenic enzymes

17α-hydroxylase deficiency

17α-hydroxylase (CYP17A1) catalyzes two reactions, the 17-hydroxylase and the 17,20-lyase reaction. Pathogenic variants in *CYP17A1* (including splice site alterations, point mutations, small insertions/deletions, and in rare cases, large deletions) result in impairment of both reactions.

Complete 17OHD (OMIM#202110) leads to disrupted steroidogenesis in both the adrenals and the gonads, as it is expressed in both, leading to puberty failure. In addition, both 46,XX and 46,XY will present with female external genitalia due to absent testosterone and dihydrotestosterone; however, 46,XY individuals, due to preservation of anti-Mullerian hormone from the testes do not have an internal Mullerian structure (75). In this form of CAH, in contrast with the others, there is some glucocorticoid production from corticosterone and thus adrenal crisis is very rare. Over the years, accumulation of DOC causes hypokalemia and hypertension. In general, the typical presentation of 17OHD is a girl in adolescent with no secondary sexual characteristics and low-renin hypertension (76, 77). Non-classic forms of 17OHDy have not been well described (75).

11β-hydroxylase deficiency

About 0.2-8% of all CAH cases are due to 11βOHD (OMIM#202010) (72). 11βOH encoded by *CYP11B1*, catalyzes the conversion of 11-deoxycortisol and 11-deoxycorticosterone (DOC) to cortisol and corticosterone, respectively. Impaired cortisol and corticosterone production leads to overproduction of the precursors that are later converted to androgens causing virilization in females (78). In addition, patients present with mild to moderate hypertension. Although deoxycorticosterone is not as a potent mineralocorticoid as aldosterone, when it accumulates it leads to retention of salt and hypokalemic hypertension (79).

3β-hydroxysteroid dehydrogenase type 2 deficiency (also known as HSDB)

This form of CAH is very rare accounting for <0.5% of the cases (OMIM#201810) (80). 3β-hydroxysteroid dehydrogenase exists in two isoforms; 3βHSD1, encoded by *HSD3B1* (the homologous type I gene) and is expressed in the placenta and peripheral tissue (liver, skin, brain) and 3βHSD2, encoded by *HSD3B2* and is found in the adrenals and gonads (72). Inactivating variants in *HSD3B2* impair steroidogenesis in both gonads and adrenals, leading to deficiency of glucocorticoids and mineralocorticoids as well as overproduction of dehydroepiandrosterone (DHEA). Subsequently, DHEA is converted to testosterone by 3βHSD1 and thus both 46,XX and 46,XY present with ambiguous genitalia at birth (81). In rare cases, 46,XX can present with virilization, including incomplete fusion of the labia, enlarged clitoris and hyperpigmentation of the genital region due to the increased testosterone (72).

Steroidogenic acute regulatory protein deficiency

StAR is a mitochondrial protein that regulates cholesterol influx between the outer and inner mitochondrial membrane, a critical and first step of steroid hormone synthesis (82). Inactivating variants in *STAR* gene cause Lipoid Congenital Adrenal Hyperplasia (LCAH, the most severe form of CAH, OMIM#201710) (83). The adrenals are filled with lipid globules derived from cholesterol and thus the name of the disease. Patients with LCAH have impaired production of mineralocorticoids, glucocorticoids and sex steroids. LCAH appears to be more common in the Korean, Japanese and Palestinian Arab populations (83–92), while certain variants have much higher frequency in specific ethnic groups, including p.Q258X variant in Japanese and Koreans (86, 89) as well as p.R182L variant in Palestinian Arabs (83). Both 46,XX and 46,XY present with female or ambiguous genitalia and adrenal crises during the neonatal period. Hyperpigmentation is also frequent due to the increased levels of ACTH. However, milder forms of the disease have been described in the literature that presented with late-onset adrenal insufficiency and male genitalia (93).

Diagnosis

21OHD can be diagnosed *via* the measurement of 17-hydroxyprogesterone; this measurement does not need necessarily to be performed after ACTH stimulation. Hormonal testing is also used to diagnose the rarer forms of CAH. Because carriers cannot be reliably identified using biochemical methods, genetic testing is necessary to detect heterozygous individuals, who can then be offered genetic counseling. Specifically for the salt-wasting form of CAH, the United States and more than 35 countries around the world have

implemented newborn screening programs for early detection and treatment (94).

Conclusion

Adrenocortical hyperplasias are rare in children. Advances over the past two decades have led to a better understanding of their molecular background, including the characterization of the cAMP/PKA signaling pathway as the main component of their pathogenesis. CAH are a group of rare diseases with genetic and clinical heterogeneity and can be life-threatening in their most severe form. The molecular analysis of CAH is useful in confirming the diagnosis and provides a powerful tool in genetic counseling.

Author contributions

GP: Writing-original draft and editing; CAS.: Conseptualization, supervision, writing, review and editing.

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Conflict of interest

Author CS holds patents on the *PRKAR1A*, *PDE11A* and *GPR101* genes and/or their function and has received research funding from Pfizer Inc. on the genetics and treatment of abnormalities of growth hormone secretion. CAS is receiving compensation by ELPEN, Inc. Neither Pfizer, Inc nor ELPEN, Inc had any role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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

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Long-term survival outcomes of pediatric adrenal malignancies: An analysis with the upstaged SEER registry during 2000-2019

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Objective: To investigate the clinicopathological characteristics and long-term survival outcomes of pediatric adrenal malignancies.

Method: This study retrospectively analyzed children with pathologically confirmed pediatric adrenal malignancies from Surveillance, Epidemiology, and End Results Database from 2000 to 2019. Kaplan-Meier curve was used to assess the overall survival (OS) and cancer-special survival (CSS), and the Log-Rank method was used to calculate statistical differences. Cox proportional hazards model and Fine-and-Grey model were used to calculate the hazard ratio (HR) of all-cause mortality risk and the sub-distribution HR (sHR) of disease-specific mortality risk, respectively, and their corresponding 95% confidence intervals (CI).

Results: 1601 children were included in the study in which 1335 (83.4%) neuroblastoma, 151 (9.4%) ganglioneuroblastoma, 89 (5.6%) adrenocortical carcinoma, and 26 (1.6%) were diagnosed with other types malignancies. Metastatic disease accounted for the largest proportion (69.3%), and the proportion of metastases diagnosed by neuroblastoma was higher than that of adrenocortical carcinoma and ganglioneuroblastoma (73.9% vs. 45.7% vs. 47.2%). The 5-year OS and CSS of all cohort were 69.5% and 70.5%, respectively. Adrenal cortical carcinoma had the worst prognosis, with 5-year OS and CSS of 52.5% and 53.1%, respectively. Patients in recent years had no better OS and CSS than in previous years at diagnosis. The tumor stage remained the main prognostic predictor. Compared to metastatic adrenal tumors, the risk of all-cause mortality (adjusted HR: 0.12, 95% CI: 0.06-0.25, $P < 0.001$) and the risk of disease-specific mortality (adjusted sHR: 0.11, 95% CI: 0.05-0.25, $P<0.001$) was significantly lower for patients with localized diseases. Additionally, higher age, adrenal cortical carcinoma, and lack of complete tumor resection are independent risk factors for poor prognosis. Furthermore, it was found that the prognosis of patients who received chemotherapy was worse than those who did not, mainly because the former mostly had

metastasis at the presentation and complete resection of the tumor cannot be achieved.

Conclusion: The clinicopathological characteristics of pediatric adrenal malignancies have not changed significantly in the past two decades, while the prognosis of patients has improved. Early diagnosis of disease and complete resection of local tumors are the keys to improving prognosis.

KEYWORDS

child, adrenal malignancy, treatment, survival, prognosis

Introduction

Pediatric malignancies of adrenal glands are rare but have become one of the leading causes of death in pediatrics (1, 2). Their onset is usually stealthy, discovered incidentally, or identified by recognizing symptoms associated with excess hormone secretion (3, 4). Neuroblastoma, ganglioneuroblastoma, and adrenal cortical cancer account for most pediatric adrenal malignancies. Adrenal cortical cancer rarely occurs and has a poor prognosis (5–8). Pediatric malignant patients have benefited from the rapid progress in tumor treatment as the optimization of multimodality therapy and early tumor screening has dramatically improved the survival rate of pediatric malignant tumors (9–13). Given the low incidence of pediatric adrenal tumors, the small sample size, and the short follow-up time of prior studies on pediatric adrenal malignancies, the clinicopathological characteristics, treatment status, and prognosis are not well understood (2, 4, 11–15). Here, we used the Surveillance, Epidemiology, and End Results (SEER) database to study pediatric malignancies of adrenal glands to explore the long-term follow-up survival outcomes and prognostic risk analysis of pediatric adrenal malignancies.

Patients and methods

Data source

All data were obtained from the SEER database of 18 registries (https://seer.cancer.gov/). Since SEER databases were anonymized and were not associated with human research, therefore, the need for ethics approval was waived by the Ethics Review Board of our institute. As a retrospective study, the patients diagnosed with primary adrenal malignancy from 2000 to 2019 were identified. Patients ≤19 years and having adrenal cortical carcinoma, adrenal ganglioneuroblastoma, adrenal neuroblastoma, and other non-common tumors were identified. All diagnoses were confirmed by histology and not by autopsy or death certification, and all patients had a detailed cause of death and follow-up data. Cases with missing values were excluded.

Study variables

The study mainly included the following variables for data analysis: year of diagnosis (2000-2004, 2005-2009, 2010-2014, and 2015-2019), age at diagnosis (0-4 years, 5-9 years, 10-14 year and 15+ year), gender (male and female), race (white, black and other), median household income ($0-$59999, $60000-$69999 and $70000+), residence locality (metropolitan and non-metropolitan), tumor size, tumor stage (distant, localized and regional), metastatic site (bone, brain, liver, lung), surgery and approaches (none, local tumor destruction/excision, radical surgery with or without other organs and simple/partial surgical removal), metastatic surgery Treatment (yes or no), chemoradiation (yes or no).

Statistical analysis

The continuous variables were described as mean ± standard deviation (SD) for data with a normal distribution and compared with the student t-test. For non-normal distribution, the Wilcoxon rank-sum test was used to compare the data, and the data were described as median and interquartile range (IQR). Classification variables were represented by frequency (%) and compared by the chi-square test. OS and CSS were the primary endpoints of interest for this study. Kaplan-Meier was used to calculate OS and CSS. The hazard ratio (HR), the Sub-distribution hazard ratio (sHR), and the corresponding 95% confidence Interval (95% CI) of all-cause death and adrenal tumor-specific death were calculated by Cox proportional hazards model and Fine and Grey model, respectively. Logistic regression analysis was done to calculate odds ratios (OR) and their corresponding 95% CI for factors associated with treatment

choice. All analyses were conducted using R (Version 42.0). $P < 0.05$ (two-sided) was considered statistically significant.

Results

Baseline characteristics

Our study included 1601 patients with adrenal-derived malignant tumors. The primary histological type was neuroblastoma (1335 cases, 83.4%). Ganglioneuroblastoma and adrenal cortical carcinoma were the other two main pathological types, accounting for 9.4% and 5.6%, respectively, whereas other rare cancers accounted for only 1.6% (Table 1 and Figure 1A). The incidence of neuroblastoma did not change much from 2000 to 2019 (Figure 1B). Most cases had an onset in the age group of 0-4 years, accounting for 79.9% of the cases. Neuroblastoma accounted for the largest proportion (86.4%) in 0-4 years old. However, ganglioneuroblastoma and adrenal cortical carcinoma accounted for 55.6% and 41.5%, respectively; furthermore, the proportion of other rare cases was 19.2% in the older age groups (Table 1).

The median tumor size (102mm) of adrenal cortical carcinoma was larger than other tumor types (neuroblastoma- 75mm, ganglioneuroblastoma- 61mm and others- 70mm). Most of the children had metastatic diseases at the time of diagnosis (Figure 1C). This condition has not changed much in these two decades (Figure 1D). Patients with neuroblastoma present diststant metastasis accounted for 73.9%, while Ganglioneuroblastoma and adrenal cortical carcinoma accounted for 45.7% and 47.2%, respectively. Bone was the most common metastasis site (about 80.0% of all metastasis cases, data did not present here). Figure 2A shows that about 70% of children under 9 years old had disease metastasis at the time of treatment (0 – 4 years old: 70.1%, 5 – 9 years old: 71.1%); although the proportion of children over 10 years old with metastasis decreased, it still more than the general children with disease metastasis (10-14 years old: 60.3%; 15-19 years old: 55.9%). However, we found that with the increase in age, the proportion of adrenal cortical carcinoma patients with metastasis increased (Figure 2B). The proportion of metastasis from adrenal neuroblastoma is higher in all age stages. In contrast, the proportion of metastatic lesions in Ganglioneuroblastoma is lower (Figures 2C, D).

Most children received surgical treatment (8.68% local tumor destruction/excision, 55.0% radical surgery with or without other organs, and 18.1% simple/partial surgical removal), and a few did not (18.2%). At the same time, 77.6% and 31.0% of patients received chemotherapy and radiotherapy, respectively. Additionally, 13.0% of patients also received surgical treatment for distant lymph node or organ metastasis, and most (76.3%) patients underwent either surgery or radiotherapy and chemotherapy within 1 month after diagnosis (Table 1).

Survival outcomes

Our results showed that OS and CSS didn't change over the years (200-2019) (Figures 3A, B). The OS and CSS of neuroblastoma and ganglioneuroblastoma was better than adrenal cortical carcinoma (Figures 3C, D). The 5-year OS of the total population was 69.5% (95% CI: 67.1% – 72.0%, Table 2). The 5-year OS of metastatic patients was 59.3%, while that of local tumor patients was 89.3%. (Table 2). Table 3 summarizes the risk factors of disease-specific death in 5-year CSS of different subgroups. Our results show that the overall 5-year CSS was 70.5% (95% CI: 68.1% -73.0%). The multivariate COX model showed that the age, histological type, tumor size, tumor stage, and treatment were independent risk factors for both, all-cause mortality and disease-specific death. Although in the recent year of the diagnosis had a lower risk of mortality compared to the prior years, the year of diagnosis was neither a significant risk predictor of all-cause mortality (Figure 4A) nor disease-cause mortality (Figure 4B).

It's puzzling that the survival analysis showed that chemotherapy did not benefit patients but increased the risk of death. However, it should be noted that chemotherapy was significantly correlated with the recent year of diagnosis, younger age of patients at diagnosis, metropolitan patient's, larger tumor size, tumor with distant disease, nonsurgical treatment performed (Supplementary Table 1).

Considering the differences in treatment and prognosis between adrenocortical carcinoma and adrenal neuroblastoma/ganglioneuroblastoma, we will perform survival analysis for the two subgroups separately. We found that OS in the adrenal neuroblastoma/ganglioneuroblastoma subgroups correlated with patient age (the older the age, the worse the OS), race (worse in blacks than whites), and tumor stage (worse in metastatic cases than in patients without metastases) (Supplementary Table 2). However, only tumor stage and complete tumor resection were independent prognostic factors for adrenocortical carcinoma (Supplementary Table 3).

Discussion

Neuroblastoma is the most common pathological type of adrenal malignancy in children (7, 16), and approximately 46% of neuroblastomas arise from the adrenal gland (17). The primary site of the adrenal neuroblastoma is often concealed and extensive, but its biological behavior shows high malignancy and rapid growth and is prone to multiple early metases (18). Our study found that ganglioneuroblastoma and neuroblastoma accounted for more than 80% of metastatic lesions. Since the clinical symptoms and signs of pediatric neuroblastoma are not specific, they are hard to diagnose. Hence, diagnosis is often missed, delaying treatment and losing the best time for the

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TABLE 1 Baseline characteristics of the study cohort.

		By histology			
	ALL N=1601	Neuroblastoma N=l335 (83.4%)	Ganglioneuroblastoma N=l51 (9.4%)	Adrenal cortical carcinoma N=89 5.6%	Other N=26 (1.6%)
Year at diagnosis					
2000-2004	374 (23.4%)	315 (23.6%)	34 (22.5%)	21 (23.6%)	4 (15.4%)
2005-2009	421 (26.3%)	352 (26.4%)	42 (27.8%)	19 (21.3%)	8 (30.8%)
2010-2014	411 (25.7%)	339 (25.4%)	36 (23.8%)	29 (32.6%)	7 (26.9%)
2015-2019	395 (24.7%)	329 (24.6%)	39 (25.8%)	20 (22.5%)	7 (26.9%)
Age at diagnosis. Median (IQR)	2.00 [0.00;4.00]	1.00 [0.00;3.00]	4.00 [2.00;7.00]	9.00 [2.00;15.0]	15.0 [8.25;17.0]
Age at diagnosis					
0-4 year	1279 (79.9%)	1153 (86.4%)	84 (55.6%)	37 (41.6%)	5 (19.2%)
5-9 year	190 (11.9%)	136 (10.2%)	41 (27.2%)	9 (10.1%)	4 (15.4%)
10-14 year	73 (4.56%)	32 (2.40%)	18 (11.9%)	20 (22.5%)	3 (11.5%)
15+ year	59 (3.69%)	14 (1.05%)	8 (5.30%)	23 (25.8%)	14 (53.8%)
Sex					
Female	722 (45.1%)	580 (43.4%)	77 (51.0%)	53 (59.6%)	12 (46.2%)
Male	879 (54.9%)	755 (56.6%)	74 (49.0%)	36 (40.4%)	14 (53.8%)
Race					
White	1234 (77.1%)	1026 (76.9%)	108 (71.5%)	78 (87.6%)	22 (84.6%)
Black	214 (13.4%)	179 (13.4%)	27 (17.9%)	6 (6.74%)	2 (7.69%)
Other	153 (9.56%)	130 (9.74%)	16 (10.6%)	5 (5.62%)	2 (7.69%)
Median household income					
$0-$59999	406 (25.4%)	335 (25.1%)	44 (29.1%)	22 (24.7%)	5 (19.2%)
$60000-$69999	498 (31.1%)	412 (30.9%)	49 (32.5%)	28 (31.5%)	9 (34.6%)
$70000+	697 (43.5%)	588 (44.0%)	58 (38.4%)	39 (43.8%)	12 (46.2%)
Residence					
Metropolitan	1448 (90.4%)	1214 (90.9%)	131 (86.8%)	79 (88.8%)	24 (92.3%)
Nonmetropolitan	153 (9.56%)	121 (9.06%)	20 (13.2%)	10 (11.2%)	2 (7.69%)
Size. Median (IQR)	73.0 [50.0;105]	75.0 [49.0; 105]	61.0 [43.5;84.5]	102 [70.8;134]	70.0 [50.5;102]
Stage					
Distant	1109 (69.3%)	986 (73.9%)	69 (45.7%)	42 (47.2%)	12 (46.2%)
Localized	270 (16.9%)	167 (12.5%)	58 (38.4%)	34 (38.2%)	11 (42.3%)
Regional	222 (13.9%)	182 (13.6%)	24 (15.9%)	13 (14.6%)	3 (11.5%)

(Continued)

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TABLE 1 Continued

	By histology				
	ALL N=1601	Neuroblastoma N=l335 (83.4%)	Ganglioneuroblastoma N=l51 (9.4%)	Adrenal cortical carcinoma N=89 5.6%	Other N=26 (1.6%)
Bone metastasis *					
Bone	375 (47.5%)	342 (52.5%)	22 (29.3%)	6 (12.5%)	5 (35.7%)
No	414 (52.5%)	310 (47.5%)	53 (70.7%)	42 (87.5%)	9 (64.3%)
Brain metastasis *					
Brain	44 (5.6%)	42 (6.4%)	2 (2.7%)	0 (0%)	0 (0%)
No	745 (94.4%)	610 (93.6%)	73 (97.3%)	48 (100%)	14 (100%)
Liver metastasis *					
Liver	169 (21.4%)	148 (22.7%)	3 (4.0%)	16 (33.3%)	2 (14.3%)
No	620 (78.6%)	504 (77.3%)	72 (96.0%)	32 (66.7%)	12 (85.7%)
Lung metastasis *					
Lung	70 (8.9%)	48 (7.4%)	3 (4.0%)	13 (27.1%)	6 (42.9%)
No	719 (91.1%)	604 (92.6%)	72 (96.0%)	35 (72.9%)	8 (57.1%)
Metastasis site *					
No	318 (40.3%)	236 (36.2%)	51 (68.0%)	25 (52.1%)	6 (42.9%)
2+ sites	153 (19.4%)	133 (20.4%)	5 (6.7%)	11 (22.9%)	4 (28.6%)
Bone	234 (29.7%)	214 (32.8%)	17 (22.7%)	1 (2.1%)	2 (14.3%)
Liver	72 (9.1%)	65 (10.0%)	1 (13%)	6 (12.5%)	0 (0%)
Lung	12 (15%)	4 (0.6%)	1 (13%)	5 (10.4%)	2 (14.3%)
Surgical treatment					
No	292 (18.2%)	266 (19.9%)	5 (3.31%)	15 (16.9%)	6 (23.1%)
Local tumor destruction/ excision	139 (8.68%)	113 (8.46%)	16 (10.6%)	9 (10.1%)	1 (3.85%)
Radical surgery with or without other organs	881 (55.0%)	728 (54.5%)	96 (63.6%)	46 (517%)	11 (42.3%)
Simple/partial surgical removal	289 (18.1%)	228 (17.1%)	34 (22.5%)	19 (213%)	8 (30.8%)
Surgery for non-primary other distant sites					
None	1393 (87.0%)	1157 (86.7%)	135 (89.4%)	79 (88.8%)	22 (84.6%)
Yes	208 (13.0%)	178 (13.3%)	16 (10.6%)	10 (112%)	4 (15.4%)
Radiotherapy					
No	1104 (69.0%)	885 (66.3%)	116 (76.8%)	81 (910%)	22 (84.6%)
Yes	497 (310%)	450 (33.7%)	35 (23.2%)	8 (8.99%)	4 (15.4%)
Chemotherapy					
No/Unknown	358 (22.4%)	235 (17.6%)	70 (46.4%)	37 (416%)	16 (615%)

(Continued)

TABLE 1 Continued

	By histology				
	ALL N=1601	Neuroblastoma N=l335 (83.4%)	Ganglioneuroblastoma N=l51 (9.4%)	Adrenal cortical carcinoma N=89 5.6%	Other N=26 (1.6%)
Yes	1243 (77.6%)	1100 (82.4%)	81 (53.6%)	52 (58.4%)	10 (38.5%)
Months to treatment					
0 months	1222 (76.3%)	1012 (75.8%)	115 (76.2%)	76 (85.4%)	19 (73.1%)
1 months	331 (20.7%)	281 (210%)	33 (219%)	12 (13.5%)	5 (19.2%)
2+ months	48 (3.00%)	42 (3.15%)	3 (1.99%)	1 (1.12%)	2 (7.69%)

IQR, Interquartile range.
*Data with complete information of metastasis organ.

treatment. Therefore, delayed diagnosis is a major factor for the poor prognosis of neuroblastoma. With the improvement of multidisciplinary comprehensive treatment, the survival rate of patients has improved to a certain extent. However, our findings found that there was no statistically significant reduction in the risk of death in patients diagnosed recently compared with patients with pediatric adrenal malignancies diagnosed in the past.

Neuroblastoma is an embryonal tumor originating from primitive neural crest cells of the sympathetic nervous system. It affects the normal development of the adrenal medulla and paravertebral sympathetic ganglia, although its exact pathogenesis is still unclear (19). *MYCN* amplification is associated with the development of neuroblastoma. Numerous studies have shown that *MYCN* amplification correlates with neuroblastoma progressing rapidly, leading to a worse prognosis (20, 21). Therefore, drugs inhibiting *MYCN* amplification, such as retinoic acid, are used for maintenance therapy in high-risk children (22, 23).

Furthermore, the 1p, 11q loss, and 17q gain are also common chromosomal abnormalities in neuroblastoma (24–26). 11q aberration is reported in 20 to 45% of neuroblastoma, and 1p and 11q loss can render neuroblastomas in an undifferentiated or poorly differentiated state, increasing the risk of recurrence and leading to a poor prognosis (21, 25, 27, 28). Most children with

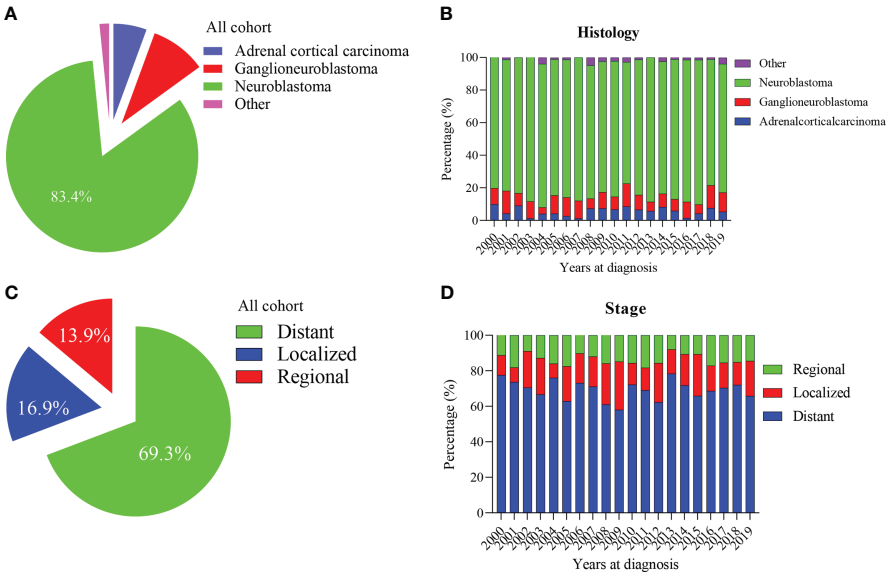

FIGURE 1
Percentage of pediatric adrenal malignancies by histology and tumor stage. **(A, B)** Percentage of histology in all cohorts and stratified by year at diagnosis. **(C, D)** Percentage of tumor stage in all cohorts and stratified by year at diagnosis.

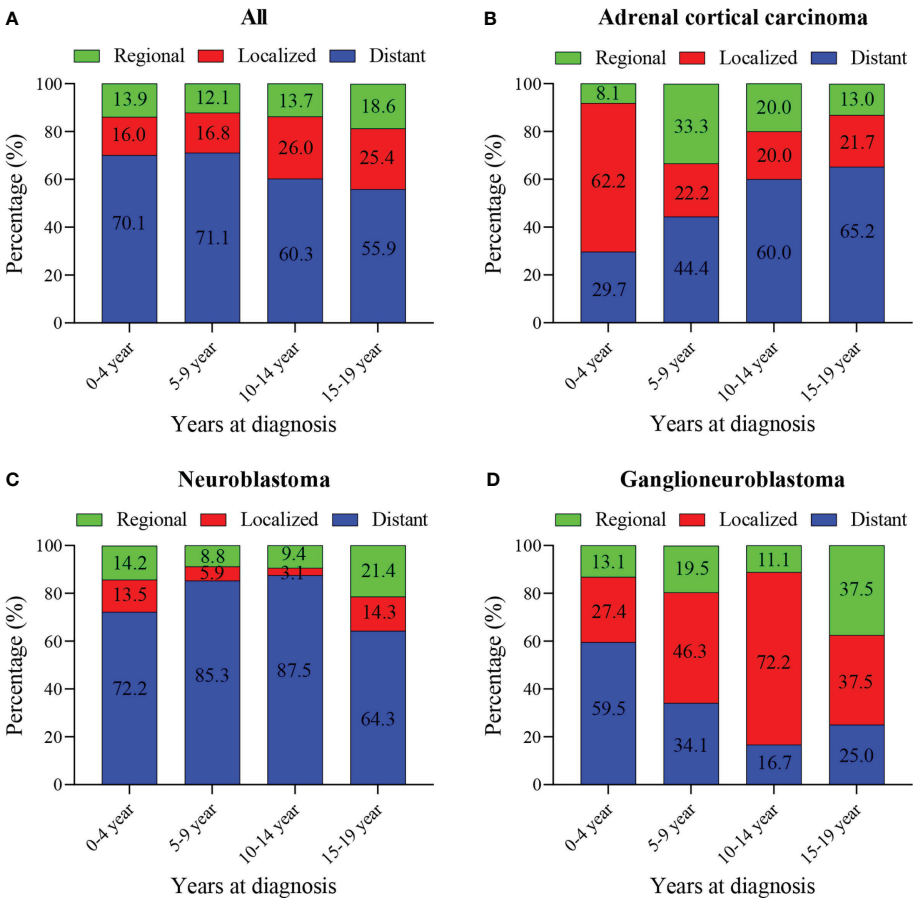

FIGURE 2
Percentage of pediatric adrenal malignancies by age at diagnosis, tumor stage and histology. **(A)** Percentage of stage in all cohorts and stratified by age at diagnosis. **(B)** Percentage of stage in Adrenal cortical carcinoma cohorts and stratified by age at diagnosis. **(C)** Percentage of stage in neuroblastoma cohorts and stratified by age at diagnosis. **(D)** Percentage of stage in ganglioneuroblastoma cohorts and stratified by age at diagnosis.

neuroblastoma develop a 17q gain (80.4%), which, together with other unfavorable prognostic factors, contributes to the poor prognosis of neuroblastoma (21, 24).

Neuroblastoma may show biochemical abnormalities in blood and urine. Neuron-specific enolase (NSE) is an acid protease unique to neurons and neuroendocrine cells and a specific marker of neuroendocrine tumors (29, 30). NSE is expressed in neurons and is highly sensitive and specific to neuroblastoma and ganglioneuroblastoma. Its elevation often indicates advanced disease progression and poor prognosis. Studies have shown that NSE has a high positive expression rate in undifferentiated neuroblastoma and has high sensitivity and accuracy in monitoring tumor recurrence, especially in metastatic tumors. Urinary vanillylmandelic acid (VMA) is also an important indicator of early diagnosis of neuroblastoma (30, 31). A vast majority of neuroblastomas can also be accompanied by abnormal metabolism of catecholamines in the body. It can directly secrete the precursor substances of VMA, increasing its concentration in the blood and the urine. Lactate dehydrogenase (LDH) is an essential enzyme in the glycolytic pathway. The tumor tissue has the characteristics of high metabolism, and the serum LDH level can be used as an important indicator representing the tumor cell burden in the whole body, which is of great significance for the prognosis of neuroblastoma (29, 32, 33).

Surgically resecting the whole tumor tissue is the key to treating pediatric adrenal tumors (17). Our study showed that achieving complete tumor resection improves patient outcomes. However, when complete tumor resection cannot be performed, local surgery did not lead to better outcomes than patients without surgery. Without adjuvant radiotherapy and chemotherapy, children with localized diseases can achieve long-term survival after complete tumor resection and no surgical complications alone. However, in the diagnosis of pediatric adrenal malignancies, the tumor is often large, adheres tightly to the surrounding tissues, and even surrounds important blood vessels such as the inferior vena cava, making it

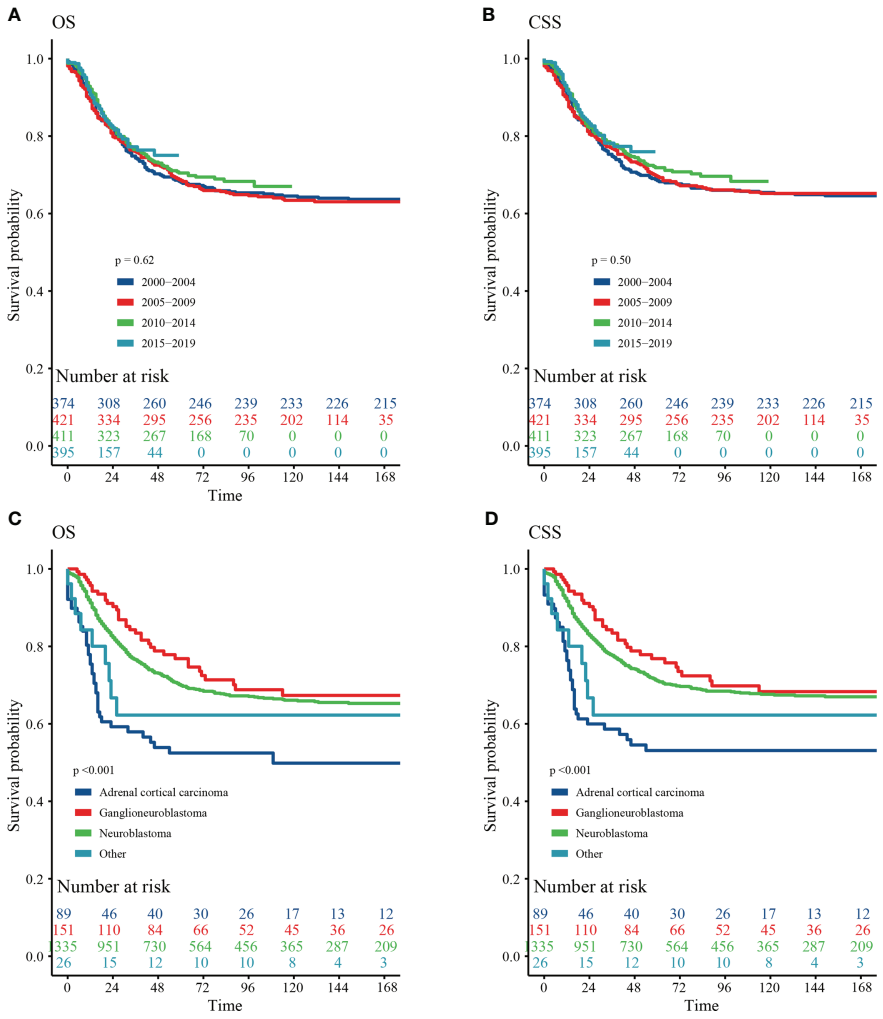

FIGURE 3
Survival curve of analyzed by Kaplan–Meier method. **(A)** Overall survival (OS) stratified by year at diagnosis. **(B)** Cancer-special survival (CSS) stratified by year at diagnosis. **(C)** OS stratified by histology. **(D)** CSS stratified by histology.

difficult to operate on the tumor and remove it altogether. Blind pursuit of complete resection may also lead to more severe complications and affect the patient's survival.

Patients with intermediate-risk tumors may receive chemotherapy to shrink the tumor before surgical removal (17, 34). Preoperative chemotherapy can significantly shrink the tumor, thicken the capsule, reduce the clinical stage of the tumor, and inhibit distant metastasis. The treatment model of neoadjuvant chemotherapy, surgical resection, adjuvant high-dose chemotherapy with hematopoietic stem cell rescue, and radiation therapy has become a classic mode of treatment for high-risk neuroblastoma (17, 35, 36). Chemotherapy can easily aggravate the degree of adhesion between the tumor and surrounding tissues and increase the difficulty of surgical resection. Moreover, chemotherapy can confuse or change the pathological staging, and special attention should be paid to the timing of surgery and chemotherapy. Prior studies have also found that surgical resection is vital in predicting outcomes for high-risk neuroblastomas that do not show a clinical response to induction treatment. However, gross total resection versus subtotal resection did not affect these outcomes (37).

Neuroblastoma is more sensitive to chemotherapy, but at the same time, it is necessary to pay attention to the toxic side effects of chemotherapy on children. Although high-intensity chemotherapy is used only for high-risk groups of neuroblastomata, the toxic side effects on children are significant and need vigilant analysis (38). An international, randomized, multi-arm, open-label, phase 3 trial found that Busulfan and melphalan improved event-free survival in children with high-risk neuroblastoma with an adequate response to induction treatment and caused fewer severe adverse events than carboplatin, etoposide, and melphalan

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TABLE 2 Five-year overall survival and predictors of all-cause mortality.

	5-year overall survival rate (95%CI)	Univariable# HR (95%CI)	P	Multivariable# HR (95%CI)	P
All cohort	69.5% [67.1%-72.0%]				
Year at diagnosis					
2000-2004	68.4% [63.8%-73.2%]	1 reference		1 reference	
2005-2009	68.8% [64.4%-73.4%]	1.02 [0.81-1.29]	0.856	110 [0.86-1.40]	0.4578
2010-2014	70.5% [66.1%-75.2%]	0.91 [0.71-1.16]	0.454	0.82 [0.63-106]	0.1317
2015-2019*	75 0% [69.2%-81.4%]	0.86 [0.64-1.17]	0.35	0.79 [0.58-110]	0.1623
Age at diagnosis					
0-4 year	72.8% [70.2%-75.4%]	1 reference		1 reference	
5-9 year	60.7% [53.4%-69.0%]	1.58 [1.22-2.03]	<0.001	1.56 [1.20-2.03]	0.001
10-14 year	53 0% [41.7%-67.4%]	2.18 [1.53-3.10]	<0.001	2.15 [146-3.17]	<0.001
15+ year	42.9% [310%-59.4%]	2.87 [2.00-4.13]	<0.001	2.54 [155-4.17]	<0.001
Sex					
Female	69.3% [65.7%-73.1%]	1 reference		1 reference	
Male	69.6% [66.4%-73.0%]	1.03 [0.86-1.24]	0.751	1.02 [0.84-1.23]	0.8728
Race					
White	71.7% [69.0%-74.4%]	1 reference		1 reference	
Black	62.5% [55.9%-70.0%]	1.37 [107-1.75]	0.013	1.28 [0.99-166]	0.0631
Other	619% [54.1%-70.8%]	1.40 [1.05-1.88]	0.023	1.31 [0.96-1.79]	0.0872
Median household income					
$0-$59999	64.8% [59.9%-70.0%]	1 reference		1 reference	
$60000-$69999	69.3% [65.1%-73.9%]	0.85 [0.68-108]	0.18	0.91 [0.70-1.19]	0.4937
$70000+	72.5% [68.9%-76.2%]	0.77 [0.62-0.96]	0.021	0.80 [0.62-1.04]	0.0907
Residence					
Metropolitan	70.4% [67.9%-73.0%]	1 reference		1 reference	
Nonmetropolitan	60.2% [52.2%-69.3%]	1.39 [1.05-1.84]	0.019	1.26 [0.91-1.74]	0.1712
Histology					
Adrenal cortical carcinoma	52.5% [42.6%-64.6%]	1 reference		1 reference	
Ganglioneuroblastoma	76.8% [69.6%-84.8%]	0.43 [0.28-0.68]	<0.001	0.38 [0.23-0.63]	0.0002
Neuroblastoma	69.9% [67.3%-72.7%]	0.51 [0.37-0.71]	<0.001	0.32 [0.21-0.49]	<0.001
Other	62.3% [45.4%-85.3%]	0.79 [0.39-157]	0.497	0.72 [0.33-1.59]	0.4207
Size. per cm		1.05 [1.03-1.07]	<0.001	1.03 [1.02-1.05]	<0.001
Stage					
Distant	59.3% [56.2%-62.5%]	1 reference		1 reference	
Localized	96.1% [93.6%-98.6%]	0.09 [0.05-0.16]	<0.001	0.12 [0.06-0.25]	<0.001
Regional	89.3% [85.0%-93.9%]	0.23 [0.15-0.34]	<0.001	0.29 [0.19-0.45]	<0.001
Surgical treatment					
No	60.9% [55.3%-67.2%]	1 reference		1 reference	
Local tumor destruction/excision	77.6% [70.5%-85.5%]	0.46 [0.31-0.69]	<0.001	0.72 [0.46-1.11]	0.1388
Radical surgery with or not other organs	69.2% [66.0%-72.6%]	0.69 [0.55-0.87]	<0.001	0.83 [0.64-1.07]	0.152
Simple/partial surgical removal	75.2% [69.9%-80.8%]	0.52 [0.38-0.70]	<0.001	0.70 [0.50-0.98]	0.0385
Surgery for non-primary other distant sites					
None	71.1% [68.5%-73.7%]	1 reference		1 reference	
Yes	58.9% [52.1%-66.6%]	1.49 [1.17-1.89]	<0.001	1.11 [0.86-1.43]	0.4085
Radiotherapy					
No	73.8% [71.1%-76.7%]	1 reference		1 reference	
Yes	59.8% [55.2%-64.7%]	1.47 [1.22-1.77]	<0.001	1.05 [0.84-1.30]	0.6871
Chemotherapy					

(Continued)

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TABLE 2 Continued

	5-year overall survival rate (95%CI)	Univariable# HR (95%CI)	P	Multivariable# HR (95%CI)	P
No/Unknown	93.2% [90.4%-96.0%]	1 reference		1 reference	
Yes	62.7% [59.9%-65.7%]	5.49 [3.75-8.05]	<0.001	2.68 [1.62-4.46]	<0.001
Months to treatment					
0 months	70.5% [67.8%-73.3%]	1 reference		1 reference	
1 months	64.2% [58.8%-70.1%]	1.26 [1.01-1.56]	0.037	0.98 [0.78-1.22]	0.8273
2+ months	78.9% [67.4%-92.3%]	0.84 [0.47-1.50]	0.565	1.12 [0.61-2.06]	0.72

HR, hazard ratio; CI, confidence interval;
#Cox proportional risk regression model;
*59 months survival rate.

TABLE 3 Five-year cancer-sQecial survival and Qredictors of mortality by adrenal cancer.

	rate (95%CI)	Univariable# sHR (95%CI)	P	Multivariable# sHR (95%CI)	P
All cohort	70.5% [68.1%-73.0%]				
Year at diagnosis					
2000-2004	68.8% [64.2%-73.6%]	1 reference		1 reference	
2005-2009	69.8% [65.5%-74.4%]	0.99 [0.78-1.25]	0.914	1.10 [0.82-1.46]	0.5229
2010-2014	71.8% [67.5%-76.5%]	0.88 [0.68-1.13]	0.32	0.92 [0.68-1.25]	0.5943
2015-2019*	76.0% [70.1%-82.3%]	0.82 [0.60-1.12]	0.215	0.83 [0.58-1.20]	0.3286
Age at diagnosis					
0-4 year	73.8% [71.3%-76.5%]	1 reference		1 reference	
5-9 year	61.6% [54.3%-70.0%]	1.59 [1.23-2.06]	<0.001	1.55 [1.16-2.09]	0.0034
10-14 year	53.0% [41.7%-67.4%]	2.29 [1.61-3.27]	<0.001	1.84 [0.24-2.39]	0.0025
15+ year	43.7% [31.6%-60.4%]	2.64 [1.79-3.89]	<0.001	1.85 [1.05-3.28]	0.0339
Sex					
Female	70.4% [66.9%-74.2%]	1 reference		1 reference	
Male	70.5% [67.3%-73.8%]	1.03 [0.86-1.25]	0.722	0.96 [0.77-1.19]	0.7076
Race					
White	72.3% [69.6%-75.0%]	1 reference		1 reference	
Black	64.4% [57.8%-71.9%]	1.33 [1.03-1.72]	0.027	1.25 [0.93-1.68]	0.1452
Other	64.7% [56.9%-73.6%]	1.32 [0.97-1.79]	0.077	1.33 [0.97-1.81]	0.0756
Median household income					
$0-$59999	66.3% [61.5%-71.5%]	1 reference		1 reference	
$60000-$69999	70.2% [66.0%-74.8%]	0.87 [0.68-1.10]	0.247	1.10 [0.82-1.48]	0.5397
$70000+	73.2% [69.7%-76.9%]	0.78 [0.62-0.98]	0.03	0.90 [0.67-1.22]	0.5113
Residence					
Metropolitan	71.4% [68.9%-74.0%]	1 reference	0.024	1 reference	0.1407
Nonmetropolitan	61.2% [53.3%-70.4%]	1.39 [1.04-1.84]		1.28 [0.92-1.77]	
Histology					
Adrenal cortical carcinoma	53.1% [43.2%-65.3%]	1 reference		1 reference	
Ganglioneuroblastoma	76.8% [69.6%-84.8%]	0.44 [0.28-0.70]	<0.001	0.44 [0.26-0.73]	0.0014
Neuroblastoma	71.1% [68.5%-73.8%]	0.51 [0.37-0.72]	<0.001	0.35 [0.23-0.53]	<0.001
Other	62.3% [45.4%-85.3%]	0.75 [0.36-1.55]	0.432	0.75 [0.29-1.89]	0.5364
Size. per cm		1.05 [1.03-1.07]	<0.001	1.04 [1.02-1.06]	<0.001

(Continued)

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TABLE 3 Continued

		Univariable#		Multivariable#	
	rate (95%CI)	sHR (95%CI)	P	sHR (95%CI)	P
Stage					
Distant	60.5% [57.5%-63.8%]	1 reference		1 reference	
Localized	96.5% [94.2%-98.9%]	0.08 [0.04-0.15]	<0.001	0.11 [0.05-0.25]	<0.001
Regional	89.3% [85.0%-93.9%]	0.23 [0.15-0.35]	<0.001	0.28 [0.18-0.45]	<0.001
Surgical treatment					
No	62.6% [57.0%-68.9%]	1 reference		1 reference	
Local tumor destruction/excision	77.6% [70.5%-85.5%]	0.47 [0.31-0.71]	<0.001	0.67 [0.41-1.10]	0.1144
Radical surgery with or not other organs	70.2% [67.0%-73.6%]	0.69 [0.55-0.87]	0.002	0.80 [0.59-1.09]	0.1531
Simple/partial surgical removal	76.0% [70.7%-81.6%]	0.53 [0.39-0.72]	<0.001	0.63 [0.42-0.94]	0.0253
Surgery for non-primary other distant sites					
None	72.0% [69.5%-74.6%]	1 reference		1 reference	
Yes	60.3% [53.4%-68.1%]	1.49 [1.16-1.90]	0.002	1.13 [0.85-1.51]	0.3915
Radiotherapy					
No	74.9% [72.2%-77.7%]	1 reference		1 reference	
Yes	60.7% [56.1%-65.6%]	1.47 [1.21-1.77]	<0.001	0.95 [0.75-1.22]	0.7048
Chemotherapy					
No/Unknown	94.0% [91.4%-96.7%]	1 reference		1 reference	
Yes	63.8% [60.9%-66.8%]	6.69 [4.36-10.27]	<0.001	2.61 [1.47-4.64]	0.001
Months to treatment					
0 months	71.3% [68.6%-74.1%]	1 reference		1 reference	
1 months	66.0% [60.6%-71.9%]	1.22 [0.98-1.52]	0.081	0.92 [0.71-1.19]	0.5188
2+ months	78.9% [67.4%-92.3%]	0.81 [0.44-1.48]	0.491	1.40 [0.68-2.87]	0.3594

sHR, Sub-distribution hazard ratio; CI, confidence interval;
#Fine and Grey regression model;
*59 months survival rate.

treatment (38). A reasonable grasp of the intensity of chemotherapy is critical to avoid the effects of insufficient intensity in high-risk children and excessive intensity in low-risk children on prognosis.

In addition to surgical resection, radiotherapy can be a crucial treatment for neuroblastoma tumors (34). Local radiotherapy has an inhibitory effect on the recurrence of neuroblastoma. For high-risk patients, radiotherapy for primary and metastatic lesions can control the patient's condition. Also, Iodine-131 metaiodobenzylguanidine (^{131}I-MIBG) and radiolabeled DOTA-conjugated peptides have been reported to be useful for neuroblastoma treatment (39, 40). Because neuroblastoma cells highly express ganglioglyceride GD2 antibodies, they can be used as a better target for immunotherapy. Immunotherapy with an anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin was associated with a significantly improved outcome compared with standard therapy in patients with high-risk neuroblastoma (41).

Adrenal cortical carcinoma in children is a rare adrenal malignancy, and its incidence is lower than that of neuroblastoma and ganglioneuroblastoma (10). In our study, adrenal cortical carcinoma patients only accounted for 5.6% of total patients. However, the degree of malignancy was very high in adrenal cortical carcinoma; the disease developed rapidly and had an infiltrative growth. The diagnosis is that the tumor volume is large and more likely to invade surrounding organs and tissues, making them hard to operate on (42). However, our study found that metastases in adrenocortical carcinoma are less common at the time of diagnosis than in neuroblastoma. The pathogenesis is unclear and can be associated with epigenetic alterations, manifesting as germline TP53 mutations or chromosome 11p abnormalities (8, 43). Compared to adult adrenal cortical carcinoma, pediatric adrenal cortical carcinoma has distinct features. It is closely associated with germline TP53 mutations, which are present in 53% of cases but only in < 10% in adults' adrenal cortical carcinoma (44). At the time of onset, there may be indicators of excessive secretion of adrenal corticosteroids, such as hypertension, precocious puberty in childhood (manifested as male feminization, female virilization, penis enlargement, pubic hair development, deepening of the voice, breast development, etc.) and Cushing syndrome (manifested as a full moon face, bloody appearance, central obesity, acne, purple striae, hypertension, secondary diabetes, and osteoporosis, etc.). For pediatric adrenocortical carcinoma, 90% of patients presented

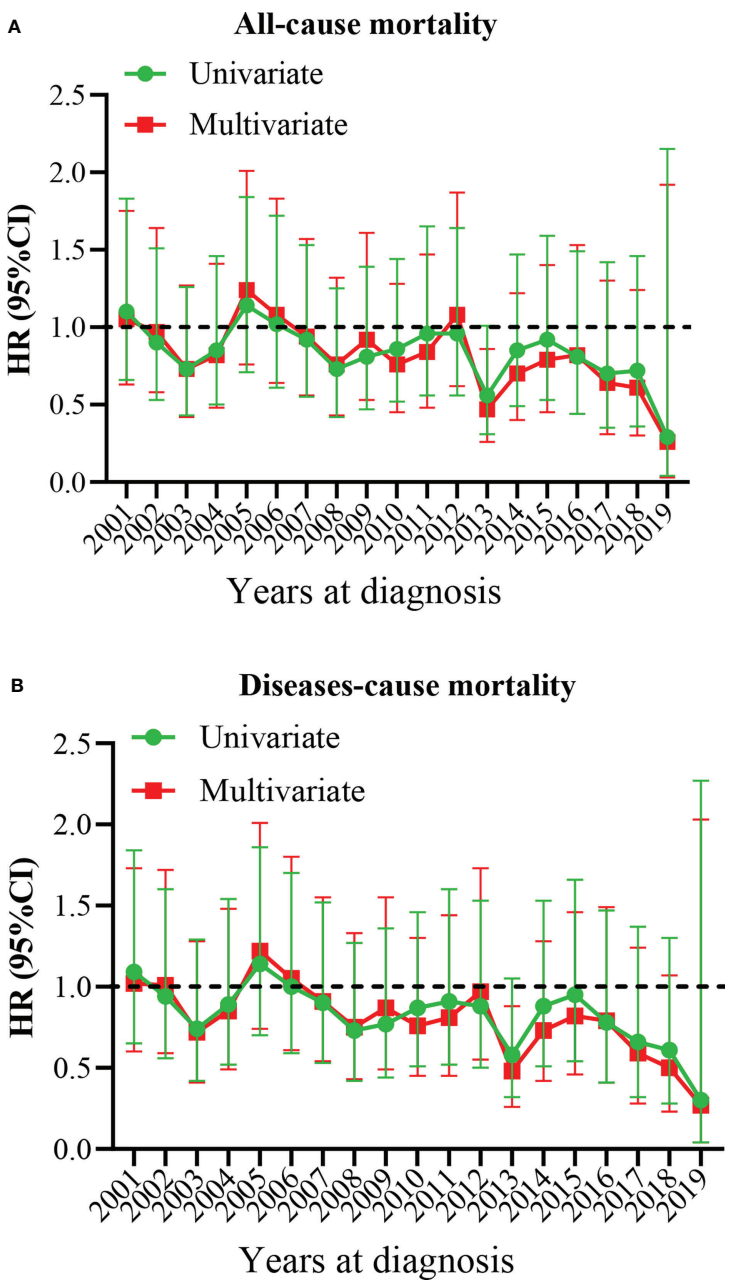

FIGURE 4
Multivariate hazard ratio (HR) analysis for all-cause mortality and diseases-cause mortality. **(A)** All-cause mortality. **(B)** Diseases-cause mortality. The year 2000 at diagnosis was considered as a reference.

with evidence of hormonal hypersecretion, and there was an association between the endocrine phenotype and stage (13). The above symptoms may be considered as a warning sign of adrenocortical carcinomas and can be further studied as prognosis indicators.

Currently, the preferred criteria for the diagnosis of pediatric adrenocortical carcinoma are those described by Wieneke et al (45). It mainly includes the following 9 criteria: tumor weight > 400 g; tumor diameter > 10.5 cm; Extension into the periadrenal soft tissue or adjacent organs; invasion into vena cava; vascular invasion; capsular invasion; the presence of tumor necrosis; mitoses > 15 per 20 high-power fields (4 mm2); and presence of atypical mitosis. Adrenocortical carcinoma can be diagnosed if 4 or more of the 9 items are satisfied. If less than or equal to 2 items, it is diagnosed as benign, and if any 3 items are satisfied, it is diagnosed as uncertain malignant potential. In addition, Ki67

has been proposed as an auxiliary biomarker to differentiate childhood adrenal adenoma from adrenal carcinoma and to predict tumor behavior. A Ki67 index of less than 10% is associated with benign disease, and a marker index of greater than 15% is associated with a higher risk of malignancy or adverse outcomes. The p53 gene is an important tumor suppressor gene in the human body. Its wild type makes cancer cells apoptotic, thereby preventing canceration. It has the function of helping cells to repair defects in genes (5, 46). The mutant type of p53 will increase canceration. Multiple studies have found that p53 is overexpressed in adrenocortical carcinomas, whereas it is normal in adrenal adenomas. Adrenocortical carcinomas are present in excess among carriers of germline p53 mutations, and, p53-associated Adrenocortical carcinomas occur predominantly in the pediatric age group (47). Unfortunately, the specific diagnostic criteria, Ki67 index, and p53 mutation of adrenocortical carcinoma could not be provided in our study. This is a limitation of this study that needs to be pointed out. However, the pediatric adrenocortical carcinoma patients included in our study were all pathologically diagnosed under rigorous scrutiny.

Like neuroblastoma, the treatment of adrenocortical carcinoma is still a surgery-based comprehensive treatment. Complete tumor resection to achieve negative margins is an important prognostic factor (8). Stage I patients are curable with surgery alone (48). Retroperitoneal lymph node dissection has failed to improve outcomes in patients with larger tumors (stage II), and its role as an independent treatment strategy is uncertain (13). Combination of surgery and chemotherapy shows good outcomes in patients with stage III adrenocortical carcinoma, but mitotane- and cisplatin-based regimens lead to higher toxicity in patients with metastatic disease, and efficacy in patients with metastatic disease was still poor and should be revised to maximize risk-benefit (13).

Drug therapy is often required to control tumor growth and excessive hormone secretion for patients who cannot achieve complete tumor resection and for patients with recurrence. Mitotane is currently the most effective drug to control adrenal hormone secretion, and it is used alternately with steroid hormones during use. It can be used as a single drug for adjuvant therapy after complete resection of an early tumor, or it can be used in combination with chemotherapy drugs for advanced childhood adrenal cortical carcinoma (13). Mitotane combined with chemotherapy improves the prognosis of patients with advanced adrenocortical carcinoma. However, not all patients can bear the drug's side effects and can complete all the courses of the treatment as prescribed due to its severe toxicity and side effects (8, 13, 49). The common toxicities of mitotane are nausea, vomiting, diarrhea, and abdominal pain. Drowsiness, lethargy, ataxic gait, depression, and vertigo have also been reported in a few cases. Common chemotherapeutic agents used alone or in combination to treat adrenocortical carcinoma include 5-FU, etoposide, cisplatin, carboplatin, cyclophosphamide, doxorubicin, and streptozotocin. Chemotherapy regimens used to treat childhood adrenocortical carcinoma consist primarily of etoposide and cisplatin, with or without doxorubicin and mitotane. Despite the diverse treatment modalities of chemotherapy, the prognosis for advanced cases remains poor (8). Unfortunately, new therapeutic strategies targeting tumor-specific aberrant pathways have not been studied in pediatric adrenocortical carcinoma. And Radiation therapy in pediatric adrenocortical carcinoma needs further exploration (50).

According to The Children's Oncology Group ARAR0332 Protocol (13), which is a prospective single-arm risk-stratified interventional study to describe the outcome of stage III or IV pediatric adrenocortical carcinoma patients treated with mitotane and chemotherapy. In the study, eight cycles of chemotherapy, and mitotane for 8 months were used for stages III and IV treatment; at the same time, according to clinical practice, surgical treatment of primary tumors and metastases may be considered as appropriate. The chemotherapy regimen was cisplatin 50 mg/m^2/dose for 1-2 days, etoposide 100 mg/m^2/dose for 1-3 days, and doxorubicin 25 mg/m^2/dose for 4-5 days per cycle. Filgrastim was administered daily at 5 mcg/kg/dose starting on day 6 until neutrophil recovery. Treatment with mitotane is administered daily to adjust the plasma concentration of mitotane to 14-20 μg/mL. During the treatment, nearly one-third of patients were unable to complete their scheduled treatment. Finally, 38 patients have evaluated for toxicity or feasibility analysis and found that 4 patients had mitotane feasibility event (10.5%), and 12 patients had chemotherapy feasibility event (31.6%). It can be seen that it is necessary to modify the treatment plan to improve the tolerability of the treatment. What's more, combining surgery and chemotherapy has a good prognosis in stage III patients and a poor prognosis in stage IV patients; with a median follow-up for OS of 60 months, the 5-year OS estimates for stages III, and IV were 94.7%, and 15.6%, respectively.

Note that mitotane produces multiple severe side effects on the metabolic and endocrine systems in the treatment of adrenocortical carcinoma, although this effect appears to be treatable and partially reversible. One study analyzed lipid profiles, thyroid hormones, sex hormones, and adrenal function in 50 patients from the first year of mitotane treatment and after discontinuation (51). In their study, they found levels of total cholesterol, LDL, HDL, and triglycerides increased after 6 months of mitotane treatment, and total cholesterol and LDL levels were reduced when statins were given concomitantly, and mitotane was discontinued Lipid can be further reduced; at the same time, it was also found that plasma free thyroxine decreased in the mitotane treatment group, but thyroid-stimulating hormone remained unchanged. The total amount of T4 increased when mitotane was discontinued. Mitotane increases plasma sex hormone-binding globulin and luteinizing hormone and increases the level of testosterone in male patients; in addition, the adrenal function can be recovered after six months of discontinuation of mitotane. Therefore, during the treatment of adrenocortical

carcinoma with mitotane, in addition to causing adrenal insufficiency, special attention should be paid to the interference of mitotane on lipid metabolism and endocrine, and it should be corrected to normal levels in time. Patients taking mitotane may require high-dose hydrocortisone replacement therapy, and reducing mitotane can interfere with steroid metabolism (52).

Because of the heterogeneity and rarity of pediatric adrenocortical carcinoma, the short follow-up time, and the fact that most patients are diagnosed at an advanced stage of the disease, it is often challenging to identify prognostic factors. Multiple studies have found that age and tumor stage are two independent predictors (4, 13, 53, 54). Other risk factors include tumor size/tumor volume/weight, surgical treatment, presence of virilization, Cushing syndrome, and hypertension. Germline TP53 status and the presence of a somatic ATRX mutation were also associated with the outcome (8, 13).

This study was retrospective and had a few limitations. Firstly, the research data comes from the SEER database, which has not recorded specific radiotherapy, chemotherapy, or targeted treatment plans and lacks laboratory test results (such as NSE, VMA, LDH, cortisol, etc.) and medical data (mitotane dosage, side effects, need for substitutive treatment with cortisone, fludrocortisone and/or levothyroxine). Additionally, due to missing values, there may be some selection bias in the process of screening cases in the study.

Conclusion

This study retrospectively performed a prognostic analysis of pediatric adrenal malignancies using a large sample size. Neuroblastoma, ganglioneuroblastoma, and adrenocortical carcinoma were the three most common pediatric adrenal malignancies. Patients with neuroblastoma and ganglioneuroblastoma often accompany metastatic lesions at presentation, making treatment challenging. Complete surgical resection of the tumor is the key to ensuring a good prognosis. Radiation and chemotherapy should be given to patients whose tumors cannot be entirely removed by surgery. The prognosis of pediatric adrenal malignancies is related to the age of the patient (older children have a poorer prognosis), type of the pathology (cortical cancer has a poor prognosis), tumor size (the larger the tumor, the worse the prognosis), and the tumor stage (the higher the stage of the patient). Notably, our study demonstrated that chemotherapy patients had a worse prognosis than those who did not. This was mainly related to the fact that patients receiving chemotherapy tend to reside in non-metropolitan regions, the pathological type was neuroblastoma, the tumor diameter was larger, the stage was higher, and the patients had not received surgery. Although the field of oncology have progressed with respect to treatment of pediatric adrenal malignancies, there still no statistically significant reduction in the risk of all-cause mortality and tumor-specific mortality in patients with recently diagnosed pediatric adrenal malignancies compared with patients in the past diagnosis period. Therefore, more experimental studies are needed in this regard urgently and efficiently to save lives

Data availability statement

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

Author contributions

ZL, XM, and SC conceived the research. ZL wrote the manuscript. ZL, YY, and YL analyzed the data and prepared the figures and tables. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.977105/full#supplementary-material

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Pediatric adrenocortical carcinoma

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Adrenocortical carcinoma (ACC) is a rare endocrine malignancy of the adrenal gland with an unfavorable prognosis. It is rare in the pediatric population, with an incidence of 0.2-0.3 patients per million in patients under 20 years old. It is primarily associated with Li-Fraumeni and Beckwith-Wiedemann tumor predisposition syndromes in children. The incidence of pediatric ACC is 10-15fold higher in southern Brazil due to a higher prevalence of *TP53* mutation associated with Li-Fraumeni syndrome in that population. Current treatment protocols are derived from adult ACC and consist of surgery and/or chemotherapy with etoposide, doxorubicin, and cisplatin (EDP) with mitotane. Limited research has been reported on other treatment modalities for pediatric ACC, including mitotane, pembrolizumab, cabozantinib, and chimeric antigen receptor autologous cell (CAR-T) therapy.

KEYWORDS

pediatric adrenal tumors, adrenocortical cancer (ACC), pediatric adrenocortical carcinoma, endocrine tumors, adrenal tumor

Introduction

Adrenocortical Carcinoma (ACC) is a rare endocrine malignancy with an overall unfavorable prognosis. It is rare amongst children, with an incidence of 0.2-0.3 patients per million, in patients under 20 years of age (1, 2), accounting for less than 0.2% of all pediatric malignancies (3). Pediatric ACC displays a bimodal distribution with peaks under the age of 5 and after 10 years and presents more frequently in females than males (4, 5). Pediatric ACC incidence is 10-15 times higher in southern Brazil, likely due to a higher prevalence of the R337H *TP53* mutation (6, 7). Pediatric ACCs are often linked to cancer predisposition syndromes, with most childhood ACCs associated with germline mutations (8, 9).

Pediatric patients with ACC often present differently than adult patients. One key difference from adult ACC is that pediatric ACCs are more often functional, often presenting with excess androgen production (5, 10, 11). Pediatric ACC patients have a 5-

year survival rate reported to be between 30% to 70%, depending on disease presentation (11–13). Outcomes in patients with metastatic disease are poor, with a 5-year survival rate estimated to be less than 20% (1, 10, 11, 14, 15). Prognosis of childhood ACC is highly variable and difficult to predict in clinical practice (9).

Genetics

ACCs that develop in children can either be sporadic or linked to a cancer predisposition syndrome. The genomic landscape of pediatric ACC is characterized by copy-neutral loss of heterozygosity of chromosomes 11 and 17, which is associated with germline *TP53* pathogenic variants, insulin-like growth factor-2 overexpression, and somatic mutations in *ATRX* and *CTNNB1* (16).

Pediatric ACCs are most commonly associated with Li-Fraumeni Syndrome, an autosomal dominant familial cancer syndrome associated with a number of malignancies including sarcoma, breast cancer, brain tumors, leukemia, lymphoma, and adrenocortical carcinomas (17–19). Li-Fraumeni Syndrome occurs in the context of germline mutations in the *TP53* tumor suppressor gene, which encodes for p53, a transcription factor that helps preserve genomic integrity and activates apoptosis in cells with DNA damage (20). Germline mutations in the gene encoding the p53 tumor suppressor located at 17p13.1 have been found in approximately 50% of children with ACC (21, 22). However, germline p53 mutations are much less common in adults with ACC (23).

In southern Brazil, there is a fifteen-fold increase in the incidence of pediatric ACC compared to other global populations (6). This is due to a unique germline missense mutation in *TP53* (R337H). This mutation exists at a high prevalence in this population (0.3%), but with a low penetrance of approximately 2%, which contrasts with the classical presentation of Li-Fraumeni Syndrome that presents with 100% penetrance (24–26). This R337H mutation affects the protein's oligomerization domain, resulting in pH-dependent instability, predisposing these individuals to adrenocortical tumors (23, 26–29). Additionally, R337H mutation carriers are at increased risk of developing other tumors associated with Li-Fraumeni Syndrome, as well as adult ACC (12, 27). A newborn screening program for R337H mutations in southern Brazil exists and has been successful at early detection of ACCs in this population (24).

Pediatric ACCs have also been associated with Beckwith-Wiedemann syndrome, a systemic overgrowth disorder characterized by macroglossia, macrosomia, organomegaly, and abdominal wall defects (29, 30). This syndrome is a result of genetic defect caused by uniparental disomy in the 11p15 chromosomal region resulting in *IGF2* growth factor overexpression (31). Normally, the *IGF2* gene is only expressed from the paternal allele due to imprinting (32, 33), but disomy leads to expression from two copies of the gene. In instances of normal functionality, IGF2 activates type 1 IGF receptors, which stimulates cell survival (34). IGF2 plays a major role in fetal adrenal growth and steroidogenesis (35, 36). While IGF2 overexpression alone is not sufficient for adrenal carcinogenesis, it does promote ACCs (36, 37).

Other hereditary tumor syndromes associated with ACC that are less common in the pediatric population include multiple endocrine neoplasia 1 (MEN1), Lynch syndrome, familial adenomatous polyposis (FAP), Neurofibromatosis type 1 (NF1), and Carney complex (Table 1). MEN1 is an autosomal dominant hereditary tumor syndrome characterized by tumors

TABLE 1 Hereditary tumor syndromes associated with ACC.

Syndrome	Associated Gene mutations	Major clinical features	Prevalence %-ACC
Li-Fraumeni syndrome	*TP53*	Breast cancer, leukemia, lymphoma, brain tumors, sarcomas, adrenocortical carcinoma	3-7% in adults 50-80% in children
Beckwith-Wiedemann syndrome	*IGF2, CDK1C, H19* locus changes on 11p15	Wilms tumor, hepatoblastoma and neuroblastoma	<1%
Multiple Endocrine Neoplasia 1	*MEN1*	Hyperparathyroidism, pituitary tumors, parathyroid tumors, pancreatic neuroendocrine tumors (PNETs), collagenoma, angiofibroma	1.4%
Lynch syndrome	*MSH2, MSH6, MLH1, PMS2*	Colorectal carcinoma, endometrial carcinoma, ovarian cancer, pancreatic cancer, brain cancer	3%
Familial adenomatous polyposis	*APC*	Colorectal cancer, duodenal carcinoma. Thyroid cancer, desmoid tumor	<1%
Neurofibromatosis type 1	*NF1*	Malignant peripheral nerve sheet tumor, pheochromocytoma, neurofibroma, optic glioma	<1%
Carney complex	*PRKAR1A*	Primary pigmented nodular adrenocortical disease (PPNAD), Sertoli cell tumors, thyroid adenoma, myxoma, pituitary tumors, schwanoma	<1%

affecting the parathyroid, pituitary, and pancreatic islet (38). Adrenal lesions occur in 20-55% of MEN1 patients, however, these lesions are usually adrenocortical adenomas or hyperplasia; ACC is extremely rare in this population (39). Lynch syndrome is an autosomal dominant inherited cancer predisposition syndrome characterized by an elevated risk of developing colorectal and endometrial cancers (40). The prevalence of Lynch syndrome amongst ACC patients is estimated to be 3.2% (41). FAP is an autosomal dominant hereditary tumor syndrome characterized by the development of adenomas in the rectum and colon followed by colorectal cancer if not treated at an early stage (42). Although rare, ACCs in patients with FAP have been reported in the literature (43–45). NF1 is an autosomal dominant genetic syndrome due to mutations in the *NF1* tumor suppressor gene and is characterized by café au lait spots, dermal and plexiform neurofibromas, pheochromocytomas, optic gliomas, and malignant peripheral nerve sheath tumors (46, 47). Rare instances of ACC in patients with NF1 have been reported in the literature (48–51). Carney complex is an autosomal dominant tumor syndrome characterized by skin pigmentary abnormalities, myxomas, endocrine tumors, and schwannomas (52). ACC in the setting of Carney complex is very rare, but has been reported in two cases in the literature (53–55).

Clinical characteristics

Pediatric ACC displays a bimodal age distribution with peaks under the age of 5 and after 10 (4), with almost half (46%) of diagnoses occurring in children less than 4 years of age (1). There is a female preponderance for this condition both in childhood and in adulthood. Previous studies have shown the growth-promoting role of estrogen on the ACC cell line NCI-H295 and may explain the basis for this predisposition (56). There is a 2:1 female to male ratio amongst diagnoses of pediatric ACC (1).

Based on international TNM staging, approximately 44% of patients are stage I, 25% stage II, 13% stage III, and 17% stage IV (57). However, differences may exist based on age. Based on data from a SEER study of pediatric ACC patients, 52% of all staged patients presented with localized tumors; however, this was 76% in those aged 4 years and younger compared to 31% in those over the age of 4 (1). Additionally, the same study showed a significantly greater tumor size in older patients. Metastases are found in approximately 25% of pediatric ACC patients (57). The majority of metastases are found in the lungs and liver (14, 57). Other reported sites of metastases include the bone, kidneys, and CNS (57).

Pediatric ACCs are almost always functional, with approximately 95% of tumors exhibiting hormone production, compared to less than 50% of adult ACCs (11, 58, 59). Consequently, diagnoses in children usually follow the presentation of symptoms (12). Amongst all pediatric adrenocortical tumors, the most common presentation is virilization due to excess androgen secretion alone or in combination with hypercortisolism in over 80% of patients (11, 12). Other presentations include Cushing syndrome (15-40%), feminization or gynecomastia due to excess estrogen production (7%), hyperaldosteronism (1-4%), or a mixture of symptoms (60). Cushing's syndrome without virilization is uncommon (5.5%) (11). Approximately 10% of pediatric patients with ACC have nonfunctional tumors; they are uncommon amongst young children, and are usually only found in adolescents (11, 60).

Michalkiewicz et al. created a registry for pediatric ACCs and provide a descriptive analysis of 254 patients registered on the International Pediatric Adrenocortical Tumor Registry. 254 patients younger than 20 years of age with newly diagnosed or previously treated ACCs were registered. The most common presenting sign (84.2%) was virilization. Cushing's syndrome without virilization was uncommon (5.5%). Tumors were completely resected in 83% of patients. Patients with disseminated or residual disease received mitotane, cisplatin, etoposide, and/or doxorubicin, and rarely, radiation therapy. At a median follow-up of 2 years and 5 months, 157 patients (61.8%) survived without evidence of disease and 97 patients (38.2%) had died. The 5-year event-free survival estimate was 54.2%. It was concluded that childhood ACCs occur predominantly in females and almost always causes clinical signs. Complete resection is required for cure. Residual or metastatic disease carries a poor prognosis (11). Wieneke's index has been validated in some studies is a histopathological tool which have shown to predict clinical outcome in pediatric ACC patients (Table 2) (61). Pediatric patients with age <4 y on presentation shows favorable prognosis while metastasis at presentation have guarded prognosis (14).

TABLE 2A Wieneke's criteria.

Wieneke's Criteria for malignancy
Tumor weight >400g
Tumor size >10.5cm
Extension into periadrenal soft tissues and/or adjacent organs
Invasion into vena cava
Venous invasion
Capsular invasion
Presence of tumor necrosis
>15 mitoses per 20 high power field (400X)
Presence of atypical mitotic figures

TABLE 2B Clinical outcomes based on Wieneke's criteria.

Two criteria	Benign long-term clinical outcome
Three criteria	Intermediate/atypical/uncertain malignant potential
Four or more criteria	Poor clinical outcome

Treatment

The treatment for pediatric ACC has largely been extrapolated from adult ACC. The European Cooperative Study Group for Pediatric Rare Tumors has published consensus guidelines for the diagnosis and treatment of childhood adrenocortical tumors, derived from the guidelines and data obtained from adult studies (11). Surgery is the primary form of therapy, with an aggressive surgical approach recommended if feasible (62). Results from a recent prospective single-arm risk stratified interventional study with 77 patients showed that surgery has been the mainstay treatment and had shown to have excellent prognosis in stage I ACC (86.2% 5-year event free survival) (63). However, tumor spillage is a frequent concern in these patients with its occurrence in approximately 21% of initial resections and 43% of resections after recurrence (11). Due to this risk of tumor rupture, laparotomy and a curative procedure are recommended, rather than a fine needle aspiration (64). An open adrenalectomy is the standard care for resection of ACC, as laparoscopic resections are associated with a high risk of rupture and peritoneal carcinomatosis (62, 65).

Mitotane is a commonly used single agent in the adjuvant setting following a complete resection of ACC in the adult population and is approved for the treatment of ACC (66). Mitotane inhibits steroidogenesis and has direct adrenolytic functions, inducing permanent atrophy of the fasciculata and reticular zones of the adrenal cortex (67, 68). Mitotane has shown to improve outcomes in the intermediate risk pediatric ACC population if it can be given for more than 6 months and if therapeutic levels (greater than 14mg/L) can be attained (14, 69). In the pediatric ACC population, it has been shown to improve outcomes in stage III and IV, although it is poorly tolerated (63). In a review of 11 children with advanced ACC who were treated with mitotane and a cisplatin based chemotherapeutic regimen, 7 patients showed measurable responses, suggesting that neoadjuvant use can be considered in patients where complete surgical resection is not an option (68). Additionally, a retrospective analysis of 177 patients with adrenocortical carcinomas showed a significant increase in recurrence-free survival amongst patients who received adjuvant mitotane therapy (66). In a interventional open label Phase III trial with pediatric ACC, Stage III ACC patients was shown to have a good clinical outcome with adjuvant chemotherapy with EDP and mitotane but was associated with toxicity. An optimal treatment regimen need to be curated for these patients (63).

Pembrolizumab is a molecular therapy that targets the programed death receptor 1(PD-1) on lymphocytes and has shown to be effective in tumors expressing PD-L1. In a study of Pembrolizumab therapy in pediatric patients with PD-L1 positive tumors, two out of four pediatric ACC patients enrolled showed a partial response (70).

Cabozantinib is a multi-tyrosine kinase inhibitor that has been studied in ACC patients. In a study of 16 patients with advanced ACC treated with cabozantinib, three patients developed a partial response and five patients had stable disease with a median progression free survival of 16 weeks and a median overall survival of 58 weeks (71). Currently, there is an ongoing trial for Cabozantinib-S-Malate for patients with rare tumors including pediatric ACC (Table 3) (72).

B7-H3-specific chimeric antigen receptor autologous T-Cells (CAR-T) have shown success in treating patients with relapsed pediatric acute lymphoblastic leukemia as well as *in vivo* success in solid tumor types (73). Currently there is a B7-H3-CAR-T cell trial testing the viability of this therapy in pediatric solid tumor types, including pediatric ACC (Table 3).

TABLE 3 Clinical trials for pediatric ACC.

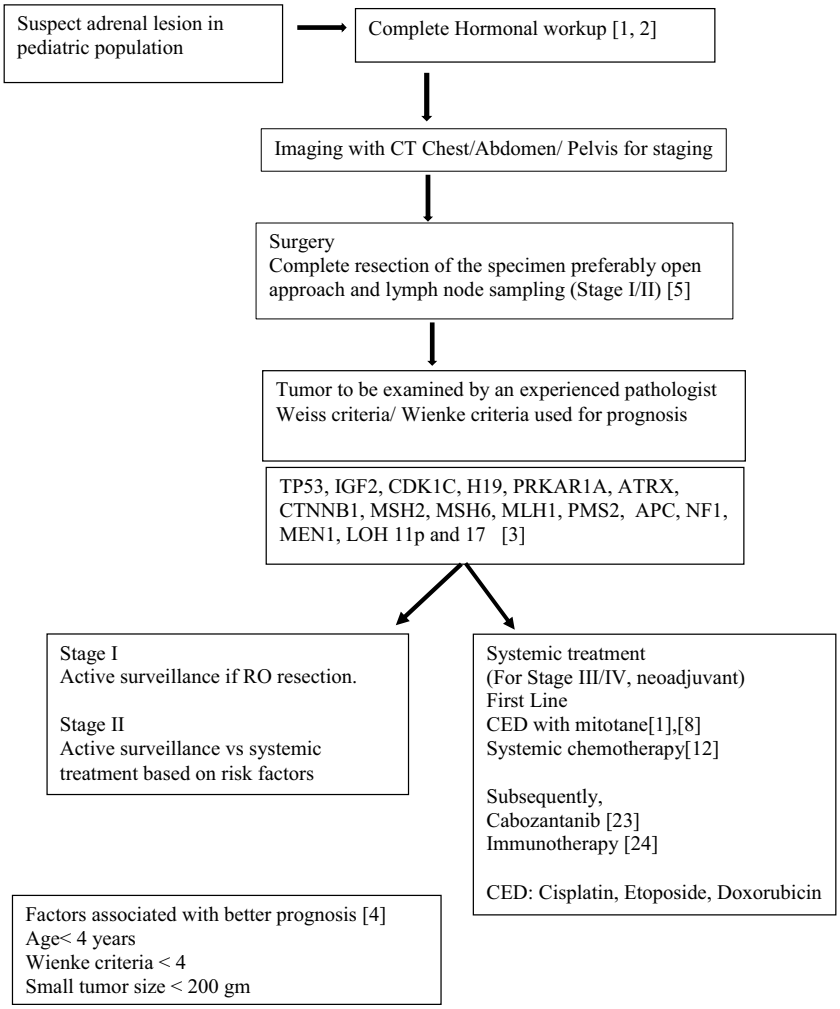
Therapy	Study Design	# of Patients	Eligibility Criteria	NCT	Status
Cisplatin	Phase 3	78	Histologically confirmed ACC	NCT00304070	Active, Not recruiting. Results available
Cabozantinib-S-Malate	Phase 2	109	Ewing sarcoma, rhabdomyosarcoma, osteosarcoma, Wilms tumor, medullary thyroid carcinoma, renal cell carcinoma, hepatocellular carcinoma, hepatoblastoma, adrenal cortex carcinoma, pediatric solid tumors with specified molecular alterations	NCT02867592	Active, Not recruiting, Results pending.
B7-H3-CAR T Cells	Phase 1	32	Solid tumor, ≤21 years old	NCT04897321	Recruiting

The use of radiation therapy in pediatric ACC patients has not been widely studied. Since most pediatric ACC cases carry TP53 mutations that predispose them to cancer, radiation can increase the likelihood of developing a secondary tumor. Driver et al., reported that amongst five long term survivors of pediatric ACC in their study, three died of secondary sarcomas that arose in the radiation field (74).

Future direction/therapeutics

Understanding the oncogenesis and tumor biology of ACC in pediatric patients has been fallen behind compared to adult ACC. The treatment protocols are primarily based on the data from adult patients with adrenocortical cancer. Surgery with lymph node dissection, chemotherapy with EDP (mitotane, cisplatin, etoposide, and/or doxorubicin) and radiation in some cases are the current preferred treatment options in pediatric ACC (Scheme 1).

It has been shown that the similar histopathology in pediatric and adult populations have different prognosis and the prognostic markers used in adult ACC might not be applicable for pediatric cases (75). Pediatric ACC arises from the fetal zone of adrenal gland and almost always presents with signs of hormone excess (14). As described previously germline mutation (p.R337H) of the *TP53* gene and *IGF1R* overexpression is seen specifically in pediatric population (75). The expression of *FATE1* gene has a negative prognostic significance in adult but not in pediatric population. Pediatric ACC is also more frequently associated with cancer predisposition syndromes (76). Ki-67 index and Weiss score are not predictive in pediatric ACC (7). Moreover, even with the differences with adult ACC, the current ongoing



SCHEME 1
Schema showing Current guidelines and therapeutics strategies.

trials for adrenocortical cancer may show some future directions for treatment of pediatric ACC. Unfortunately, however, much of the preclinical and clinical trial data we have comes from adult ACC and no pediatric cell line for this tumor exist, limiting our ability to study the pediatric ACC population (77). As such, much of the investigation into treatment modalities for pediatric ACC patients has been extrapolated from adult in-vitro and in-vivo studies.

Targeted molecular therapies have proven to be efficacious in a number of malignancies. Their use in ACC has been studied with limited success. IGF2 overexpression has been found in approximately 90% of ACC cases, making it a potential target for precision molecular therapy (78). Additionally, in pediatric ACC, IGF1R overexpression is associated with a worse prognosis (75, 79). Preclinical studies of IGF2/IGF1R antagonists in ACC xenograft models have been promising, showing a dose dependent growth inhibition (80). Unfortunately, targeted therapies for these targets have not yet been substantiated *in vivo*. The IGF1R inhibitor lisitinib failed to show improvement in overall or progression free survival in an adult Phase III clinical. However, 5/90 patients showed a stabilization of disease (81).A phase II trial on the IGF1R inhibitor cixutumumab in combination with mitotane was terminated due to slow accrual and limited efficacy (82).

The activation of β-catenin signaling has been found in approximately 40% of ACC tumors (83, 84). Studies done on the NCI-H295R in-vitro model of ACC have shown that inhibition of β-catenin can inhibit tumor proliferation and promote apoptosis (76, 85, 86). However, the clinical utility of Wnt/β-catenin signaling inhibitors has not yet been demonstrated. Wnt/β-catenin signaling is essential for stem/progenitor cell maintenance (83). As such, Wnt/β-catenin signaling inhibition can result in a number of on-target toxicities, which has prevented these agents from advancing to phase III clinical trials (87, 88).

The Ki-67 proliferation index of has demonstrated a major prognostic role in ACC (89). This finding combined with genomic findings in ACC tumors suggest a role for cell cycle activation in advanced ACC (78, 90, 91) The cell cycle inhibitors, gemcitabine, capecitabine, and 5-fluorouracil have shown moderate efficacy and tolerability in patients with advanced ACC. Further investigation of broad and targeted kinase cell cycle inhibitors may be warranted in patients with advanced ACC.

Promising data exists supporting the use of certain modalities of radiotherapy for treating ACC. A study on Iodine-131 Iodometomidate (^{131}I MTO) targeted radionuclide therapy demonstrated one partial response and five patients with stable disease in a cohort of 11 patients with unresectable ACC (92). In ACC patients with somatostatin expressing tumors, Yttrium-90/^{177}Lu-DOTATOC has shown potential as a treatment option. In a study of 19 patients with advanced ACC, eight patients showed radiometabolic uptake of Yttrium-90/^{177}Lu-DOTATOC and two patients showed strong uptake in addition to overall disease control lasting 4 and 12 months (93).

The use of single cell sequencing of tumor specimen from primary and metastasis in larger cohort of patient population to understand of heterogeneity in ACC and use of liquid biopsies will help in more personalized treatment approach for pediatric ACC. The continuation of global consortiums and international collaborations like International Pediatric Adrenocortical Tumor Registry (IPACTR) are necessary to build on the existing knowledge of pediatric ACC (11).

Conclusions

ACCs are a rare tumor in the pediatric population with a poor prognosis. Current treatment algorithms for pediatric ACC patients are based on research in adult populations. Investigation of cisplatin, cabozantinib and CAR-T cell therapy for pediatric ACC patients is ongoing. Research of treatment modalities for childhood ACC is limited and the need for more investigation into the treatment of this unique population exists. Due to the differences in ACC between pediatric and adult populations, we believe additional research in the treatment of childhood ACC is warranted.

Author contributions

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The use of temozolomide in paediatric metastatic phaeochromocytoma/paraganglioma: A case report and literature review

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There is increasing evidence to support the use of temozolomide therapy for the treatment of metastatic phaeochromocytoma/paraganglioma (PPGL) in adults, particularly in patients with *SDHx* mutations. In children however, very little data is available. In this report, we present the case of a 12-year-old female with a SDHB-related metastatic paraganglioma treated with surgery followed by temozolomide therapy. The patient presented with symptoms of palpitations, sweating, flushing and hypertension and was diagnosed with a paraganglioma. The primary mass was surgically resected six weeks later after appropriate alpha- and beta-blockade. During the surgery extensive nodal disease was identified that had been masked by the larger paraganglioma. Histological review confirmed a diagnosis of a metastatic SDHB-deficient paraganglioma with nodal involvement. Post-operatively, these nodal lesions demonstrated tracer uptake on ^{18}F-FDG PET-CT. Due to poor tumour tracer uptake on ^{68}Ga-DOTATATE and ^{123}I-MIBG functional imaging studies radionuclide therapy was not undertaken as a potential therapeutic option for this patient. Due to the low tumour burden and lack of clinical symptoms, the multi-disciplinary team opted for close surveillance for the first year, during

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which time the patient continued to thrive and progress through puberty. 13 months after surgery, evidence of radiological and biochemical progression prompted the decision to start systemic monotherapy using temozolomide. The patient has now completed ten cycles of therapy with limited adverse effects and has benefited from a partial radiological and biochemical response.

KEYWORDS

paraganglioma, pheochromocytoma, temozolomide, succinate dehydrogenase (SDH), SDHB = SDH enzyme complex subunit B

Case description

A 12-year-old girl presented to a clinic appointment with a two-year history of sweating, intermittent flushing and occasional headaches. After monitoring her heart rate using a wearable device, she and her parents noted tachycardia even at rest, with a heart rate ranging from 120 to 145 beats per minute.

Upon presentation, she was tachycardic, hypertensive (207/184 mmHg) and a left parasternal heave was palpable. She was noted by the assessing clinician to be of short stature, with a current height of 136.4 cm (0.4th centile) while her target height was near the 70th centile. In the absence of any pubertal development, she was also diagnosed with delayed puberty.

Diagnosis, treatment, and outcomes

A magnetic resonance imaging (MRI) scan of the abdomen was performed, which showed a 6 cm x 3 cm right-sided suprarenal mass abutting the liver, inferior vena cava and hilum, but without features of direct invasion on imaging (Figure 1A). An echocardiogram showed left ventricular hypertrophy, indicating that the patient's hypertension was likely longstanding. The patient was started on an alpha- and beta-blockade with phenoxybenzamine and atenolol respectively and admitted to manage her hypertension. Her ongoing care was subsequently transferred to our tertiary centre.

To further characterise the suprarenal mass, a ^{68}Ga-DOTATATE positron emission tomography (PET-CT) was performed. The mass demonstrated heterogenous ^{68}Ga-DOTATATE uptake (Figures 1C, D), in keeping with a somatostatin receptor positive tumour such as a paraganglioma or phaeochromocytoma, with the former diagnosis suspected in this case. In addition, the patient's urinary metanephrine concentration was 64591 nmol/l, significantly elevated above the reference range.

Surgical removal of the mass was planned. Magnetic resonance (MR) spectroscopy was performed (Figure 1B), demonstrating that the tumour had a high succinate concentration in keeping with a succinate dehydrogenase (SDH)-deficient paraganglioma (1). Due to the increased risk of malignant or aggressive behaviour from an SDH-deficient paraganglioma, the agreed pre-operative plan was to aim for complete surgical resection.

Six weeks after the patient's presentation, an open resection of the mass was performed. As the mass was adherent to the gallbladder and liver, a cholecystectomy and partial caudate lobe resection were necessary. Additionally, the mass was found to be invading the inferior vena cava, requiring resection and reconstruction of the retro-hepatic vena cava with a deceased donor aortic graft. Intra-operatively, extensive nodal disease (not identified on pre-operative imaging) was noted including para-aortic and hilar nodes; some of the latter involved the first order bile ducts at the hilar plate which were not amenable to resection.

Histological analysis of the tumour confirmed an *SDHB*-deficient paraganglioma. Extensive lymph node involvement was also shown, confirming the diagnosis of a malignant SDH-deficient paraganglioma. Post-operatively a restaging ^{18}F-fluorodeoxyglucose (FDG) PET-CT was performed (Figure 1E). This demonstrated FDG-avid deposits in the porta hepatis and the aortocaval region suspicious for metastatic nodal disease. Debulking surgery was discussed but was not believed to be feasible without major liver resection and attendant significant morbidity. At that time, the patient was asymptomatic, the tumour volume was low, and plasma metanephrines had almost normalised (Table 1). After discussion at a multi-disciplinary team meeting (MDT), close surveillance was

Abbreviations: CVD, cyclophosphamide, vincristine, dacarbazine; FDG, fluorodeoxyglucose; GBM, glioblastoma multiforme; MIBG - metaiodobenzylguanidine; MDT, multidisciplinary team; MRI, magnetic resonance imaging; MTIC, methyl-triazene-1-yl-imidazole-4-carboxamide; PET, positron emission tomography; PPGL, phaeochromocytoma/paraganglioma; PPRT, peptide receptor radionuclide therapy; *SDHB*, succinate dehydrogenase complex subunit B; *SDHx*, succinate dehydrogenase; SPECT, single-photon emission computerised tomography; SSTR, somatostatin receptor.

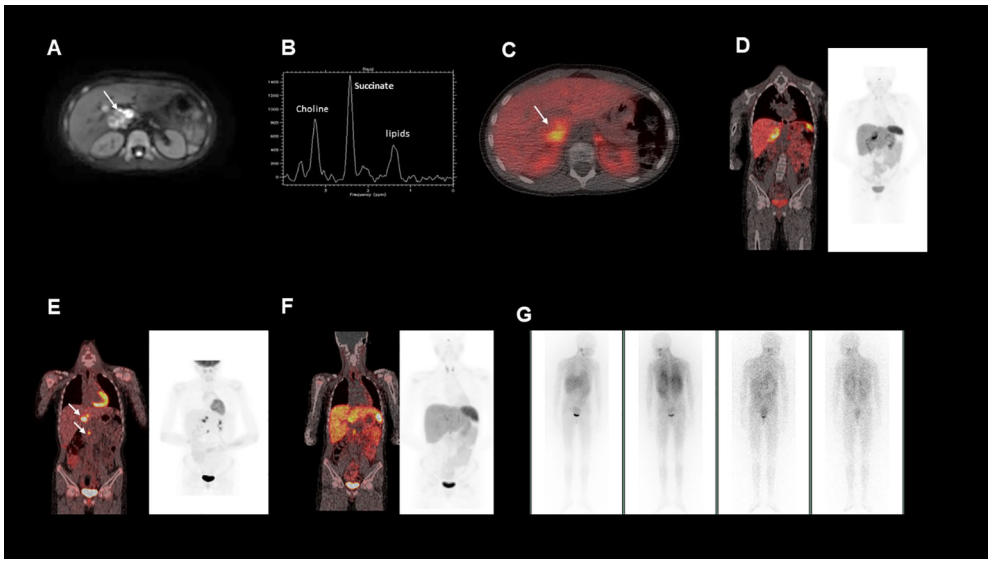

FIGURE 1
(A), This is an axial diffusion-weighted MRI image showing a right sided heterogeneous suprarenal mass. **(B)**, MRI spectroscopy acquired from a voxel placed within the right suprarenal mass demonstrated a high succinate concentration. **(C, D)**, show fused axial and coronal images from a ^{68}Ga-DOTATATE PET/CT scan showing heterogeneous uptake in the right suprarenal mass. **(E)**, Fused coronal image from a post operative ^{18}FDG-PET/CT, demonstrating FDG avid nodal metastases near the liver hilum and aortocaval region **(F)**, Fused coronal image from a post operative ^{68}Ga-DOTATATE PET/CT, demonstrating minimal uptake in the identified nodal metastases **(G)**, Post-operative ^{123}I-MIBG whole body scan, which showed no significant MIBG uptake in the known metastatic deposits.

recommended over systemic treatment to allow the patient to advance through puberty and adolescence, a critical period of growth and physical, emotional and social development.

Germline genetic analysis confirmed a pathogenic *SDHB* variant c.136C>T p. (Arg46*) and predictive testing has identified several *SDHB* carriers on the maternal side of the family (Figure 2). Paired Whole Genome Sequencing (PWGS) detected somatic loss of the short arm of chromosome 1 (1p), consistent with loss of heterozygosity at the SDHB locus.

Over the subsequent 13 months, the patient remained largely asymptomatic, though she became tachycardic towards the end of this period and her plasma normetanephrine levels continued to increase (Table 1). She underwent normal pubertal development, achieved menarche and grew in height: she is

TABLE 1 Table showing concentration of plasma metanephrine and plasma normetanephrine at various timepoints.

Date	Plasma metanephrine (pmol/l)	Plasma normetanephrine (pmol/l)	Notes
04/09/2020			Operation to remove tumour
23/09/2020	<180	1346	3 weeks post operation
04/01/2021	<180	2888	4 months post operation
17/02/2021			Doxazosin 1mg od restarted
25/03/2021	<180	3128	Doxazosin increased to 4mg od
25/06/2021	<180	3641	9 months post operation
04/10/2021	301	5268	Prior to 1st cycle of temozolomide
04/02/2022	<180	3485	Prior to 5th cycle of temozolomide
14/04/2022	<180	3787	Prior to 7th cycle of temozolomide
28/07/2022	<180	2474	After 10th cycle of temozolomide

Reference range for plasma metanephrine: <600 pmol/l - catecholamine secreting tumour unlikely; between 600 and 900 pmol/l – equivocal; >900 pmol/l - high likelihood of catecholamine secreting tumour.
Reference range for plasma normetanephrine: <1000 pmol/l - catecholamine secreting tumour unlikely; between 1000 and 2500 pmol/l – equivocal; >2500 pmol/l - high likelihood of catecholamine secreting tumour.
Bold was used to show dates for which biochemical values were available; non-bold text was used for important dates without biochemical values.
Also included are the dates of the patient's operation, and the restarting of alpha-blockade.

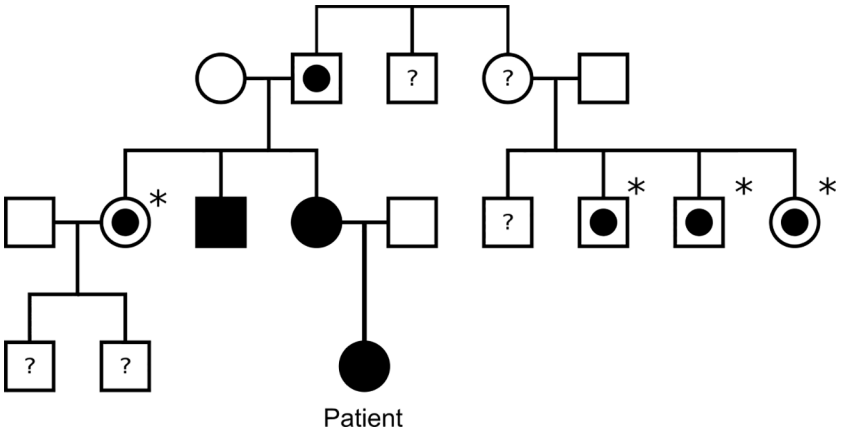

FIGURE 2
Family tree of the patient's family. Black square/circle: male/female with paraganglioma and confirmed *SDHB* mutation. White square/circle with circle within: male/female with confirmed *SDHB* mutation, but no known paraganglioma. Asterisk: indicates patient has not undergone tumour screening. White square/circle: male/female with no known *SDHB* mutation or paraganglioma. Question mark: indicates patient has not undergone genetic testing for *SDHB*.

now at the 9th centile and still growing. Her alpha-blockade with doxazosin was restarted, initially at 1 mg once daily, then subsequently increased to 4 mg per day. Additionally, surveillance CT imaging showed evidence of disease progression (Figure 3).

Due to the patient's biochemical and radiological progression, consideration of systemic therapy was discussed at an MDT meeting. An ^{123}I-metaiodobenzylguanidine (MIBG) single positron emission computerised tomography (SPECT-CT) scan was carried out (Figure 1G), but showed poor ^{123}I-MIBG uptake, indicating that the patient would be unlikely to benefit from MIBG therapy. The ^{68}Ga-DOTATATE PET-CT was repeated (Figure 1F) to evaluate whether the patient's metastatic nodal disease would be suitable for peptide receptor radionuclide therapy (PPRT) with ^{177}Lu-DOTATATE, but due to heterogeneous uptake, this was not deemed the best option.

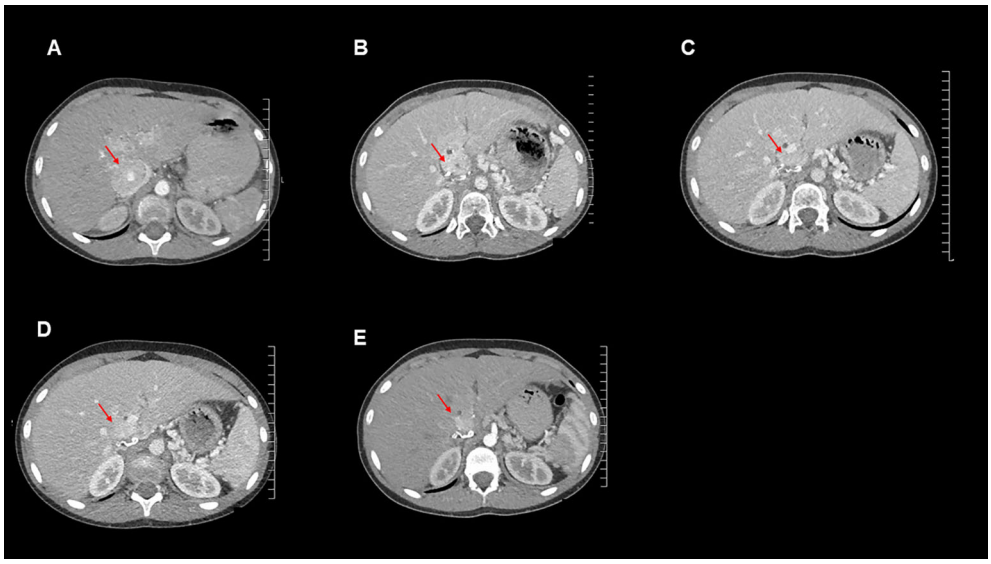

FIGURE 3
Axial contrast-enhanced CT images for this patient from the following time points: **(A)**: September 2020 (post operation), **(B)**: October 2021 (temozolomide therapy commenced), **(C)**: January 2022, **(D)**: April 2022, **(E)**: July 2022.

To assess the likely response of the tumour to temozolomide (1), O6-methylguanine methyltransferase (MGMT) methylation studies were undertaken and demonstrated MGMT promoter hypomethylation (mean methylation 2%) in the patient's tumour cells. Despite this, temozolomide was still agreed as the best therapeutic option by the MDT. There is a good evidence base for the tolerability and safety of temozolomide in children (2, 3) and the toxicity profile is favourable to conventional chemotherapy regimens such as cyclophosphamide, vincristine, dacarbazine (CVD) (1). In addition, temozolomide was thought to be less likely to induce significant pubertal delay, and given the patient's relatively low tumour burden was believed to have sufficient efficacy in this case.

Oral temozolomide was commenced in cycles of 5 days every 28 days, at a dose of 150 mg/m^2/day for the first cycle and 200 mg/m^2/day for each subsequent cycle, taken with ondansetron. This was initially well-tolerated by the patient, however in later cycles the patient suffered significant nausea, which was subsequently managed with dexamethasone and levomepromazine. In addition, the patient had several episodes of mild thrombocytopenia, necessitating total cumulative delays of 5 weeks over the 10 cycles, and dose reductions to 65% and 75% on three and two occasions respectively.

Imaging and measurement of plasma metanephrines were performed at 3-monthly intervals. These showed reduction in plasma metanephrine levels (Table 1) and decreases in size of the metastatic lesions on surveillance CT scans, indicating a response to temozolomide therapy (Figure 3). The MDT intend to continue to treat with temozolomide as long as the patient tolerates the drug and continues to improve clinically and biochemically.

Discussion

Phaeochromocytomas and paragangliomas in children

Phaeochromocytomas and paragangliomas (PPGL) are neuroendocrine tumours arising from neural crest cells. These can be functional (catecholamine-secreting) or non-functional. The term phaeochromocytoma refers exclusively to tumours arising within the adrenal medulla, whilst tumours arising in sympathetic and parasympathetic paraganglia elsewhere in the body are referred to as paragangliomas. PPGL are rare tumours in children, with an estimated overall incidence of 0.6 per 100,000 person-years (4). Of these, around two thirds are extra-adrenal and a third are adrenal (5). In addition, the prevalence of metastatic PPGL in children is significantly higher than in adults, comprising around half of cases (5, 6). PPGL typically present with signs and symptoms attributable to catecholamine excess, e.g. hypertension, flushing, sweating, headaches or palpitations (7). They may also present as an incidental finding on cross-sectional imaging or with symptoms of pain due to mass effect (8).

Familial PPGL and *SDHB* mutations

Up to 80% of paediatric PPGL cases are thought to have a hereditary basis (5). These include hereditary tumour syndromes such as neurofibromatosis type 1, multiple endocrine neoplasia types 2A and 2B, and von Hippel-Lindau syndrome (9). Germline pathogenic variants in one of the genes encoding the succinate dehydrogenase complex (*SDHx*) also predispose to the development of PPGL. Such pathogenic variants are autosomal dominant, and require biallelic inactivation for tumorigenesis to occur (10). Higher levels of succinate in *SDHx*-deficient cells lead to increased stabilisation of hypoxia-inducible factor 1α (HIF-1α), as well as DNA hypermethylation. This can lead to neoplasia by driving angiogenesis, anaerobic metabolism and promoting hypermethylation (11).

The succinate dehydrogenase complex is made up of 4 subunits encoded by individual genes (*SDHA*/*B*/*C*/*D*). Of these, pathogenic variants in the *SDHB* gene confer the highest malignant potential, such that the majority (80%) of metastatic PPGL in children occur in patients with *SDHB* variants (12). *SDHB* variants more commonly lead to paragangliomas than phaeochromocytomas. Furthermore, they are implicated in the development of renal cell carcinoma (RCC), gastrointestinal stromal tumours (GISTs) and pituitary adenomas (13).

Functional imaging modalities in PPGL

Several functional imaging modalities based on PET or SPECT are used for diagnosis and to guide therapy in PPGL. ^{68}Ga-DOTATATE PET-CT is thought to be the most sensitive modality for imaging PPGL (14), especially for the detection of metastases (15). In addition, positive uptake of ^{68}Ga-DOTATATE can indicate amenability to therapy with somatostatin receptor analogues, or peptide receptor radionuclide therapy with ^{177}Lu-DOTATATE (16). Cases of absent or heterogeneous ^{68}Ga-DOTATATE uptake, as noted here, have been reported in more aggressive *SDHx*-deficient PPGL and is hypothesised to reflect a degree of tumour dedifferentiation and loss of SSTR expression (14).

PET-CT can also be carried out using ^{18}F-FDG, a tracer which acts as a marker of glucose metabolism. This is useful for assessing the metabolic activity of PPGL or suspected metastases, but is less sensitive than ^{68}Ga-DOTATATE imaging for detecting metastases in patients with secretory PPGL because of brown fat activation by catecholamine secretion and ^{68}Ga-DOTATATE PET/CT is a more specific tracer for PPGL (17).

^{123}I-MIBG is a tracer which binds to the noradrenaline transporter and can be used in conjunction with SPECT-CT imaging. Its sensitivity is also inferior to ^{68}Ga-DOTATATE in detecting metastatic disease (14), and particularly in *SDHx*-

deficient PPGL (15). It nonetheless remains a useful theranostic imaging modality, predicting whether a tumour may be suitable for treatment with to ^{131}I-MIBG therapy.

Therapeutic options in metastatic PPGL

In addition to symptom management with alpha- and beta-blockers, there are several therapeutic approaches for metastatic PPGL. Radionuclide therapy with ^{131}I-MIBG or ^{177}Lu-DOTATATE are commonly used (18). Both these approaches are only possible if respective functional imaging modalities show sufficient uptake of analogous tracers (see above).

The first-line cytotoxic chemotherapy regime in metastatic PPGL has traditionally been cyclophosphamide, vincristine, and dacarbazine (CVD) (18). Of these, dacarbazine has been hypothesised to be the only active agent against PPGL (19). Dacarbazine is converted to an active alkylating agent, methyl-triazene-1-yl-imidazole-4-carboxamide (MTIC), which is also the active metabolite of temozolomide (20). Temozolomide is thought to have favourable pharmacokinetics and a better toxicity profile when compared to dacarbazine (21), whilst having similar efficacy (22).

Two small studies (19, 20) and several case reports (23) have found temozolomide to be an effective chemotherapy in a number of patients with metastatic PPGL. Reported response to temozolomide was shown to be greater if the patient had a germline *SDHB* mutation (19, 20). When used for glioblastoma multiforme (GBM), the efficacy of temozolomide is known to be correlated with the methylation status of the MGMT promoter: hypermethylation of the MGMT promoter is associated with a better response, likely due to a decreased ability of tumour cells to repair damage to DNA (24). This association has also been borne out by a small study of the use of temozolomide in metastatic PPGL, in which 80% of responders to temozolomide therapy had MGMT promoter hypermethylation. In addition, the study also found a correlation between *SHDB* mutations and MGMT promoter hypermethylation (19), suggesting one possible mechanism by which *SDHB* mutations may confer susceptibility to temozolomide.

Use of temozolomide in children with metastatic phaeochromocytoma/paraganglioma

Various options for systemic therapy were considered in this case. The mainstay of chemotherapy for PPGL has traditionally been CVD (cyclophosphamide, vincristine, dacarbazine), but this is associated with significant side effects such as myelosuppression, peripheral neuropathy and gastrointestinal toxicity (25). Systemic radionuclide therapy using ^{131}I-MIBG or ^{177}Lu-DOTATATE is also possible, but only if functional imaging has confirmed sufficient tracer uptake (see introduction). ^{131}I-MIBG is an effective palliative treatment for PPGL (26), but can cause severe haematological toxicity (27). If the tumour is SSTR positive, PPRT with ^{177}Lu-DOTATATE is an option. This has reduced haematological toxicity compared to ^{131}I-MIBG therapy, and necessitates shorter inpatient stays (16). In SSTR-positive cases, there may also be a role for combination or maintenance therapy with somatostatin analogues such as octreotide or lanreotide (26).

There are few published reports on the use of temozolomide in children with PPGL, with the current evidence base being limited to two case reports in which temozolomide was trialled in the treatment of adolescents with metastatic paragangliomas alone (28) and in combination with olaparib (29). A more recent study of temozolomide use in metastatic *SDHx*-deficient PPGL includes a child aged 13 within its cohort (20). There are several reports of the use of temozolomide in adults with PPGL which point particularly towards the benefits of temozolomide therapy in *SDHB*-deficient PPGL (19, 20, 23). The tolerability of temozolomide in these patients was also thought to be superior to standard CVD therapy, with few patients experiencing adverse effects (19, 23). There is also a good evidence base for temozolomide being well-tolerated in other paediatric cancers such as GBM (2).

The partial response to temozolomide means that this case adds to existing literature by showing that temozolomide can also be an effective treatment option for a metastatic *SDHB*-related paraganglioma in a child. Moreover, the increased tolerability of temozolomide in children compared to standard chemotherapy regimens such as CVD may make the risk-benefit profile preferable to these agents, particularly during the adolescent period when consideration also needs to be given to growth and puberty, in addition to education and overall psychological wellbeing. Indeed, a key consideration in the management of this case was the timing of systemic chemotherapy. Alkylating agents such as temozolomide are linked to premature ovarian failure and pubertal delay (30, 31), therefore a balance had to be struck between the benefits of chemotherapy, and allowing the patient to grow and undergo puberty. The onset of temozolomide therapy was therefore delayed to minimise these effects and was eventually commenced 15 months after diagnosis. Cryopreservation was not performed in this case, as the patient was not eligible based on local criteria.

Given the lack of evidence around its use in children with PPGL, the optimal duration of treatment with temozolomide is unclear. Most of the data surrounding the use of temozolomide concerns its use as an adjuvant to radiotherapy in the treatment of GBM (32), whereas in this case its use is palliative. An Anglo-French study of temozolomide monotherapy for GBM in children used up to 24 cycles of chemotherapy, and found that haematological toxicity was the main side-effect, with myelosuppression necessitating delays and dose reductions in 17% and 22% of all cycles respectively (3). In this case, thrombocytopenia delayed several cycles and caused dose reductions, reflecting these findings. Ongoing therapy will therefore need to balance the impact of temozolomide on disease progression against potential toxicity and reduced tolerability.

Conclusion

This report demonstrates that temozolomide therapy can be an effective therapeutic option for children with *SDHB*-deficient metastatic paraganglioma. Further work is needed however, to establish whether temozolomide is preferable to standard chemotherapy with CVD in children. The case also identifies a potential pitfall in the use of SSTR functional imaging in metastatic paraganglioma, in that metastatic lesions may be missed if there is dedifferentiation and loss of SSTR expression.

Data availability statement

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

Ethics statement

Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

Author contributions

CU drafted and revised the initial manuscript, Figure 2 and Table 1. RC, AH and JN helped supervise the writing process and edit the manuscript. VK, CJ and A-ML provided input about the operative aspects of the case. BF, IH, MM, LA and FG contributed information and figures from the imaging undertaken in the case, and helped edit the manuscript. RA and PT provided input about the genetics of the case. LH provided information about the histopathology of the case. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Low number of neurosecretory vesicles in neuroblastoma impairs massive catecholamine release and prevents hypertension

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Introduction: Neuroblastoma (NB) is a pediatric cancer of the developing sympathetic nervous system. It produces and releases metanephrines, which are used as biomarkers for diagnosis in plasma and urine. However, plasma catecholamine concentrations remain generally normal in children with NB. Thus, unlike pheochromocytoma and paraganglioma (PHEO/PGL), two other non-epithelial neuroendocrine tumors, hypertension is not part of the usual clinical picture of patients with NB. This suggests that the mode of production and secretion of catecholamines and metanephrines in NB is different from that in PHEO/PGL, but little is known about these discrepancies. Here we aim to provide a detailed comparison of the biosynthesis, metabolism and storage of catecholamines and metanephrines between patients with NB and PHEO.

Method: Catecholamines and metanephrines were quantified in NB and PHEO/PGL patients from plasma and tumor tissues by ultra-high pressure liquid chromatography tandem mass spectrometry. Electron microscopy was used to quantify neurosecretory vesicles within cells derived from PHEO tumor biopsies, NB-PDX and NB cell lines. Chromaffin markers were detected by qPCR, IHC and/or immunoblotting.

Results: Plasma levels of metanephrines were comparable between NB and PHEO patients, while catecholamines were 3.5-fold lower in NB vs PHEO affected individuals. However, we observed that intratumoral concentrations of

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metanephrines and catecholamines measured in NB were several orders of magnitude lower than in PHEO. Cellular and molecular analyses revealed that NB cell lines, primary cells dissociated from human tumor biopsies as well as cells from patient-derived xenograft tumors (NB-PDX) stored a very low amount of intracellular catecholamines, and contained only rare neurosecretory vesicles relative to PHEO cells. In addition, primary NB expressed reduced levels of numerous chromaffin markers, as compared to PHEO/PGL, except catechol O-methyltransferase and monoamine oxidase A. Furthermore, functional assays through induction of chromaffin differentiation of the IMR32 NB cell line with Bt2cAMP led to an increase of neurosecretory vesicles able to secrete catecholamines after KCl or nicotine stimulation.

Conclusion: The low amount of neurosecretory vesicles in NB cytoplasm prevents catecholamine storage and lead to their rapid transformation by catechol O-methyltransferase into metanephrines that diffuse in blood. Hence, in contrast to PHEO/PGL, catecholamines are not secreted massively in the blood, which explains why systemic hypertension is not observed in most patients with NB.

KEYWORDS

neuroblastoma, pheochromocytoma, catecholamine, metanephrine, biomarkers, neurosecretory vesicles, chromaffin cell differentiation

Introduction

Neuroendocrine neoplasms encompass a large diversity of epithelial and non-epithelial neoplasms differing for their incidence, localization, morphology, biology and available treatments. Despite these facts, these tumors share common characteristics, including the potential ability of hormones and biogenic amine production and secretion (1–4). Pheochromocytoma (PHEO), paraganglioma (PGL) and neuroblastoma (NB) are non-epithelial neuroendocrine neoplasms arising from the adrenal medulla and ganglia of the sympathetic nervous system (SNS), which typically produce and secrete catecholamines (CATs; dopamine, DA, norepinephrine NE, epinephrine, E) (5). However, whereas PHEO and PGL typically occur in adults and are composed of differentiated chromaffin cells or sympathetic neurons, respectively, NB is a pediatric neoplasm resulting from the abnormal differentiation and maturation of sympathetic progenitors derived from the embryonic trunk neural crest (6). NB is the second most common solid tumor in children, and can occur anywhere in the sympathetic chain, but most commonly in the abdominal region and adrenal medulla. Fifty percent of NB cases are diagnosed before the age of 2 years and 90% before the age of 5 years. Hypertension, a typical clinical symptom of CAT-producing tumors, has a low prevalence in NB, ranging from 2.2% to 10% in two recent reports and is mainly related to renal artery compression, which frequently resolves after tumor resection (7, 8).

CAT metabolism has been reviewed extensively elsewhere (2, 3). Briefly, CAT production occurs primarily in adrenal chromaffin cells and sympathetic nerves. It starts in the cytoplasm from L-tyrosine, which is converted to dihydroxyphenylalanine (DOPA) by the rate-limiting enzyme tyrosine hydroxylase (TH), and DOPA is further converted to DA by L-aromatic amino acid decarboxylase (DDC). In chromaffin cells, DA is internalized by vesicular monoamine transporters (SLC18A1 and SLC18A2, formerly VMAT1 and VMAT2, respectively) into neurosecretory vesicles where it is converted to NE by dopamine beta-hydroxylase (DBH). Subsequently, chromaffin adrenergic cells expressing the enzyme phenylethanolamine-N-methyltransferase (PNMT) synthesize E from NE. Following sympathetic stimulation, CATs stored in neurosecretory vesicles are secreted into the bloodstream to reach their receptors and trigger the described "fight or flight response" (9). A small proportion of the CATs that leak from the neuroendocrine vesicles is converted in the cytoplasm to metanephrines (MNs) by the COMT (catechol O-methyltransferase) enzyme. MNs refers to the three metabolites methoxytyramine (MT), normetanephrine (NMN), and metanephrine (MN), generated from DA, NE and E, respectively. NE and DA in the cytoplasm can also undergo oxidation to dihydroxyphenol glycol (DHPG) and 3,4-

dihydroxyphenylacetic acid (DOPAC), respectively, by monoamine oxidase A (MAOA). MNs, DHPG and DOPAC diffuse freely across the membrane and are released into the bloodstream (10, 11).

Few reports have been published on the mechanisms responsible for CATs synthesis and release in NB cells compared with normal chromaffin cells. The majority of studies have focused on PHEO/PGL and it has been shown that the excess concentration of CATs found in these tumors and in the plasma of the affected patient was a consequence of the overexpression of TH and DBH enzymes involved in CATs synthesis (12, 13). NB were shown to produce CATs, especially, DA and NE (14, 15) but no E due to the lack of expression of PNMT in NB (16, 17). In contrast to PHEO/PGL, NB contain few storage/neurosecretory vesicles and low amount of CATs in tumor tissues (18). Nevertheless, NMN and especially MT, which arise from NE and DA, respectively, represent potent biomarkers of NB in plasma and urine (19). In this study, we explore the metabolism of CATs in NB versus PHEO/PGL and decipher at the molecular level the mechanism that prevents NB from inducing hypertension, an expected clinical sign for a CAT-producing neoplasm.

Material and methods

NB and PHEO/PGL tissues and plasma collection

PHEO/PGL tissue samples were carefully selected by the surgeon or pathologist to be free of remaining healthy adrenal tissue. NB tumor material was collected from patients with high-risk L2 and M stage NB diagnosed at the Hemato-Oncology Unit of the University Hospital of Lausanne, Switzerland, enrolled in the European International Collaboration for Neuroblastoma Research (SIOPEN) HR-NBL1 study, after informed consent and in accordance with local institutional ethical regulations. For plasma collection, samples were collected through a forearm venous cannula with the patient held in a supine position for at least 15 minutes before collection. Patients' relatives or nurses were informed of the need to fast 24 hours before blood collection, when possible. All samples were collected on ice and centrifuged within 30 minutes of puncture at 2500g for 10 minutes at 4°C. Plasma was stored at -80°C until analysis. The NB patient cohort for plasma collection consists of cases at diagnosis representing of all stages, ages, MYCN amplified and non-amplified cases. Quantification of MNs and CATs in plasma was performed as part of the NB or PHEO/PGL diagnostic exclusion test. The complete list of patient samples used for the various analyses is reported in Supplementary Tables 1A, B. The available clinical data for the PHEO patients included in our study are reported in Supplementary Table 2, with the newly proposed three cluster classification (20). This study was approved by the local ethics committee of the canton of Vaud (reference numbers: 2017-01865, 95/04 and 26/05).

NB xenograft

All *in vivo* procedures were performed in accordance with the guidelines of the Swiss Ordinance on Animal Protection and the Ordinance on Animal Experiments of the Federal Veterinary Office (FVO). The animal testing protocols were approved by the Swiss FVO (authorization number: VD2995). All reasonable efforts were made to reduce suffering, including anesthesia for painful procedures.

The NB-PDX material used in this study was derived from a previous study (17), except for the NB12-BM-2 model. The latter NB-PDX was generated from NB cells isolated from bone marrow aspirate of a patient at the time of diagnosis (male, 18 months at diagnosis, stage 4, MYCN amplified) and maintained *in vitro* for a limited number of passages in neuronal basal medium (<5). Primary NB cells ($1*10^6$) were suspended in 200 µl of Dulbecco Modified Eagle DMEM medium (Invitrogen, Luzern, Switzerland) and BD Matrigel Basement Membrane matrix (1:1; BD Biosciences, San Diego, CA, USA) and implanted subcutaneously (s.c.) into the flanks of athymic Swiss nude mice (Charles River Laboratories, France). Tumor growth was monitored using calipers every 3 days. Mice were sacrificed when tumors reached a volume of approximately 900 mm3. NB12-BM-2 correspond to the second *in vivo* passage of subcutaneous transplants. Tumor fragments were divided into pieces for paraffin-embedded tissue formation, or collected in 0.1 M perchloric acid for quantification of CATs and MNs, or snap-frozen in liquid nitrogen for protein or RNA extraction. NB xenograft fragments were also dissociated using the Mouse Tumor Dissociation Kit (Miltenyi Biotec GmbH, Germany) according to the manufacturer's instructions and filtered through CellTricks (50 µm; Partek, Inc, St Louis, MO, USA).

RNA extraction and real-time qPCR

RNA extraction was performed from fragmented tumor tissue with a micropotter using Trizol (Invitrogen, Luzern, Switzerland) and for cell lines using the RNAeasy kit with DNaseI treatment according to the manufacturer's instructions (Qiagen, Hombrechtikon, Switzerland). The synthesis of cDNA was performed from 1 µg of RNA using the PrimeScriptTM RT reagent kit (Takara Bio Inc, Japan). Real-time qPCR analyses for tumor tissues were performed in 384 wells using Sybergreen (Roche, Basel, Switzerland) as follows: 10 min at 95°C, 40 cycles of 15 sec at 95°C, 1 min at 60°C with the Applied Biosystems 7900HT SDS (Thermo Fischer Scientific, Reinach, Switzerland). Normalization of gene expression was performed on the three reference genes (RG) TBP, EEIF1A1, and GAPDH using the ΔCt

method with Ct_RG = (Ct_ TBP+Ct_GAPDH+Ct_EEIF1A1)/3 and mRNA expression ratio = $2^{-(Ct\_GeneX-Ct\_RG)}$.

Real-time qPCR for *in vitro* assay were performed in duplicates using the QuantiFast SYBR® green reagent (Qiagen, Hilden, Germany). Cycling conditions were: 5 min at 95°C, 40 cycles of 10 sec at 95°C, 30 sec at 60°C, and 1 sec at 72°C with the Rotor Gene 6000 real-time cycler (Corbett, Qiagen). Normalization of gene expression was performed on the two reference genes (RG) HPRT1 and SDHA using the ΔCt method with Ct_RG = (Ct_ HPRT1+Ct_SDHA)/2 and mRNA expression ratio = $2^{-(Ct\_GeneX-Ct\_RG)}$.

Primers were chosen with the primer designing tool from the National Center for Biotechnology Information (NCBI) and described in the Supplementary Table 3.

Immunoblotting

Tumor tissues were disaggregated and lyzed with a micropotter in lysis buffer (1x PBS, 0.5% triton X-100, and 1x protease inhibitor cocktail (Complete mini, EDTA-free, Roche, Mannheim, Germany) to represent 20% w/v. The tissue lysates were then sonicated and centrifuged at 2000 g for 30 seconds to remove pellets. Samples were fractionated by SDS-PAGE under reducing conditions using precast gels (Bio-Rad, Reinach, Switzerland). The loaded volumes were 10 µl from a 20% w/v extract. Next, proteins were transferred to a nitrocellulose membrane (Amersham Milian, Geneva, Switzerland) and probed with primary antibodies against SYP (1/1000, Cusabio Tech, ref. CSB-PA0004215 Chemie Brunschwig, Basel, Switzerland), TH (1/1000, Millipore, Zug, Switzerland ref AB-152), DBH (1/5000, generated against full-length recombinant human DBH protein (21) and ACTB (AC-15, 1/5000 Sigma-Aldrich Chemie, Buchs, Switzerland). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were from Bio-Rad (Cat. Nos. 170-6516 and 170-6515, respectively and diluted 4000x). Immunoreactive bands were revealed by a chemiluminescence assay (PerkinElmer, Schwerzenbach, Switzerland) and the signal was processed by a digital image analyser (ImageQuant LAS-4000, General Electric, Glattbrugg, Switzerland) and quantified using ImageJ software.

Tumor dissociation and primary cell culture

Tumor tissues were cut into small pieces and digested with collagenase (1mg/ml) (Sigma) in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), with shaking at 37°C until complete dissolution of tumor pieces. Cells were washed three times by centrifugation (235g for 2 minutes) and suspended in DMEM supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin G, and 100µg/ml streptomycin sulfate (Sigma) and seeded into 24-well plates. After 48 h of incubation in a humidified incubator at 37°C and 5% CO_2, the cell medium was collected, the cells were washed in PBS and lysed in 100µl of lysis buffer (0.1% tween 20) before quantification of CATs and MNs. Normalization of CATs and MNs quantification to protein levels was performed using a BCA assay (Thermofischer, Reinach, Switzerland) according to the manufacturer's protocol.

CAT and MN quantification

Tumor tissues and cells (primary and from cell lines) were disaggregated in lysis buffer (0.1% tween 20) and sonicated using a Branson Sonifier 450 (Branson, Danbury, CT, USA) at full power for 30 seconds. CATs in plasma (free forms) and MNs in tissue and cultured cells (free forms) and MNs in plasma (total forms, which consist of free and SO4-conjugated forms) were extracted using activated alumina (for CATs) or solid-phase extraction (for MNs) and quantified by ultra-high pressure liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) (22–24).

Cellular differentiation and exocytosis

The established human NB cell lines (SH-SY5Y and IGR-NB8) were obtained from their home laboratory and IMR32 from ATCC. Authentication of the SH-SY5Y and IMR32 cell lines used for the functional assay was performed by microsatellite short tandem repeat analysis before starting the transduction experiments (Microsynth, Switzerland). SH-SY5Y and IMR32 cell lines were incubated for 4 days with Bt2cAMP (Dibutyryl cAMP, N6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate sodium from Sigma) at 500nM in DMEM supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin G, and 100µg/ml streptomycin sulfate (Sigma) in 6-well plates in a humidified 5% CO_2 incubator at 37°C. Cell medium was collected and cells were resuspended in cold PBS, washed in PBS, and lyzed in 100µl of 0.1% TX-100 before quantification of CATs and MNs or used for RNA extraction as described above. For exocytosis experiments, the cell medium was removed and incubated with pre-warmed Krebs buffer (25) containing 56mM KCl or 100 µM nicotine for 45 minutes. After incubation, the cell medium was collected and the cells were resuspended and lyzed before quantification of CATs and MNs.

Electron microscopy and vesicle counting

Cells were fixed in glutaraldehyde solution (EMS, Hatfield, PA, USA) 2.5% in Phosphate Buffer (PB 0.1M pH7.4) (Sigma, St

Louis, MO, USA) during 1 hour at room temperature (RT). Then they were directly postfixed by a fresh mixture of osmium tetroxide 1% (EMS, Hatfield, PA, US) with 1.5% of potassium ferrocyanide (Sigma, St Louis, MO, US) in PB buffer during 1 hours at RT. The samples were then washed three times in distilled water and spin down in low melting agarose 2% in H_2O (Sigma, St Louis, MO, US), let to solidify on ice, cut in $1mm^3$ cube and dehydrated in acetone solution (Sigma, St Louis, MO, US) at graded concentrations (30%-40min; 50%-40min; 70%-40min; 100%-2x1h). This was followed by infiltration in Epon (Sigma, St Louis, MO, US) at graded concentrations (Epon 1/3 acetone-2h; Epon 3/1 acetone-2h, Epon 1/1-4h; Epon 1/1-12h) and finally polymerized for 48h at 60°C in oven. Ultrathin sections of 50nm were cut on a Leica Ultracut (Leica Mikrosysteme GmbH, Vienna, Austria) and picked up on a copper slot grid 2x1mm (EMS, Hatfield, PA, US) coated with a polystyrene film (Sigma, St Louis, MO, US). Sections were poststained with uranyl acetate (Sigma, St Louis, MO, US) 2% in H_2O during 10 minutes, rinsed several times with H_2O followed by Reynolds lead citrate in H_2O (Sigma, St Louis, MO, US) during 10 minutes and rinsed several times with H_2O. Two montages (8x8 tiles) per conditions with a pixel size of 9.48nm over an area of 120x120µm were taken with a transmission electron microscope Philips CM100 (Thermo Fisher Scientific, Waltham, MA USA) at an acceleration voltage of 80kV with a TVIPS TemCam-F416 digital camera (TVIPS GmbH, Gauting, Germany). The stereology analysis was performed using 3Dmod and its stereology plugin (IMOD software) (26). Briefly, a grid (500nm spacing) was applied on each montage and each intersection was defined as being part of the vesicles, nucleus and cytoplasm, allowing to determine the percentage of vesicles volume per cell volume.

Immunohistochemistry

Immunohistochemical analyses were performed on 3 µm thick microtomal sections obtained from formalin-fixed, paraffin-embedded tissue samples using specific commercially available antibodies directed against Chromogranin A, (CHGA) (mouse monoclonal, clone LK2H10, Ventana, Roche Diagnostic Corporation, Indianapolis, IN, USA), Synaptophysin (SYP) (rabbit monoclonal, clone SP11, Ventana), Tyrosine Hydroxylase (TH) (mouse monoclonal, clone 1B5, Novocastra, Newcastle, UK) and SLC18A2 (rabbit polyclonal, Chemicon, Temecula, CA, USA). Immunostains were performed manually as previously described (27), slides were observed under a light microscope (DM2000, Leica Microsystems, Wetzlar, Germany), and the presence of a positive immunoreaction was visualised as cytoplasmic brown stain. The results were scored semi-quantitatively as the percentage of positive cells in relation to the total, as well as with respect to intensity (+, weak; ++, moderate; +++, strong).

Statistics

The measurement data were explored statistically and graphically using Prism (v. 9.1.0, GraphPad Software, Inc. La Jolla, CA, USA). Methods used are described in the figure legends.

Result

NB tissues display massively reduced concentrations of MNs and CATs compared to PHEO/PGL

Because NB secretes MNs into the blood, as does PHEO/PGL, it would be expected to produce and contain massive amounts of CATs and MNs. However, early studies reported a small amount of CATs stored in NB tissues, compared with PHEO/PGL (14). As accurate quantification of CATs metabolites was not technically feasible at the time, we measured CATs and MNs levels by UHPLC-MS/MS in tumor tissues and in plasma of NB and PHEO/PGL patients. In order to compare the biosynthesis and metabolism of CATs and MNs in NB and PHEO/PGL patients we first determined the total amount of CATs (DA, NE and E) and MNs (MT, NMN, MN) stored in both type of tumors and released into the blood. In plasma, we observed comparable values of MNs (geo. mean NB: 151.8 and PHEO/PGL: 195.4 nmol/l, 1.3x), in contrast to CATs that were 3.5 fold higher in PHEO/PGL than in NB (NB: 3.8 and PHEO/PGL: 13.5 nmol/l) (Figure 1 and Supplementary Figures 1A-B). Although in plasma global MNs values were found in similar concentrations for NB and PHEO patients, MT levels were more elevated while MN levels were reduced in NB relative to PHEO. In tumor tissues, drastic reductions in MNs (-103x) and CATs (-1671x) concentrations were observed in NB as compared to PHEO/PGL, with 0.53 versus 54.9 nmol/g for MNs and 5.4 versus 8992 nmol/g for CATs, respectively (Figure 1 and Supplementary Figures 1C-D). This confirms, in a large cohort of patients, the striking difference in CATs and MNs concentrations in NB versus PHEO/PGL.

Chromaffin markers and neurosecretory vesicle content are reduced in NB compared with PHEO/PGL

The lower concentration of CATs detected in NB tissues compared with PHEO/PGL may result from reduced production of CATs, more efficient conversion to MNs, and/or a lower amount of neurosecretory vesicles (NVs) in the cytoplasm. In the latter scenario, newly synthesized DA is metabolized to MT by COMT and a fraction is converted to NE by DBH present in

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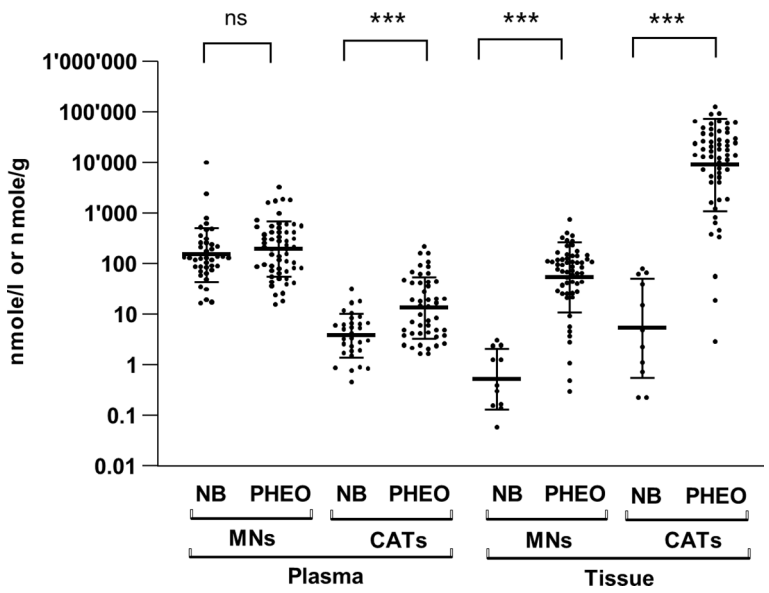

FIGURE 1
Plasma and tissue concentrations of CATs and MNs in NB and PHEO. Sum of CATs/MNs values and geo mean ± geo SD are plotted on a logarithmic scale and analyzed with a non-parametric Mann-Whitney test (non significant= ns, ***=p<0.001, ****=p<0.0001). Values are reported in the text. Number of patients for plasma values: MNs: NB (n=41), PHEO/PGL (n=57); CATs: NB (n=31), PHEO/PGL (n=47); and for tissue values: MNs: NB (n=11), PHEO/PGL (n=57); CATs: NB (n=11), PHEO/PGL (n=57). MNs concentrations represent total forms (free and SO_4-conjugated forms) in plasma and free forms in tumors (no conjugated forms are detected in tumor). CATs values represent free forms in both plasma and tumors. Part of these values for NB (n=22/41 for plasma MNs, n= 21/31 for plasma CATs and n=10/11 for tissue values for CATs and MNs) were already published in another study comparing CATs values in human and mice with NB (17) (Supplementary Table 1).

the rare NVs. NE is then metabolized to NMN in the cytoplasm and MT and NMN diffuse freely into the blood. To explore these hypotheses, we compared the expression levels of key enzymes involved in CAT metabolism (TH, DBH, DDC, PNMT, COMT, and MAOA) and NV markers in NB and PHEO/PGL in four NB primary tumor datasets and two PHEO/PGL series using the R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl). We choose as NV markers in addition to SLC18A1/2, synaptophysin (SYP), which is an integral NV membrane protein, as well as chromogranin A and B (CHGA/B) and secretogranin 2 (SCG2), three prohormones co-released with CATs. These proteins are commonly used as general neuroendocrine markers in IHC analyses (18, 28–32). This *in silico* analysis in large tumor datasets demonstrated that the expression levels of TH, DDC, and DBH were reduced in NB compared to PHEO/PGL, inversely to MAOA levels. As previously demonstrated, PNMT levels were almost undetectable in NB and highly variable in PHEO/PGL due to the adrenergic and noradrenergic phenotypes of these tumors (16), while very similar levels of COMT were found in NB and PHEO. Regarding NV markers, the levels of SYP, SLC18A1/2, CHGA/B and SCG2 were also lower in NB compared to PHEO/PGL (Figure 2A). These data were confirmed by qPCR mRNA quantification in a cohort of tissues from NB and PHEO/PGL with again a strong decrease in TH, DDC, DBH expression levels in NB (Figure 2B).

We also validated these observations at the protein level, as PHEO/PGL tissues gave a stronger signal by IHC for TH and particularly for the vesicular markers SYP and SLC18A2 and the secretory granular marker CHGA compared with NB and NB-PDX tissues (Figure 2C). By immunoblotting, it was confirmed that SYP was highly expressed in PHEO/PGL while only a weak signal was detected in 5 NB and 6 NB-PDX biopsies (Figure 2D). It is noteworthy that NB-PDX tumors were recently described by our group as representing a reliable tool to study CAT metabolism in NB (17). Overall, these data suggest not only a reduced production of CATs but also a reduced amount of NV in NB compared to PHEO/PGL.

To directly confirm that these different expression levels of NV markers correlate with the amount of NV in the cytoplasm of NB and PHEO/PGL, we performed electron microscopy on isolated cells from PHEO/PGL biopsy and NB-PDX tissue. We observed and counted numerous densely nucleated vesicles corresponding to CATs storage NVs in PHEO (P86) cells with a mean ± SD of 124 ± 22.03 NVs/cellular field (n=20). This is in contrast to NB cells where the cytoplasm was almost devoid of NVs in all 3 cell types studied (mean ± SD for NB12-BM2: 1.1 ± 0.98 NV/cellular field (n=44), for NB4-BM-8: 0.8 ± 0.65 NV/

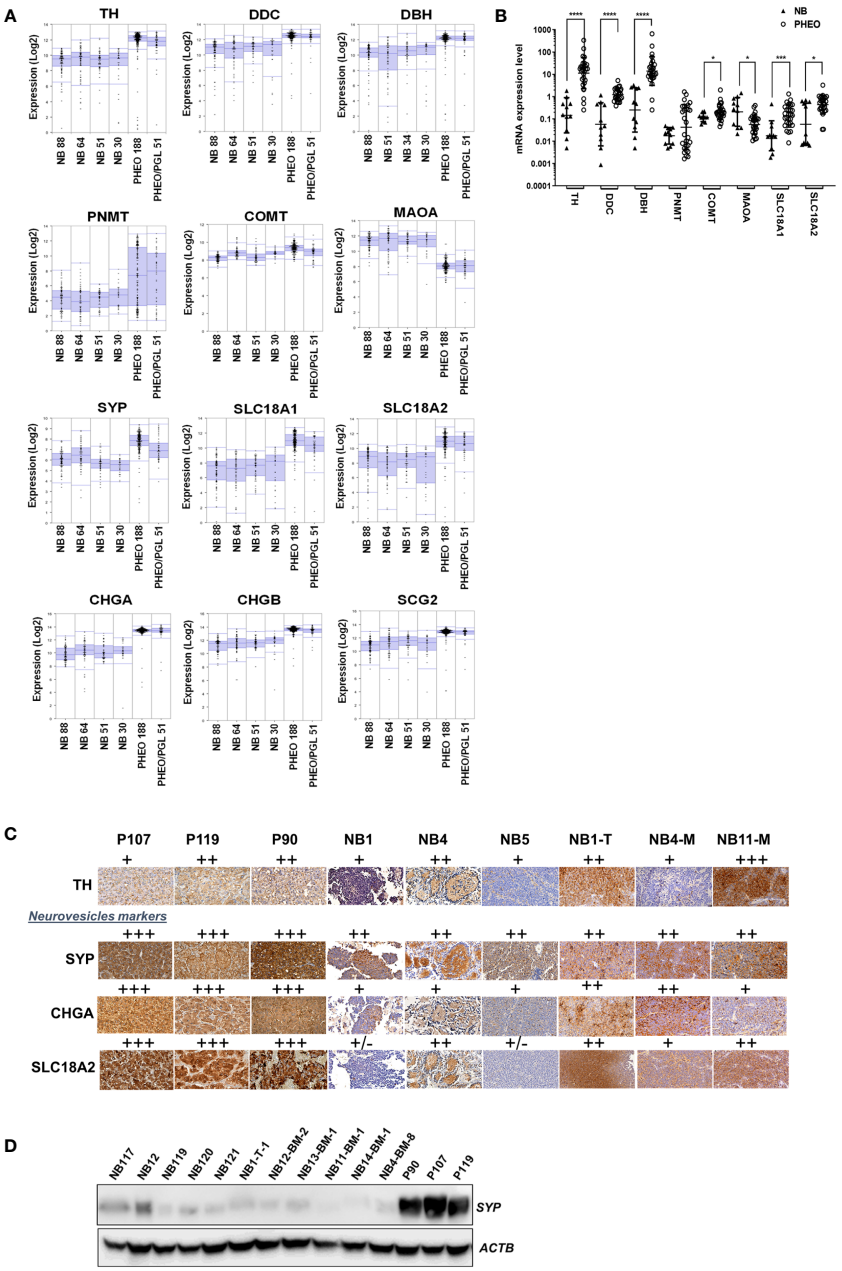

FIGURE 2
Expression level of genes involved in CATs metabolism and NV markers in NB and PHEO. **(A)** Expression levels (log2) of the indicated genes in 4 NB transcriptomic datasets (Versteeg n=88, Hiyama n= 51, Delattre n=34, Lastowska n=30) and 2 PHEO/PGL datasets (Favier n=188, and Korpershoek n=51) analyzed by microarray using the R2: Genomics Analysis and Vizualization Platform (http://r2.amc.nl, MegaSampler analysis: Human Genome U133, Plus 2.0; MAS5.0 data normalization). **(B)** mRNA quantification by RT-qPCR of the main enzymes involved in CATs metabolism and NV markers from NB (n=10) and PHEO/PGL (n=28) tissues. Individual values and geo means ± SD are plotted and analyzed with a non-parametric Mann-Whitney test. *p* values are reported only when statistically significant (<0.05), *=$p<0.05$, ***=$p<0.001$, ****=$p<0.0001$). The values for NB were already published in another study comparing primary NB and NB-PDX (17) (Supplementary Table 1). **(C)** Representative images of IHC staining the detection of TH, SYP, CHGA and SLC18A2 protein expression on three PHEO/PGL tissue: P90, P107, P119, three NB biopsies from patients: NB1, NB4 and NB5 and three NB-PDX: NB1-T-1, NB4-BM-8, NB11-BM-1. The results were semi-quantitatively scored (+, faint; ++, moderate; +++, strong). **(D)** Immunoblotting for the detection of SYP in tissue samples from five NB biopsies (4 after chemotherapy and one, sample NB-121 at the time of diagnosis), six NB-PDX : NB1-T-1, NB12-BM-2, NB13-BM-1, NB11-BM-1, NB14-BM-1, and NB4-BM-8, and from three PHEO/PGL: P90, P107, P119. Volumes loaded were 10µl from a 20% wt/vol extracts. ACTB was used as loading control.

cellular field (n=39), and for NB11-BM-1: 1.25 ± 1.47 NV/ cellular field (n= 43) (Figure 3).

Low amounts of CATs in the cytoplasm of NB cell lines and primary cells, and in PDX

Given the very low amounts of CATs and MNs metabolites stored in NB tissues (Figure 1), we sought to further investigate the molecular basis for this finding. Thus, the concentrations of CATs and MNs were measured in cell lysates and supernatants of 4 noradrenergic NB cell lines: IGR-NB8, SH-SY5Y, LAN-1, IMR32, and a PHEO/PGL cell line (PC12), as well as dissociated primary cells from two PHEO/PGL biopsies (P86, P88) and two NB-PDX (NB11-BM-1, NB12-BM-2) (17) cultured *in vitro*. We observed trace amounts near or below our limit of quantification for CATs in the culture medium of all NB cell lines and PDX-derived cells. This is in contrast to the substantial amounts of CATs detected in the cell medium of PC12 (mainly due to the concentration of DA) and PHEO/PGL primary cells (P86 and P88) (Figure 4A). The differences between NB and PHEO/PGL were even greater when considering the amounts of CATs and MNs stored in the cytoplasm of the cells (Figure 4B). Interestingly, NB primary cells (NB11-BM1 and NB12-BM2) had a higher MNs content than CATs, whereas the opposite ratio was observed for PHEO/PGL primary cells where a massive concentration of CATs was detected, giving further evidence that a significant amount of CATs is not stored inside NVs and available for processing into MNs in NB.

Induction of NV genesis allows the NB cell line to protect and secrete CATs

Although CATs are actively synthesized in NB cells, the scarcity of NVs prevents the protection of CATs from degradation by MAOA and COMT enzymes, resulting in pathological values for MNs in the blood of patients. This could also explains the very low amount of CATs measured in NB tissues and the normal values of CATs in the blood of most NB patients. To address this hypothesis, we therefore investigated whether an increase in NV synthesis correlated with an increase in CATs concentration and whether newly synthesized NVs were functional in terms of CATs secretion upon pharmacological stimulation. To this end, SH-SY5Y and IMR32 cells were treated with Bt2cAMP to induce differentiation into a noradrenergic phenotype, as previously described (33, 34). We observed an increase in intracellular DA and NE levels after 4 days of treatment compared to untreated cells. For SH-SY5Y: a 9-fold increase for DA and 15.8-fold increase for NE was recorded and for IMR32 the fold change was 4.8 and 9.9 for DA and NE, respectively, while no E was detected in both cell lines due to the absence of PNMT in NB cells (17) (Figure 5A). Intracellular concentrations of NMN and MT were also increased in both cell lines: SH-SY5Y: 1.5-fold for

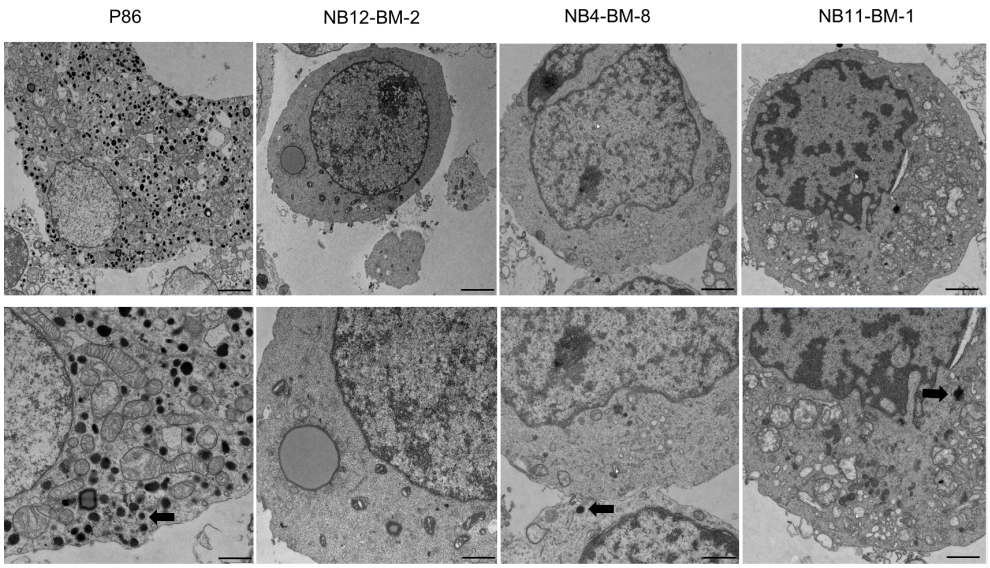

FIGURE 3
Analysis of the amount of neurosecretory vesicles in primary NB and PHEO cells by electron microscopy. Representative images of electron micrographs of PHEO/PGL and NB-PDX cells dissociated from primary tumor biopsies and xenografts, respectively. Lower panels: zoom of upper panels showing in details the cytoplasm. Thick arrows shows the electron dense content that correspond to CATs in neurosecretory vesicles. Scale bars: 1µm (upper panels, 1st and 2nd column), 2µm (upper panels, 3rd and 4th column) and 500nm (lower panels).

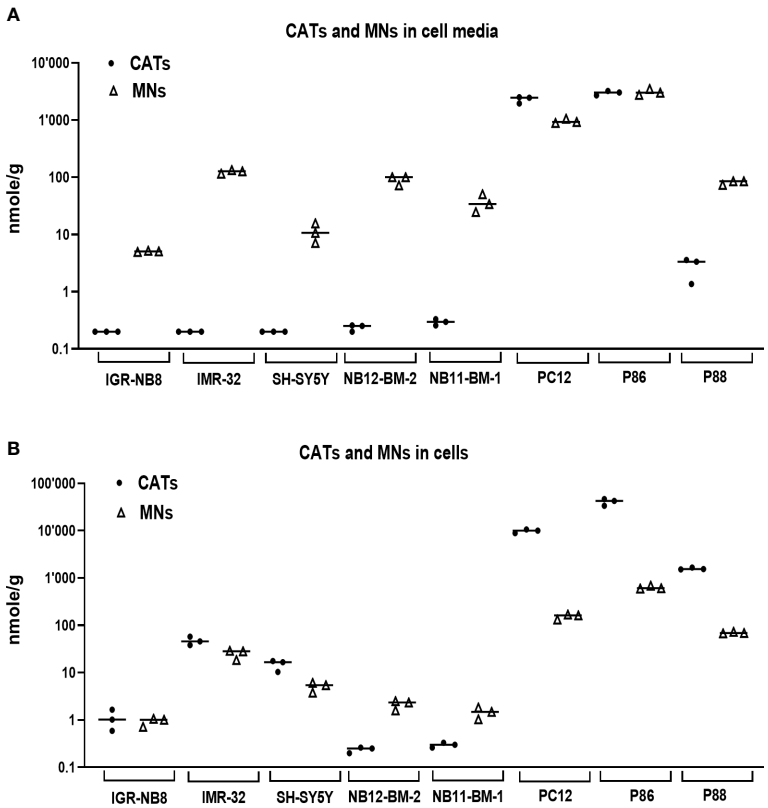

FIGURE 4
CATs and MNs concentrations in NB and PHEO/PGL cells *in vitro* and their culture media. **(A)** Quantification of sum of CATs (dots) and MNs (triangles) in cell culture medium (in nmol/g of protein in cell lysates) of three NB cells lines, two primary cell sample from NB-PDX (NB11-BM-1, NB12-BM-2), the PC12 cell lines, and two PHEO/PGL biopsies (P86 and P88). Assays were performed in triplicate and corresponding values are plotted in the graph with indication of the mean: IGR-NB8: 0.2 and 5.1 nmol/g for CATs and MNs respectively; IMR32: 0.2 and 125.2; SH-SY5Y: 0.2 and 11.1; NB12-BM-2: 0.24 and 91; NB11-BM-1: 0.3 and 36.4; PC12: 2300 and 957; P86: 2990 and 3087; P88: 2.8 and 81.3. Values under our limit of quantification were set at 0.025, 0.125 and 0.075 nmol/g for respectively E, NE and DA, which represent half of their lower limit of quantification (LLOQ). **(B)** Quantification of intracellular CATs and MNs (in nmol/g of protein) in the corresponding cell lysates from **(A)** Mean values: IGR-NB8: 1.1 and 0.9 nmol/g for CATs and MNs respectively; IMR32: 47.1 and 25.3; SH-SY5Y: 14.9 and 5.1; NB12-BM-2: 0.24 and 2.2; NB11-BM-1: 0.3 and 1.5; PC12: 9872 and 154.6; P86: 40910 and 634; P88: 1576 and 70.

MT and 6.2-fold for NMN. For IMR32, the increase was 4.5-fold for MT and 13.8-fold for NMN (Figure 5B). In the incubation medium, very low levels of CATs were detected for SH-SY5Y cells with or without BT2cAMP, whereas differentiation induced an increase in CATs concentration for IMR32 cells (DA: 18.3x, NE: 2x). The levels of CATs released into the culture media were also very low compared with MNs, which were increased upon Bt2cAMP treatment (SH-SY5Y fold change: 11.1X for MT and 9.7X for NMN; and IMR32: 6.1X for MT and 15.3X for NMN) (Figures 5C, D).

The molecular basis of the NB cell response to differentiation was then analyzed by measuring the mRNA expression levels of TH and DBH and NV markers (SYP, CHGA/B, SLC18A1/2) (Figure 6A), as well as the protein levels of TH, DBH, and SYP (Figures 6B, C). The expression of TH was significantly increased in both cell lines, which was confirmed at the protein level. The level of DBH mRNA was significantly increased in the IMR32 line after Bt2cAMP treatment, but DBH protein expression was below the limit of detection. This explains the low amount of NE and NMN produced by this cell line compared with DA and MT (Figures 5A, B). Overall, mRNA expression levels of NV markers were higher in treated cells compared with controls, with the exception of CHGA mRNA, which was slightly reduced by Bt2cAMP treatment in SH-SY5Y cells, and SYP protein level, which was reduced in SH-SY5Y cells.

Because several NV markers were upregulated after pharmacological differentiation of both cell lines, we performed

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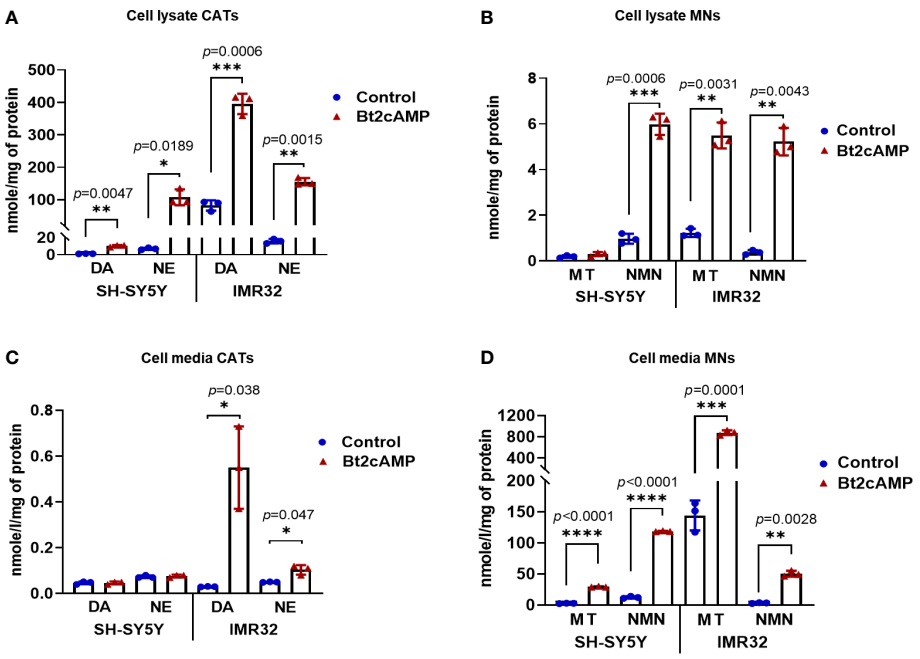

FIGURE 5
Induction of CATs synthesis through Bt2cAMP treatment of NB cells. Concentrations of CATs and MNs in SH-SY5Y and IMR32 cell lysates **(A, B)** and cell culture media **(C, D)** after 4 days with or without Bt2cAMP incubation. Assays were performed in triplicate and corresponding values are plotted in the graph with mean and SD. **(A)** CATs: SH-SY5Y: DA: 10.21 vs 1.14 nmol/g (treated vs control), NE: 107.5 vs 6.82 nmol/g; IMR32: DA: 395.5 vs 82.13 nmol/g, NE 155.43 vs 15.67 nmol/g, respectively. **(B)** MNs: SH-SY5Y: MT: 0.29 vs 0.19 nmol/g, NMN: 5.98 vs 0.97 nmol/g; IMR32: MT: 5.49 vs 1.22 nmol/g, NMN: 5.22 vs 0.37 nmol/g, respectively. **(C)** CATs: IMR32: DA: 0.55 vs 0.03 nmol/g, NE 0.1 vs 0.05 nmol/g, respectively **(D)** MNs: SH-SY5Y: MT: 29.37 vs 2.65 nmol/g, NMN: 118.75 vs 12.29 nmol/g; IMR32: MT: 877.89 vs 144.35 nmol/g, NMN: 50.49 vs 3.29 nmol/g. Individual values and means ± SD are plotted and analyzed with a Welch's t-test. *p* values are reported only when statistically significant (<0.05), *=$p<0.05$, ***=$p<0.001$, ****=$p<0.0001$).

electron microscopy studies to morphologically assess a possible increase in NV size and/or concentration in the cell cytoplasm. Using a stereological method for NV quantification (see Materials and Methods), we measured a 2- and 2.5-fold increase in NV volume in IMR32 and SH-SY5Y cell lines, respectively, after treatment with 500 nM Bt2-cAMP for 4 days compared with controls (Figure 6D, Supplementary Figure 2).

We next investigated whether differentiation led to proper internalization of CATs into newly formed NVs, and thus whether differentiated cells could respond to exocytosis stimuli, such as KCl or nicotine (25). Because SH-SY5Y cells do not produce enough CATs to be reliably quantified in the cell medium, even after pharmacological differentiation, the tests were performed on IMR32 cells only. The percentage of NE exocytosis was statistically significantly increased after treatment with KCl or nicotine by 4 and 5.4-fold, respectively (Figure 6E).

Discussion

Both NB and PHEO/PGL are non-epithelial neuroendocrine neoplasms arising from sympathoadrenal tissues. Plasma MNs represent reliable biomarkers for these CATs-producing tumors (19, 35), however, NB tissues have been shown to contain low amounts of CATs, contrasting with the massive concentrations in PHEO/PGL (14, 36). In this study, we performed an extensive comparative analysis of the biosynthesis, metabolism, and storage of CATs and MNs in NV for both tumor types. First, using UHPLC-MS/MS to quantify the metabolites of CATs and MNs in tumors and plasma, we showed that intratumoral concentrations of CATs in NB are several orders of magnitude lower than those in PHEO/PGL, confirming early studies performed with less sensitive methods (14, 36). Furthermore, we demonstrated that the amount of MNs is also greatly reduced in NB compared to PHEO/PGL tumor tissues. In contrast, in plasma, we observed that CATs were slightly higher in PHEO/PGL-affected individuals, whereas overall MNs levels were comparable between NB and PHEO/PGL patients. However, the relative profiles of MNs were nevertheless distinct in the plasma of the two tumor types, with higher concentrations of MT and reduced levels of MN in NB compared to PHEO/PGL, as expected due to the noradrenergic phenotype of NB (17).

Next, we analyzed and compared the expression levels of various chromaffin markers, including enzymes involved in

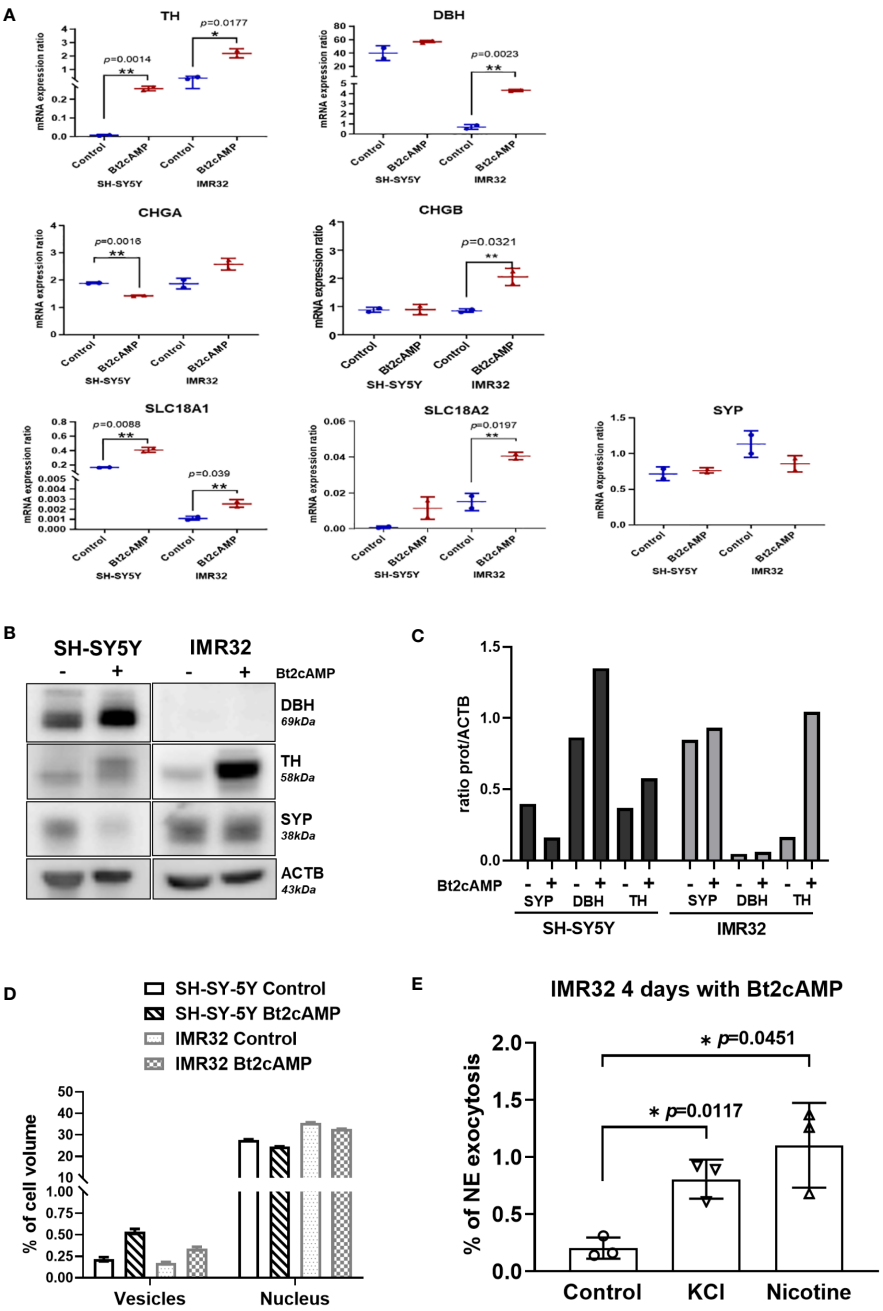

FIGURE 6
NV induction and exocytosis in cAMP treated cells. **(A)** mRNA quantification of TH, DBH, CHGA, CHGB, SLC18A1, SLC18A2 and SYP in SH-SY5Y and IMR32 cell lines treated without (Control) and with 500nM Bt2cAMP for 4 days. Fold change: TH: 42.6 in SH-SY5Y and 6 in IMR32, DBH: 1.42 in SH-SY5Y, 6.2 in IMR32, CHGA: 0.75 in SH-SY5Y and 1.38 in IMR32, CHGB: 1.5 in IMR32 cells, SLC18A1: 2.5 in SH-SY5Y and 2.3 IMR32; SLC18A2: 12.8 in SH-SY5Y and 2.7 in IMR32. Assays were performed in triplicate and individual values and means ± SD are plotted and analyzed with an unpaired t-test. *p* values are reported only when statistically significant (<0.05). *=$p<0.05$, **=$p<0.01$. **(B)** Immunoblotting for SYP, DBH and TH expression in cells treated as in **(A, C)** Quantification of immunoreactive signal normalized with ACTB protein expression using the Image J software. **(D)** Bare graph representing the counting of NVs (vesicles) and nucleus volume per cell using stereologic method on 2 fields of 120x120 µm per condition (approx. 30 and 40 cells/fields for SH-SY5Y and IMR32 cells, respectively). **(E)** Quantification of NE following chemical stimuli (56mM KCl or 100 µM nicotine) from IMR32 cells incubated 4 days with 500 nM Bt2cAMP. Values are expressed in % of NE found in cell medium regards to total NE (cytoplasmic plus cell medium concentrations). NE: values for control, KCl and nicotine: 0.2, 0.81 and 1.1% respectively. Assays were performed in triplicate and individual values and means ± SD are plotted and analyzed with a Welch's t-test. *=$p<0.05$.

CATs biosynthesis (TH, DDC, DBH, PNMT) and transformation (COMT and MAOA), as well as markers of CATs storage vesicles (SYP, SLC18A1/2, CHGA, CHGB and SCG2) between NB and PHEO/PGL. Our data revealed reduced expression of most of these markers in NB, with the exception of MAOA and COMT. Low MAOA expression in PHEO/PGL compared to healthy tissue has been previously reported (12), but the consequence of this higher expression in NB has not been studied in detail. As NB and PHEO express a similar amount of COMT, this may explain why MNs are also reliable biomarkers for NB despite their reduced capacity for CATs production and storage. Interestingly, this set of genes, as well as other chromaffin markers, were identified as genes specifically overexpressed in NB and PHEO/PGL compared to various cancerous and normal tissue settings in a large-scale *in silico* analysis of transcriptomic data (37). However, although their differential expression analysis between NB and PHEO/PGL confirmed the downregulation of many chromaffin markers in NB (except MAOA: overexpressed, COMT: not differentially expressed), the authors did not report this fact in their manuscript.

The low expression levels of chromaffin markers in NB as a CATs-producing tumor suggest a reduced capacity for CATs synthesis and storage compared with PHEO/PGL. Here, we also demonstrate that the low amount of CATs stored in the cytoplasm of NB cells is mainly due to a low amount of NVs produced in the majority of tumor cells, as illustrated by electron microscopy analysis showing the paucity of NVs identified in NB cells, which is in accordance with previous studies (14, 18, 38).

One of the reasons for the low concentration of NV could come from a downregulation of several proteins involved in NV biogenesis with notably myosin 1b and F-actin (39), or as more recently demonstrated CHGA (40, 41). It is interesting to note that this last protein is present in high concentration in PHEO and in lower concentration in NB, although CHGA represents a histochemical marker of these two types of tumors. Thus, a low concentration of CHGA in NB could be one of the reasons rather than a consequence of the lower number of NV in NB compared to PHEO.

A recent study in primary PHEO cells demonstrated an increase in many proteins involved in vesicular exocytosis or CATs synthesis as well as a higher number of exocytotic events in PHEOs compared with chromaffin cells at the single cell level for the same stimulation (42). This suggests in cells secreting high concentrations of CATs an increase in the number of NVs rather than an increase in the storage capacity of each NV. In this case, a larger storage of CATs will necessarily imply more of these NVs in the cytoplasm while an insufficient number will imply rapid degradation to MNs and/or DHPG. It is noteworthy that NB and PHEO express similar amounts of COMT. This may explain the high level of MNs in NB despite a low amount of NVs, as the CATs that escape from the few NVs are efficiently transformed into MNs.

Whereas PHEO/PGLs derive from fully differentiated chromaffin cells of the adrenal medulla, pediatric solid tumors, such as NB, are thought to arise from developmental defects affecting the normal sympathoadrenergic differentiation and maturation program (43). Therefore, the reduced amount of noradrenergic markers and neurosecretory vesicles observed in NB compared to PHEO/PGL may result from blockade in the differentiation program in sympatho-adrenal progenitors at the origin of NB, although partial dedifferentiation cannot be excluded. Indeed, recent single-cell transcriptomic analysis of primary NBs and adrenal glands confirmed that NBs are predominantly composed of cells with transcriptional signatures of adrenal neuroblasts/sympathoblasts, which are distinct from adrenal chromaffin cells, although a small proportion of chromaffin cells have been identified in several high-risk NBs (44, 45).

Differentiation with retinoic acid (13-cis RA) is part of the maintenance phase of current treatment protocols for high-risk NB. It has been shown to inhibit cell proliferation and induce differentiation characterized by increased expression of various neuronal markers and neurite outgrowth *in vitro* (46, 47). However, retinoic acid has been shown to be ineffective in inducing differentiation to a noradrenergic/chromaffin phenotype, as the CATs concentration remains low (48, 49). A recent study provided a mechanistic explication for these observations, as it was that retinoic treatment reprograms the enhancer landscape and alters the noradrenergic core regulatory circuitry (NOR-CRC) of NB cells, by reducing the expression of the transcription factors Phox2b, GATA3, and MYCN (50). In contrast, Bt2cAMP has been shown to induce noradrenergic differentiation in NB cell lines, as evidenced by increased CATs synthesis and TH expression (33, 34, 49). Here, our functional studies using Bt2cAMP as a differentiating agent resulted in an increased in NV number and volume as well as upregulation of the NV marker SLC18A1/2. Bt2cAMP also induced the synthesis of TH, the rate-limiting enzyme for CATs synthesis, and DBH, leading to the concomitant increase in CATs and MNs biosynthesis, as revealed by their higher concentrations in the cell cytoplasm and in the culture media. Furthermore, we demonstrated that these newly produced NV mediated by Bt2cAMP were fully functional as exocytosis stimuli led to an increase in CATs in the cell media.

A limitation of this study is that our cohort of NB and PHEO/PGL samples used for the measurement of CATs and MNs in plasma and tumor tissue, as well as NV detection, is only partially complete (some samples were not available for metabolite, mRNA, or protein quantification because of material scarcity), as detailed in Supplementary Tables 1A, B.

For a similar reason, electron microscopy detection of NVs for NB was performed in PDX-derived tumor cells and cell lines rather than using primary tumor-derived cells. However, our NB-PDX models have been shown to closely mimic primary NB in terms of CATs synthesis, metabolism and storage (17).

From a clinical point of view, our data may explain at the cellular and molecular levels the low incidence of hypertension recorded in children with NB, which contrasts with the hypertension usually associated with PHEO/PGL. Indeed, NB cells containing rare NV cannot efficiently store E and NE and release them into plasma by exocytosis. This differs from PHEO/PGL, where hypertension is a classic symptom of the disease, because of the massive and episodic exocytosis of CATs into the bloodstream. As an extension of this study, it would be interesting to evaluate whether, in NB, the amount of intratumoral CATs and NV is increased for the rare NB patients diagnosed with tumor-induced hypertension without renal artery compression.

In conclusion, we demonstrated that the metabolism of CATs in NB differs from that well-characterized in PHEO/PGL, with low amounts of chromaffin and NV markers in NB, resulting in low intratumoral and plasma concentrations. Thus, in NB cells, DA synthesized in the cytoplasm by DDC is immediately available for conversion to DOPAC *via* MAOA, to MT *via* COMT, or to NE *via* DBH in NV. Because cytoplasmic NE and DA are available for MAOA and COMT catalysis, DHPG, DOPAC, NMN, and MT are therefore produced in large quantities and these metabolites subsequently diffuse into the bloodstream (10). DHPG and DOPAC are not specific tumor markers due to their synthesis in other tissues and cell types, so they were not measured in this study. This scenario would explain the increase in plasma NMN and MT concentrations, which are used as biomarkers of NB (3). Overall, our study also updated and detailed with modern technologies the early observations on cellular CAT metabolism in NB (18, 36).

Data availability statement

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

Ethics statement

The protocol for this study was approved by the local Ethics committee and all families signed an informed consent. Animal experimentation protocols were approved by the Swiss Federal Veterinary Office.

Author contributions

AM-M and KA conceived the study. KA, KB, SU, PE, AM-M and JD performed the experiments. MP, EG, AM-M and KA interpreted the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1027856/full#supplementary-material

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Intratumoral immunotherapy of murine pheochromocytoma shows no age-dependent differences in its efficacy

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Cancer immunotherapy has shown remarkable clinical progress in recent years. Although age is one of the biggest leading risk factors for cancer development and older adults represent a majority of cancer patients, only a few new cancer immunotherapeutic interventions have been preclinically tested in aged animals. Thus, the lack of preclinical studies focused on age-dependent effect during cancer immunotherapy could lead to different therapeutic outcomes in young and aged animals and future modifications of human clinical trials. Here, we compare the efficacy of previously developed and tested intratumoral immunotherapy, based on the combination of polysaccharide mannan, toll-like receptor ligands, and anti-CD40 antibody (MBTA immunotherapy), in young (6 weeks) and aged (71 weeks) mice bearing experimental pheochromocytoma (PHEO). The presented results point out that despite faster growth of PHEO in aged mice MBTA intratumoral immunotherapy is effective approach without age dependence and could be one of the possible therapeutic interventions to enhance immune response to pheochromocytoma and perhaps other tumor types in aged and young hosts.

KEYWORDS

intratumoral immunotherapy, age, pheochromocytoma, TLR ligands, pancreatic adenocarcinoma, anti-CD40 antibody

Abbreviations: AUC, area under curve; B16-F10, murine model of melanoma; BAM, biocompatible anchor for cell membrane; CT26, murine model of colon carcinoma; LTA, lipoteichoic acid; MBTA, mannan-BAM +TLR ligands+anti-CD40; MTT, pheochromocytoma mouse tumor tissue-derived cells; Panc02, murine model of pancreatic adenocarcinoma; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; PHEO, pheochromocytoma; Poly(I:C), polyinosinic:polycytidylic acid; R-848, resiquimod; TLR, tool-like receptor; TRAMP-C2, murine model of prostate adenocarcinoma; TUBO, murine model of mammary tumor; qRT-PCR, real-time polymerase chain reaction.

1 Introduction

Undoubtedly, the incidence of various cancers is increasing with age (1). The process of immune dysfunction that occurs with age, so-called immunosenescence, is characterized by increased autoimmune diseases, decreased adaptive immune response, and increased risk of infections (2). It has also been hypothesized that age-related immune system dysfunction can decrease cancer immunosurveillance and lead to immunosurveillance escape and tumorigenesis. Despite of increasing interest in intratumoral immunotherapies and age-associated differences in immune system function, there is limited number of studies that have been focused on these differences in aged mice and humans. The lack of age-related research and clinical trials that include aged patients could lead to the failure of new treatment approaches in those patients (3–5).

MBTA immunotherapy is based on intratumoral injection of mannan-BAM, polysaccharide from *Saccharomyces cerevisiae via* with a biocompatible anchor for cell membrane (BAM), a mixture of toll-like receptor (TLR) ligands, and anti-CD40 antibody. After the intratumoral injection, mannan-BAM anchors to the tumor cell surface via the BAM anchor and serves as a ligand stimulating phagocytosis via the activation of the complement lectin pathway and tumor cell opsonization (6–8). Mixture of TLR ligands, namely resiquimod, polyinosinic-polycytidylic acid, and lipoteichoic acid, supports the infiltration of immune cells into tumors, as well as their activation (9–11). Anti-CD40 is an agonistic monoclonal antibody that mimics the CD40 ligand expressed on helper T cells. After the ligation of CD40 expressed on antigen-presenting cell, such as macrophages, dendritic cells, and B cells, anti-CD40 leads to the activation of these cells, enhances antigen presentation, and induces an effective T cell anti-tumor response (12, 13). MBTA therapy has been previously tested in various subcutaneous tumor models in mice, such as melanoma (B16-F10), pancreatic adenocarcinoma (Panc02), colon carcinoma (CT26), and pheochromocytoma (PHEO, MTT cells) with the complete elimination of tumors in 62-83% of mice depending on tumor type (14–16). MBTA therapy has also shown systemic immune response in PHEO models including its metastatic model, bilateral Panc02, and bilateral CT26 model, where the therapy resulted in slower progression of non-treated distal tumors and significant prolongation of survival of treated mice (14, 16, 17). Moreover, strong infiltration of both adaptive and innate immune cells was observed in treated and distal tumors in case of bilateral PHEO model (18).

PHEO are rare catecholamine-producing neuroendocrine tumors derived from neural crest cells (19). These tumors are most common between the age 30-50 depending on catecholamine phenotypes and genetic background (20), but about 10-20% of all cases are diagnosed in pediatric patients (21). All the aforementioned studies focused on intratumoral MBTA therapy in murine PHEO have been performed in 6-8 weeks old mice which is equivalent to human 11-16 years of age (22). Therefore, in the present study, we assessed the efficacy of MBTA therapy in 6-weeks and 71-weeks old mice, which correlates to 11 and 56 years of human age, to evaluate possible age-dependent difference and more simulate a situation in patients with PHEO. To confirm our results on different tumor model, we also tested MBTA therapy in aged and young mice bearing bilateral Panc02 tumor model which more mimics the real situation with higher tumor burden.

2 Materials and methods

2.1 PHEO cell line, mice, tumor model establishment, and tumor size evaluation

Mouse tumor tissue-derived (MTT) cells, used in this study, are rapidly growing cells derived from liver metastases of mouse pheochromocytoma (MPC) cells (23, 24). Cells were maintained in Dulbecco's modified eagle media (DMEM) (Sigma-Aldrich, Saint Lous, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gemini, West Sacramento, CA, USA), and 100 U/mL of penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Waltham, MA, USA). Cells were cultured at 37 °C in humidified air with 5% CO_2. Cell lines was tested for mycoplasma using the MycoAlert™ detection kit purchased from Lonza (Walkersville, MD, USA). Both female young (6 weeks) and aged (71 weeks) C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). For establishment of PHEO tumors, mice were subcutaneously injected in the previously shaved right lower dorsal site with 3×10^6 MTT cells in 0.2 mL of DMEM without additives. For rechallenge experiment, the mice were s.c. injected in the previously shaved left lower dorsal site (opposite site) with the same number of MTT cells as mentioned above.

Tumor volume was measured every fourth day with a caliper and calculated as $V = (\pi/6)\ AB^2$ (A and B, the largest and the smallest dimension of tumor, respectively). Survival curves are based on (1): the time of death of experimental mice, (2) the time of euthanasia of experimental mice reaching the maximal allowed tumor volume 2000 mm^3, (3) tumor diameter exceeding 2 cm, (4) non-healing skin necrosis over the tumor. Materials and methods for Panc02 model are described in Supplementary Material.

2.2 MBTA therapy

Mannan from *Saccharomyces cerevisiae*, lipoteichoic acid (LTA) from *Bacillus subtilis*, and polyinosinic:polycytidylic acid (poly(I:C)) was obtained from Sigma-Aldrich (Saint Lous, MO, USA). BAM was obtained from NOF Corporation (White Plains, NY, USA). Resiquimod (R-848) was obtained from Tocris Bioscience (Minneapolis, MN, USA). Monoclonal anti-CD40 (clone FGK4.5/FGK45) was obtained from BioXCell (West Lebanon, NH, USA). Mannan-BAM synthesis was performed as previous reported (25). After development of tumors (average tumor volume 55 mm^3), mice were treated intratumorally in days 0, 1, 2, 8, 9, 10, 16, 17, 18, 24, 25, and 26 with 50 µL of the therapeutic mixture consisting of 0.5 mg R-848 (HCl form), 0.5 mg poly(I:C), 0.5 mg LTA, and 0.4 mg

anti-CD40 per mL of 0.2 mM mannan-BAM in PBS (MBTA therapy).

2.3 Real-time polymerase chain reaction

Pheochromocytoma samples (n=6/group) were dissected on day 46 after subcutaneous transplantation of tumor cells. Total RNA was extracted using PureLink RNA miniKit (Invitrogen, Walham, MA) and 1 ug of RNA was converted to cDNA using High-capacity cDNA Reverse Transcription Kit (Applied Biosystem, Walham, MA) according to manufacturer's instructions. Real-time PCR was performed on the ViiA™ 7 System (Applied Biosystems, Carlsbad, CA) using the PowerUp SYBR Green Master Mix (Applied Biosystem) with recommended standard cycling mode by manufacturer. Each sample was run in doublet. The $2^{-\Delta\Delta Ct}$ was used to calculate relative gene expression for *Cd3e*, *Cd4*, *Cd8a*, and *Cd68* after normalization to the *Gapdh* internal control. Primer sequences of targeted genes are listed in Supplementary Table 1.

2.4 Immunohistochemistry

Paraffin-embedded tumor specimens were cut into 5-µm sections, and tissue slides were deparaffinized with Histo-Clear (National Diagnostics, Atlanta, GA, USA) and rehydrated by sequential incubation in ethanol at different concentrations. Antigen retrieval was performed by incubation of the slides in boiling citrate buffer for 20 min, followed by incubation with 3% H_2O_2 and blocking buffer (Sigma-Aldrich, St. Louis, MO, USA). The slides were probed with rabbit anti-CD3 antibody [SP7] (ab16669; dilution 1:150; Abcam, Cambridge, UK) or rabbit anti-CD45 antibody [D3F8Q] (70257S; dilution 1:200, Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The signal was amplified using Anti-Rabbit EnVision+ System, HRP (Dako, Carpinteria, CA, USA) for 1h at room temperature and visualized using the liquid DAB+ Substrate Chromogen System (Dako). Sections were viewed at 10× magnification using a microscope BZ-X710 (Keyence Corporation, Osaka, Japan). The positively stained cells were counted in three randomly selected field of view by BZ-X Analyzer (1.3.1.1., Keyence Corporation) software and the mean for each sample was calculated.

2.5 Data analysis and statistics

For analyses of tumor growth and reduction during MBTA therapy, the area under the curve (AUC) was calculated, and statistical analysis was performed on AUC values using Two-way ANOVA. Kaplan-Meier survival curves were compared using a Log-rank test. Data were analyzed using GraphPad Prism version 8 (GraphPad Software, La Jolla, CA, USA) and STATISTICA 12 (StatSoft, Inc., Tulsa, OK, USA). Error bars indicate the standard error of the mean (SEM).

3 Results

3.1 Growth of subcutaneous murine pheochromocytoma (PHEO) in young and aged mice

First, we decided to evaluate the growth of murine PHEO in young and aged mice. The mice were subcutaneously transplanted with mouse MTT PHEO cells and the development of tumors was monitored. After 46 days, the incidence of tumors in aged mice was 88.2% compared to 70% in young mice. The tumors in aged mice (n=17) were significantly larger, averaging 499.81 ± 152.55 mm^3 and ranging from 53.25-2020.19 mm^3, compared to the tumors in young mice (n=20), averaging 181.83 ± 71.76 mm^3 and ranging 5.38-1000.43 mm^3 in tumor volume (p=0.031, Mann-Whitney test) (Figure 1A). Similar trend of tumor size differences in young and old animals was also observed in bilateral pancreatic adenocarcinoma, representing another hard-to-treat tumor model with high tumor burden (Supplementary Data, Supplementary Figure 4).

Given the faster growth in our aged mice and age-related changes in number of subpopulations of T cells previously reported by other groups, we next assessed T cell markers *Cd3e*, *Cd4*, and *Cd8a* in tumors from aged and young mice (n=6/group). Quantitative PCR showed that the expression levels of these markers were extensively upregulated in young mice compared to aged mice (*Cd3e*, p=0.009; *Cd4*, p=0.041; *Cd8a*, p=0.041, Mann-Whitney test) (Figures 1B, D). However, when we compared the size of the analyzed tumors and theirs Cd3e mRNA expression level, we observed that lower Cd3e mRNA level is associated with increasing tumor size (p=0.013, Spearman correlation test) (Figure 1B). Immunohistochemical analysis of CD3 in tumors (n=6/group) confirmed the significantly higher infiltration of these cells in tumors from young mice compared to tumors from aged mice (p=0.011, Mann-Whitney test) (Figure 1C). Immunohistochemical analysis of CD45, as a marker of all leukocytes, showed no significant differences between young and aged mice (data not shown). Representative pictures for CD3 and CD45 are shown in Supplementary Figures 1, 2, respectively. We also assessed Cd68 mRNA level in tumor as a marker for monocytes, including circulating and tissue macrophages (26). Our results showed that there was no difference in Cd68 mRNA level between tumors from young and aged mice (p=0.093, Mann-Whitney test) (Figure 1E).

Collectively, these data point out that incidence of murine PHEOs after the subcutaneous injection of MTT cells is higher and their growth is significantly faster in aged mice than in young mice where we observed significantly higher level of *CD3e* also confirmed by IHC analysis of CD3. However, such difference was correlated with the size of tumors.

3.2 Efficacy of intratumoral MBTA therapy of murine PHEO in young and aged mice

To evaluate the age-dependent effect of MBTA therapy, we applied MBTA therapy into subcutaneous PHEO tumors of young

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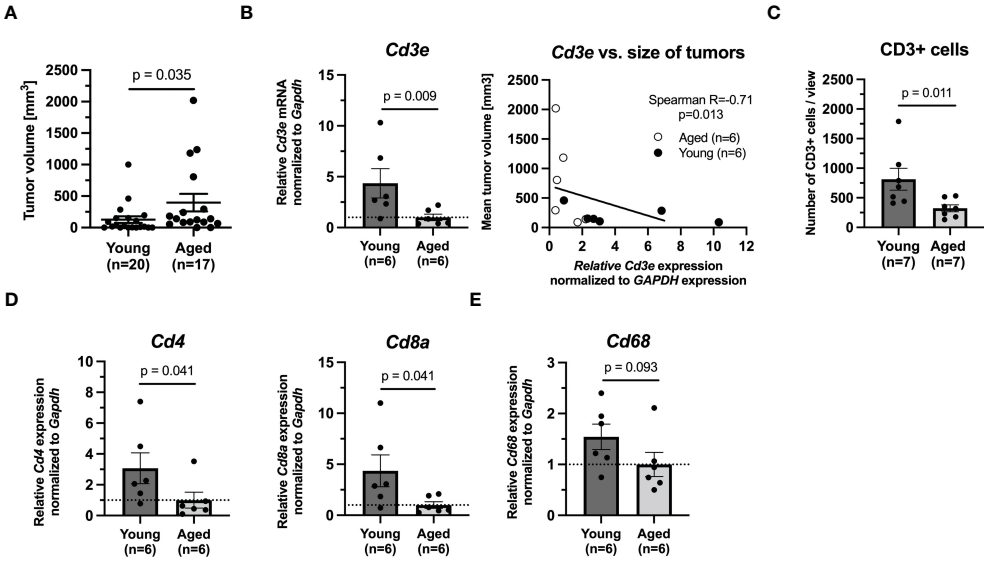

FIGURE 1
The growth of tumors in (6 weeks) and aged (71 weeks) mice bearing PHEO tumor. **(A)** The tumor volume in young and aged mice after 46 days. **(B)** mRNA levels of *Cd3e* and correlation analysis between *Cd3e* and size of tumor. **(C)** Immunohistochemical analysis of CD3 in tumors. **(D)** mRNA levels of *Cd4* and *Cd8a* as representative of T cells populations. **(E)** mRNA level of *Cd68* as representative marker for monocytes/macrophages.

and aged mice. First, the mice were subcutaneously injected with MTT cells. Therapy of aged mice started on day 23 after the injection of MTT cells when tumors were averaging 55.92 ± 6.27 mm^3 (23.44-114.96 mm^3). For young animals, therapy started on day 30 when tumors were averaging 54.65 ± 5.25 mm^3 (24.8-115.02 mm^3) (Figure 2A). The growth of PHEOs confirmed the faster growth in aged mice compared to young mice observed in first experiment. The reduction of tumor growth was similar in aged and young MBTA treated mice compared to control group (aged, $p<0.0001$; young, $p<0.0001$) (Figures 2B, C). Both aged and young MBTA treated mice demonstrated significant increase in survival compared to their control groups (aged, $p<0.0001$; young, $p=0.002$, Log-rank test) (Figure 2D). No significant difference was observed in reduction of tumor growth or survival between aged and young MBTA treated mice. The experiment was done twice with comparable outcomes (Supplementary Figure 3) This observation was also confirmed in bilateral Panc02 model where the reduction of tumor growth during MBTA therapy was similar between aged and young mice (Supplementary Data, Supplementary Figure 4).

To assess the efficacy and immune memory in both aged and young animals, the mice were followed up for possible recurrence and re-challenged with transplantation of PHEO cells. In post-treatment observation of 120 days, 7/10 of aged or young MBTA treated mice bearing PHEO achieved complete remission for the duration of study. On day 121, these mice were retransplanted again with the same number of MTT cells as described. All mice remained tumor free after 40 days from re-transplantation suggesting a long-term immunological memory after MBTA therapy (data not shown).

4 Discussion

Over the past years, immunotherapy has become one of alternative to conventional cancer treatments. Patients have benefited from inhibition of checkpoint inhibitors, especially programmed cell death protein 1/ligand 1 (PD-1/PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (27). However, these immunotherapeutics are administered systemically which can be associated with off-target and dose-related toxicities (28). Contrary, intratumoral injection enables to use much lower dosage of immunotherapeutics and thus lower systematic exposure (29). This advantage makes intratumoral administration a feasible candidate for cancer treatment in elderly patients who may have age-related immune dysfunction, collectively called immunosenescence.

A mouse model is commonly used animal model for investigation of immune system as well as for immunosenescence. Many age-related alterations in immune system have been described in both mice and humans. Changes in innate immunity include decreased phagocytosis by neutrophils, antigen presentation by macrophages and dendritic cells, and cytokine and chemokine productions (30, 31). Contrary, changes in adaptive immune cells are associated with decreased development of naïve T cells, but increased number of memory specific T cells and regulatory T cells (32, 33). In case of B cells, increased production of autoantibodies and decreased immunoglobulin production have also been described in elderly compared to young patients (34).

In the present study, we compared the growth of subcutaneous PHEO in 6-weeks (young) and 71-weeks (aged) old mice with

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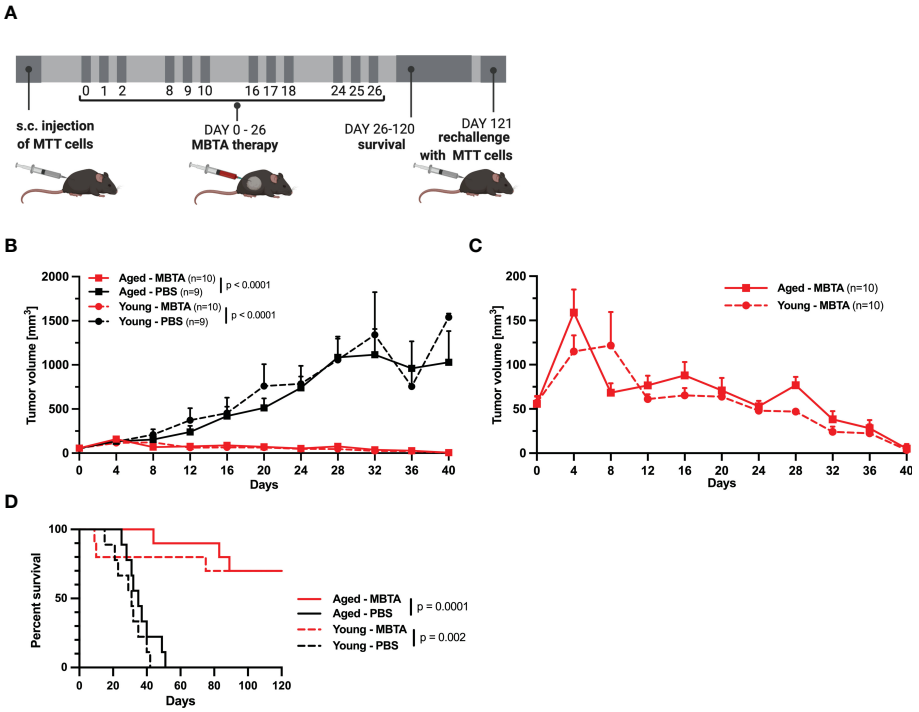

FIGURE 2
The efficacy of MBTA therapy in young (6 weeks) and aged (71 weeks) mice bearing PHEO tumor. **(A)** The schema of experimental treatment in young and aged mice. After development of tumors, the mice were randomized into treatment groups and treated on specific days. Following the 120 days for survival analysis, the mice with complete elimination of tumors were re-challenged with s.c. injection of MTT cells. **(B)** The tumor growth for all groups. **(C)** The tumor reduction for the groups of young and aged MBTA treated mice. **(D)** The survival analysis of MBTA and PBS treated mice. Days on x axis represent the days from beginning the therapy.

subsequent application of intratumoral MBTA therapy to better mimic the age of patient with PHEO which is an important factor for potential use of this approach in future clinical trial. We showed that the incidence of subcutaneous pheochromocytoma tumors is higher in aged mice, and the growth of PHEO are faster in aged mice compared to young mice. Published studies have previously shown the difference in tumor growth in aged and young mice with general acceptance of slower growth in aged mice (35, 36). However, for example, murine mesothelioma AE17 (37), prostate adenocarcinoma TRAMP-C2 (38), and colon carcinoma CT26 (39) models have showed faster growth in aged mice compared to young mice, suggesting the specificity for each tumor cell line. Indeed, in our second model, bilateral Panc02 tumor model which here better represents the real situation in patient with higher tumor burden, we observed significantly larger tumor volumes in 72-weeks (aged) animals compared to in 8-weeks (young). Previously, age-related changes in number of subpopulations of T cells have been widely reported before and T cells may have a key role in tumor development in general. In case of PHEO, we measured higher levels of mRNA of *Cd3e*, *Cd4*, and *Cd8a* in tumors from young mice, as well as CD3+ positive cells, suggesting higher infiltration of T cells in the tumors. However, such higher level of *Cd3e* negatively correlated with size of analyzed tumors between groups, suggesting that the infiltration is based on size of tumors and not age difference.

Intratumoral MBTA immunotherapy of PHEO showed no age-related differences in efficacy and was able to completely reduce tumor growth in 70% of treated both young and old animals. Such results are consistent with our previous results with young mice bearing pheochromocytoma, pancreatic adenocarcinoma, melanoma, or colon carcinoma where MTBA immunotherapy was efficient in 62-83% (14–16). Similarly, the previous studies of the efficacy of MBTA in pancreatic adenocarcinoma, Panc02 tumor model (15, 17), were done in young animals only while pancreatic cancer most often affects older adults (40). Our results of MBTA in bilateral Panc02 tumor model, which was tested in 8-weeks and 72-weeks old mice, representing 15 years and 57 years in human, showed the similar reduction of tumors and prolonged survival of treated mice as published before (17). Such results suggest that MBTA therapy is effective even in this hard-to-treat Panc02 model with two advanced tumors without any age differences. While in PHEO model the survival was comparable for both age groups, we observed the difference in survival of MBTA-treated mice between aged (1/6 mice) and young mice (3/6 mice) in Panc02 model. Such difference can be explained by different tumor size at the beginning of therapy when the tumors in aged mice were significantly bigger than in young mice.

Interestingly, despite increasing interest in intratumoral application, there is limited number of studies comparing the efficacy of intratumoral immunotherapy in young and old animals. Sharma et al. compared the intratumoral application of poly(I:C) and CpG-ODN in 2-4-months and 18-22-months old mice. Their results indicated that only intratumoral injection of CpG-ODN induced the complete rejection of mammary tumors (TUBO) and delayed prostate adenocarcinoma tumor (TRAMP-C2) growth in both young and old

mice. Contrary, intratumoral injection of poly(I:C) induced the rejection of TUBO tumors and delayed the growth of TRAMP-C2 tumors in young but not in the old mice (41). Another example of study focused on an efficacy of intratumoral immunotherapy in young and aged mice was published by Duong et al. (37). They compared the efficacy of IL-2/anti-CD40 antibody and founded this therapy less effective in aged (38%) than in young mice (90%). Even though it appears that the tolerance of the tumor growth and progression of murine PHEO in aged and young mice were different, both age groups have a similar immune response once triggered by intratumoral application of MBTA.

Although clinical trials have not showed major increases in age-related adverse events during checkpoint inhibitor therapies (42), it should be mentioned that there is still limited evidence due to underrepresentation of elderly patient in clinical trials (42, 43). While we did not observe any adverse events during MBTA immunotherapy in aged mice, 2 young mice were found dead on day 10 (after the second cycle of MBTA therapy). However, the cause of death is momentarily unknown. We assume that the cause of death may be related to the extensive activation of immune system and weight of animals which is not comparable between young and aged mice. This limitation of study should be answered in the future for further and safe use of MBTA immunotherapy.

Incidence of tumor development is continually increasing with age of patients. However, many preclinical studies are not focused on age-related differences and clinical studies do not often include elderly patients. Here, we demonstrate that intratumoral MBTA immunotherapy has same efficacy in young and old PHEO-bearing mice. However, future clinical study is necessary to confirm the efficacy of described intratumoral immunotherapy in patients.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Animal Care and Use Committee of the National Institutes of Health (ASP 18-028, pheochromocytoma model) and Ministry of Education, Youth, and Sport of the Czech Republic (Protocol No. 12098/2016-2, pancreatic adenocarcinoma model).

Author contributions

OU, KHV, JZ, and KP designed the study. OU, KHV, RL, and AF performed the experiments. OU, KHV, and HW verified, analyzed, and interpreted the data. JZ, ZZ, and KP supervised and administrated the project. All authors wrote, read, and approved the final manuscript.

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Conflict of interest

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

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Supplementary material

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HIF and MYC signaling in adrenal neoplasms of the neural crest: implications for pediatrics

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Pediatric neural crest-derived adrenal neoplasms include neuroblastoma and pheochromocytoma. Both entities are associated with a high degree of clinical heterogeneity, varying from spontaneous regression to malignant disease with poor outcome. Increased expression and stabilization of HIF2α appears to contribute to a more aggressive and undifferentiated phenotype in both adrenal neoplasms, whereas *MYCN* amplification is a valuable prognostic marker in neuroblastoma. The present review focuses on HIF- and MYC signaling in both neoplasms and discusses the interaction of associated pathways during neural crest and adrenal development as well as potential consequences on tumorigenesis. Emerging single-cell methods together with epigenetic and transcriptomic analyses provide further insights into the importance of a tight regulation of HIF and MYC signaling pathways during adrenal development and tumorigenesis. In this context, increased attention to HIF-MYC/MAX interactions may also provide new therapeutic options for these pediatric adrenal neoplasms.

KEYWORDS

pheochromocytoma, neuroblastoma, catecholamines, sympathoadrenal cell lineage, hypoxia, MYC, neural crest, paraganglioma

1 Introduction

The neural crest comprises a multipotent stem cell population that gives rise to a large variety of cell types, including sympathetic and parasympathetic ganglia and chromaffin cells. From these cell types neuroblastomas or pheochromocytomas/paragangliomas can originate. Both tumor entities can occur in the adrenal gland in some cases already soon after birth or in early childhood and are characterized by clinical and molecular heterogeneity. In both neural crest-derived neoplasms, it is discussed that increased expression and stabilization of HIF2α contributes to a more aggressive and undifferentiated phenotype (1–4). However, dependent on the context HIF2α may also

promote tumor suppressive activities, which was reported for both neuroblastoma (5–7) and non-neural crest-derived neoplasms (8–10). The occurrence of *MYCN* amplification in approximately 20% of neuroblastomas, which correlates with high-risk disease and poor prognosis (11), further underscores the importance of MYC and HIF signaling pathways in these adrenal neoplasms derived from the neural crest.

The human adrenal gland composes two distinct tissues, the outer cortex and the inner medulla under a common capsule (Figure 1A). The steroid hormone producing adrenal cortex (mineralocorticoids, glucocorticoids and androgens) arises from the coelomic mesoderm of the urogenital ridge, while the adrenal medulla arises from the neural crest and secretes the catecholamines epinephrine and less prominently norepinephrine (13, 14). During neurulation, the neural folds arise at the boundary between non-neural and neural ectoderm and subsequently coalesce in the midline to form the neural tube. The pre-migrating neural crest cells localized at the neural fold are characterized by their epithelial phenotype associated with strong cell-cell junctions. To initiate migration, neural crest cells must undergo an epithelial-mesenchymal transition (EMT) to acquire a motile phenotype and lose their cell-cell junction (15).

During migration, neural crest cells proliferate extensively to generate enough precursor cells to colonize their targets. Ventrally migrating neural crest cells colonize the paravertebral sympathetic ganglia in the trunk and the chromaffin cells of the adrenal medulla and paraganglia (16) (Figure 1B). After reaching their targets, neural crest-derived cells differentiate into the appropriate cellular subtypes. Migration and differentiation of the distinct neural crest cell derivatives is orchestrated by expression of lineage specific markers that are influenced by signals of the microenvironment such as hypoxia.

Single-cell RNA sequencing (scRNA-seq) revealed differences in the transitions between sympathoadrenal fates in humans and mice (12). In human embryos, Schwann cell precursors (SCPs; $SOX10^+$) derived from neural crest cells connect to $STMN2^+ISL1^+PRPH^-$ sympathoblasts and the $CHGA^+PENK^+PNMT^+$ chromaffin cells through a 'fork-like' transition (Figure 1C) (12). Cells within this first transition show overlaps between both expression programs in form of $SOX10^+/ISL1^+/HAND2^+$, $SOX10^+/ISL1^+$ and $SOX10^+/HAND2^+$ transitory cells (12). A second transition between sympathoblasts and chromaffin cells (markers: *SOX4*, *BEX1*, *RAMP1*, *PENK*) was identified (Figure 1C). In mice, chromaffin cells of the adrenal medulla arise from an intermediate bridge cell

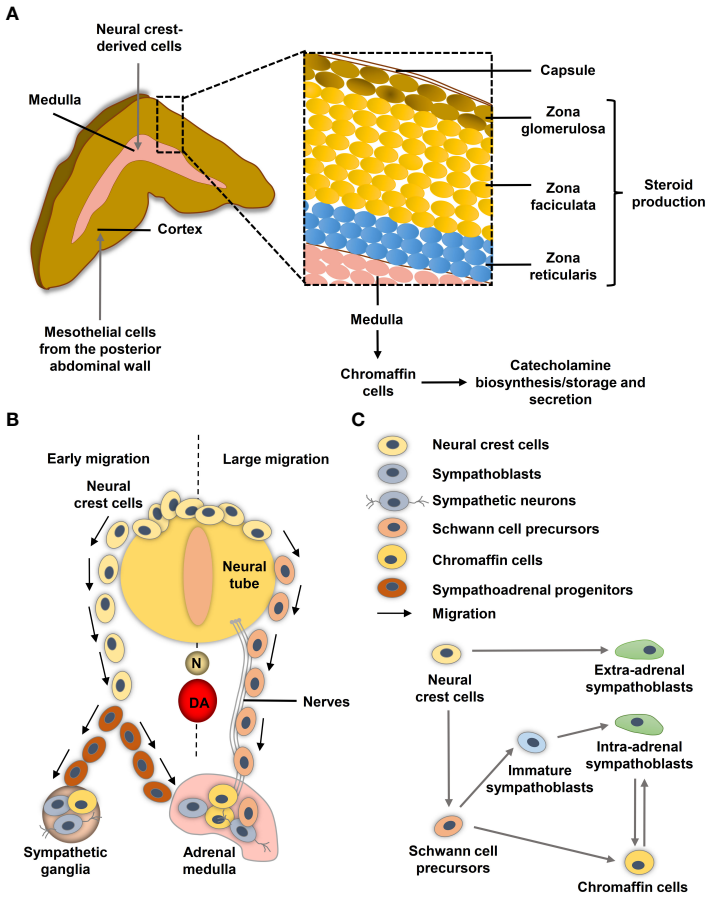

FIGURE 1
Neural crest and adrenal chromaffin cell development. **(A)** Human adult adrenal zonation. **(B)** Schematic illustration of human adrenal and sympathetic ganglia development from the neural crest. **(C)** Chromaffin cell and sympathoblast development from neural crest-derived cells in humans (adapted from (12)). DA, dorsal aorta; N, notochord.

population (transition 1) and can transit to sympathoblasts (12, 17). While cellular properties, such as high proliferative capacity and motility, are crucial during embryogenesis, they may become problematic later in life when the same properties contribute to tumor aggressiveness and metastasis. Low-risk neuroblastomas share similarities to differentiated late sympathoblasts (also named neuroblasts in the context of neuroblastomas), whereas high-risk *MYCN*-amplified neuroblastomas resemble more undifferentiated sympathoblasts (18–20). Bedoya-Reina et al. further showed that tumor cells enriched in high-risk neuroblastomas resemble a subtype of TRKB+ cholinergic progenitor population that they previously identified in human postnatal adrenal glands (20). An undifferentiated phenotype is also associated with a higher risk of metastasis in pheochromocytomas and extra-adrenal paragangliomas (21, 22), although the exact cellular origin of the various subgroups is still unclear.

In the present review, we describe current knowledge about the interplay between MYC and HIF signaling during neural crest, adrenal development and the potential role of these signaling pathways in catecholamine-producing neural crest-derived neoplasms.

2 Interplay of MYC and HIF signaling

MYC proteins are key regulators of cell fate and part of a network of interacting transcription factors, that regulate expression of various genes involved in, for example, cell proliferation, differentiation and metabolism (23). In addition to c-MYC, the MYC protein family also includes MYCN and l-MYC. These helix–loop–helix leucine zipper transcription factors heterodimerize with MYC-associated protein X (MAX) (Figure 2). After heterodimerization, MYC/MAX binds to specific DNA sequence (CANNTG) called E-box to activate or repress transcription of more than 15% of all genes in cells (24–26). MAX itself is under tight control by a network of protein-protein interactions with MAX dimerization protein (MXD1, also known as

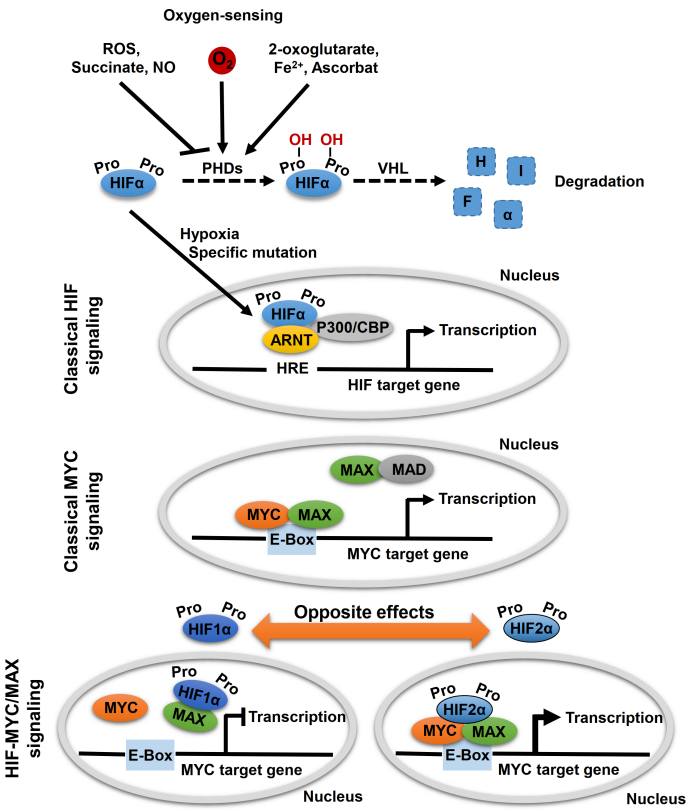

FIGURE 2
Oxygen-sensing and HIF-MYC signaling. Key molecule of oxygen-sensing system are hypoxia-inducible factors (HIFs), which regulate the transcription of a wide range of oxygen responsive genes. In presence of oxygen, HIF proline hydroxylases (PHDs) hydroxylate proline residues within HIFα subunits, which lead to proteasomal degradation of HIFα by the von Hippel-Lindau (VHL) tumor suppressor. Hypoxic conditions (absence of oxygen) or specific mutations, that affect HIFα degradation, lead to a stabilization of HIFα. Subsequently, HIFα subunits translocated to the nucleus where they form a complex with aryl hydrocarbon receptor nuclear translocator (ARNT, also known as HIFβ) and specific co-factors and bind to hypoxia-responsive elements (HREs) leading to transcription of HIF target genes (classical HIF signaling). MYC proteins encoded by MYC proto-oncogenes (*c-MYC, MYCN, l-MYC*) are localized in the nucleus and form heterodimers with myc-associated factor X (MAX), which enables recognition by the hexameric DNA sequence CACGTG (E-Box) and subsequent transcriptional activation of MYC target genes (classical MYC signaling). Moreover, other binding partners of MYC and MAX also regulate MYC target gene transcription, including MAX dimerization protein 1 (MAD) and HIFαs. Thereby, opposite effects on MYC target gene expression were described for HIF1α and HIF2α. While HIF1α dimerizes with MAX and thereby suppresses binding to the E-box, HIF2α leads to stabilization of the MYC/MAX complex and thus to activation of MYC target genes.

MAD) and MAX interactor 1 (MXI1, also known as MAD2) (27). Other factors, such as the hypoxia inducible factor (HIF) 1α and 2α, further affect binding of MYC/MAX to the E-box.

HIF proteins are the main regulators of oxygen sensing in cells (Figure 2). Under normoxic conditions (presence of oxygen), proline residues of the HIFα subunits are hydroxylated by oxygen- and α-ketoglutarate-dependent prolyl hydroxylases (PHDs). This allows for recognition of the HIFα subunits by the von Hippel–Lindau protein (VHL) and their subsequent degradation by proteasomes (Figure 2). Under hypoxic conditions (absence of oxygen), HIFα subunits are stabilized and form a complex with aryl hydrocarbon receptor nuclear translocator (ARNT, also known as HIFβ) and several co-factors, including CREB-binding protein and p300. The HIF complex binds to hypoxia-response element (HRE) of the gene promoter for transactivation, thereby regulating genes involved in angiogenesis, pH regulation, glycolysis, and glucose transport, which enable cellular adaptation to hypoxia.

The two main HIFα subunits, HIF1α and HIF2α, have mostly complementary functions, but their activity differs temporally: while HIF1α predominantly mediates the acute response to severe hypoxia, HIF2α modulates adaption to chronic or even mild hypoxia (4). In addition, HIF1α is ubiquitously expressed, while HIF2α has a more restricted expression profile (28). Both HIFαs differ in their targets; with HIF1α specifically activates genes involved in glycolysis and HIF2α preferentially activates VEGF, transforming growth factor-α (TGFα), lysyl oxidase (Lox), Oct4 and Cyclin D1 (29, 30). Evidence suggests that HIF2α preferentially promotes tumorigenesis and affects the differentiation status of different tumor entities (29). The effects on the tumor phenotype might be explained by the HIF2α-induced expression of the stem cell factor Oct4 and the transcriptional activation of c-Myc (29, 30). For example, in hepatocellular carcinoma (HCC) tumor tissue, c-Myc expression showed a positive correlation with HIF2α but not with HIF1α (31). Knockdown of HIF2α diminished expression of c-Myc in HCC cells, suppressed hypoxia-related proliferation, and induced apoptosis (31). On the other hand, Yang et al. revealed that HCC patients with high HIF2α protein levels had longer overall survival indicating a tumor suppressor function of HIF2α in these tumors (10). In colorectal cancer cell lines, HIF2α regulated expression of *c-Myc* under chronic hypoxia and thereby controls sensitivity to 5-flurouracil (32). HIF1α and HIF2α play distinct roles in colon cancer (9). It was also shown that *EPAS1* was significantly reduced in primary adenocarcinoma samples of the colon compared to histopathologically non-neoplastic tissue, while no difference was found for *HIF1α* (33). Renal cell carcinoma cells expressing almost exclusively HIF2α exhibit lower genomic instability, which correlates with enhanced c-MYC-dependent expression of genes involved in DNA repair (34). Moreover, emerging evidence suggests that MYC regulates the levels and activity of HIF1α (35–38).

In addition, other HIF-dependent mechanisms modulating MYC/MAX complex formation and promoter occupancy have also been proposed, with HIF1α and HIF2α also exhibiting opposing effects (Figure 2) (26, 39, 40). HIF1α antagonized c-MYC activity by displacing c-MYC from transcription factor Sp1 binding that is required for MYC promotor activation, while phosphorylation of HIF2α prevents HIF2α from competing with MYC for Sp1, thereby enhancing Sp1-c-Myc activity (41–44). Under chronic hypoxia, HIF1α promotes c-MYC degradation and induces expression of MAX interactor 1 (MXI1) that furthermore inhibits MYC target gene expression (45, 46). HIF1α can further bind to MAX to thereby disrupt the formation of the MYC/MAX complex (39). On the other hand, HIF2α promotes MYC activity by enhancing Sp1-c-Myc activity as mentioned above. HIF2α enhances c-MYC activity by stabilizing the MYC/MAX complex (Figure 2) (45, 47). This effect appears to be much stronger than HIF1α-mediated degradation of c-MYC in tumor cells, leading to activation of MYC under hypoxic conditions (47). Moreover, HIF has been shown to promote proteosomal degradation of MYC under chronic hypoxic conditions in dependence of the used cell system (45, 48–50).

Most of the aforementioned studies addressing the interaction between HIF and MYC focused exclusively on the effect of c-Myc. Whether the mechanisms are also applicable to MYCN remains unclear. Due to the tissue- and cell-specific differences in c-MYC and MYCN expression and the resulting different roles (e.g. during neural crest development and tumorigenesis), it can be assumed that the interaction with HIF1α and HIF2α is also different.

3 HIF signaling during neural crest and adrenal development

The ability to sense oxygen (Figure 2) is crucial for the survival of water- and air-breathing organisms. Adrenomedullary chromaffin cells as well as glomus cells of the carotid body that both arise during neural crest development synthesize and release catecholamines in response to hypoxic stress (51, 52). Chemoreceptors in the carotid body are relatively non-sensitive to hypoxia in the neonatal period and achieve adult-like sensitivity by 2-3 weeks postnatally in rats. In contrast, rat adrenomedullary chromaffin cells are most sensitive to hypoxia in the perinatal period, and this sensitivity is gradually lost until it is largely gone by 2-3 weeks postnatally (53–55). The oxygen sensitivity of these neural crest-derived cells suggests involvement of the HIF signaling pathways already during embryogenic development.

Under physiological conditions, hypoxia occurs in amniotic embryos prior to the onset of a functional blood circulation (56). In rodents and chicken embryos, low oxygen concentrations (5%) have been shown to be required until the neural tube has closed and the cells of the cranial neural crest have emigrated (57, 58). Studies in Xenopus, chicken, and quail embryos indicate that HIF1α and ARNT are expressed together and ubiquitously in the developing embryo (up to HH14; HH stage in chicken embryo), whereas HIF2α has a more pronounced expression pattern that includes tissues that do not express HIF1α (58, 59). Trunk neural crest cells that give rise to the adrenal medulla and the sympathetic ganglia form mainly after vascularization and are not affected by high oxygen or deletion of HIF1α (57, 58, 60). However, stabilization of HIFα in neural crest cells seems to be controlled by oxygen-dependent (hypoxia) and oxygen-independent mechanisms

(pseudohypoxia) (61), similar to what is already known for neuroblastoma, pheochromocytoma and paraganglioma (PPGL) (62, 63).

Inhibition of Hif1α in Xenopus laevis and zebrafish embryos results in complete blockade of neural crest migration by controlling chemotaxis and epithelial-mesenchymal transition (61). In mice selectively deficient in HIF1α in ISL^+ cells, the development of sympathetic ganglia and chromaffin cells is impaired (64). Mice with deletion in Vhl restricted to tyrosine hydroxylase (Th)-positive cells exhibit atrophy of the carotid body, adrenal medulla and sympathetic ganglia; this was associated a striking intolerance to systemic hypoxia that could lead to death (65). *HIF1α* is diffusely expressed in all cell types of the developing human adrenal medulla with a stronger accumulation in the connecting progenitor cells and early chromaffin cells (Figures 3A, B), while expression of *HIF2α (gene also known as EPAS1)* appears to be restricted to specific cell populations (Figure 3C). snRNA-seq revealed a high expression of *HIF2α* in early chromaffin cells and neuroblasts as well as in the connecting progenitor cells, whereas SCPs and late neuroblasts as well as late chromaffin cells showed only limited expression of *HIF2α* in isolated cells (Figures 3C, D) (18).

During neural crest development, HIF2α is expressed in migrating trunk neural crest cells and sympathetic neuroblasts in human, murine, and avian embryos (66). In the sympathetic ganglia of human embryos, HIF2α is expressed at embryonic week 6.5, but expression is lost at later stages (67). *HIF2α* knockout mice exhibit severe sympathetic nervous system (SNS) abnormalities associated

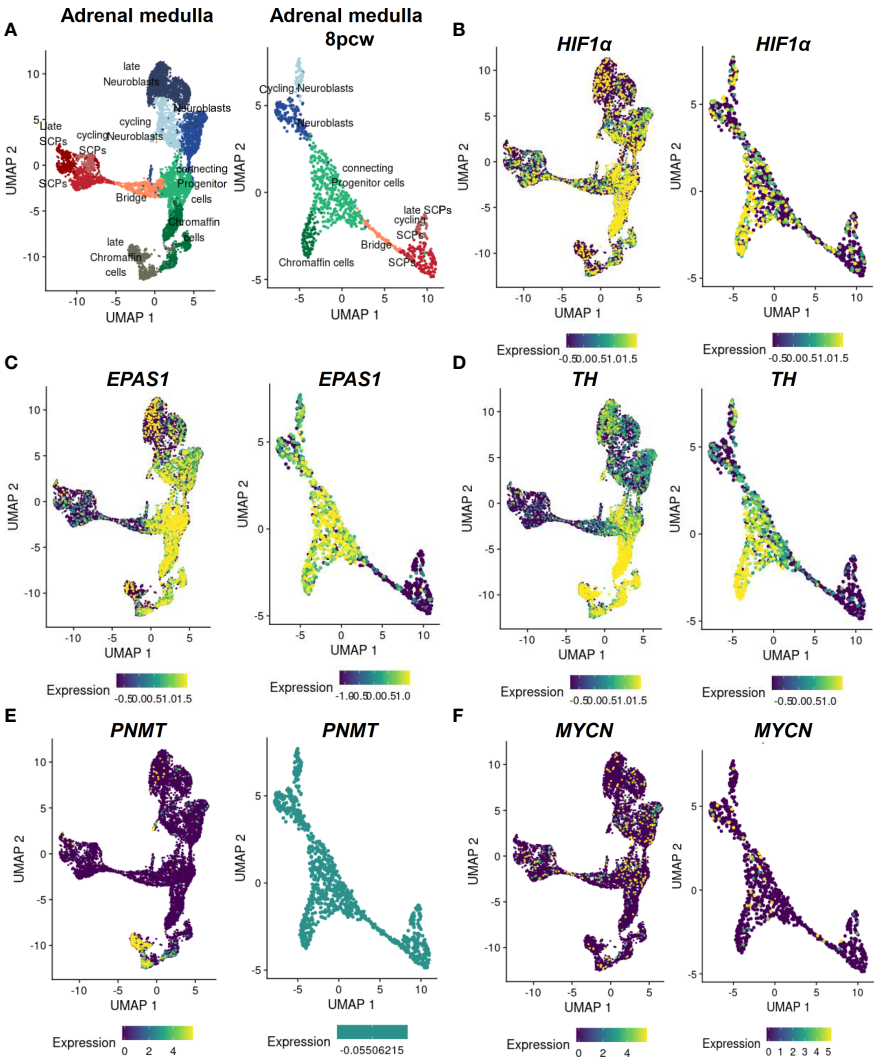

FIGURE 3
Expression of *MYCN*, *HIFs* and chromaffin cell markers in the developing human adrenal medulla. Expression patterns of genes of interest obtained by single nucleus RNA sequencing data (18) of the developing human adrenal medulla were visualized by https://adrenal.kitz-heidelberg.de/developmental_programs_NB_viz/ (last request: March 2022). **(A)** UMAP plot of adrenal medullary cells of developing adrenal and of the fetal adrenal eight weeks post conception (8pcw). Different colors highlight different cell populations. Expression pattern of **(B)** *HIF1α*, **(C)** *EPAS1*, **(D)** *TH*, **(E)** *PNMT* and **(F)** *MYCN* in the developing human adrenal and of the fetal adrenal eight weeks post conception. The color indicates the normalized gene expression (blue low expression; yellow high expression). Data on *c-Myc* and *MAX* expression were not available. UMAP: Uniform manifold approximation and projection; SCP, Schwann cell precursors.

with mid-gestation lethality (68). Both overexpression and silencing of HIF2α *in vivo* delay neural crest development, induce proliferation and self-renewal capacity of neural crest cells, and reduce the proportion of neural crest cells that migrate ventrally to sympathoadrenal sites, suggesting that HIF2α needs to be tightly controlled during normal development of the trunk neural crest (66). These data underline the importance of HIF2α in chromaffin cell and sympathoblast differentiation, which may further provide a rational for the various differentiation states of adrenal neoplasms.

In mice, inactivation of Phd3 (responsible for the hydroxylation of the HIFαs that initiates their proteasomal degradation) promotes survival of sympathoadrenal neurons, which appear hypofunctional despite increased numbers of Th-positive cells in the adrenal medulla and carotid body, due at least in part to upregulation of Hif2α but not Hif1α (69). Moreover, hypoxia induced catecholamine release by chromaffin cells, and here in particular regulated by HIF2α (68, 70), is crucial to maintain physiological homeostasis of the fetus before sympathetic innervation is fully complete (71, 72). During birth, increased catecholamine release facilitates adequate hemodynamic adjustment and stimulates surfactant production by the lungs (73, 74). A deficiency of *Phd2* in the adrenal medulla of mice results in a Hif2α-mediated reduction in phenylethanolamine *N*-methyltransferase (Pnmt) associated with a reduced epinephrine biosynthesis (75). This is also consistent with studies in *Hif2α*$^{-/-}$ mice, which showed reduced epinephrine levels in the adrenal gland (68, 76). In the developing human adrenal medulla, expression of *PNMT* is restricted to late chromaffin cells, which do not express *HIF2α* or express *HIF2α* in a restricted manner (Figure 3E) (18). Reduced oxygen promotes survival and catecholaminergic differentiation, characterized by the expression of tyrosine hydroxylase, which catalyzed conversion of tyrosine to L-DOPA (precursor catecholamine biosynthesis), in neural crest stem cells and central nervous system progenitors (77, 78). It is therefore possible that hypoxia mediates stem cell function by affecting Oct4, which is controlled by HIF2α as discussed in the previous section (30).

4 MYC signaling during neural crest and adrenal development

Mice studies revealed that c-*Myc* and *Mycn*, but not *l-Myc*, are fundamental for normal development since targeted deletions are lethal to the embryo at mid-gestation (79–81). Tissue- and cell-specific expression patterns of Mycn and c-Myc have been observed during embryogenesis. While Mycn expression is restricted to specific cell types of epithelial tissues, including those of the developing nervous system and organs characterized by epithelial-mesenchymal interaction, c-Myc expression is restricted to mesenchymal compartments (82). A time-dependent expression of c-Myc and Mycn in the developing neural crest and neural crest cells is observed in chicken embryos (83). In the neural plate during gastrulation throughout the anterior-to-posterior axis Mycn, but not c-Myc is expressed. Neither c-Myc nor Mycn is expressed in the neural plate boarder at stage HH4-7, which later form the neural crest. When the neural tube closure starts (HH8) expression of Mycn is restricted to the ventral parts, which form the central nervous system (CNS). The expression of c-Myc begins at later stage HH8 in the dorsal neural tube later forming the neural crest. In stage HH9, c-Myc expression remains restricted to the dorsal neural crest area, while Mycn is expressed in the remaining neural tube except in the dorsum (83).

In Xenopus, c-Myc is an essential early regulator of neural crest cell formation, with expression of c-Myc localized to the neural plate boundary prior to expression of early neural crest markers (84). It has been suggested that c-Myc prevents cell fate decisions during neural crest formation, possibly via the Myc target gene *Id3* (85). Knockdown of *Id3* leads to a lack of neural crest formation in Xenopus embryos, since Id3 maintains the precursor state of neural crest cells (85). Loss of c-Myc results in a drastic reduction in the number of emigrating cells of the neural crest, due to a reduced capacity for self-renewal, increased cell death, and a shorter duration of the emigration process in chicken embryos. In this regard, c-Myc appears to bind to Miz1 rather than to the E-box to activate the cell cycle (86).

In chicken and mouse embryos, Mycn is expressed in early neural crest lineage, the central nervous system, a subset of mesoderm derivatives and endodermal epithelia, in particular (87). Deficiency of Mycn in mouse embryos leads to a reduction in mature neurons of the dorsal root ganglia and sympathetic ganglia, underscoring the importance of Mycn in the development of neurons from the neural crest (81). At early stages, Mycn appears to be involved in the proliferation of progenitor populations rather than in their differentiation per se (79). Later during neural development, Mycn has been linked to the maintenance of neural fate as it is expressed by slowly proliferating neural stem cells and is involved in the expansion and differentiation of neural progenitor cells in the CNS (81, 88, 89). In addition, Mycn promotes neural fate and differentiation in the peripheral nervous system (79, 87). After neural crest EMT, Mycn is only expressed at low levels in migrating neural crest cells followed by a further downregulation before the cells aggregate to form the ganglia (90). Mycn is expressed in regions of the neural plate destined to form the central nervous system, but not in the neighboring neural crest stem cell domain (83). Mycn expression in the neural crest domain biases cells toward a more CNS neural stem cell-like fate (expression SOX2), leading to improperly specified neural crest cells; this may play a role of priming in neuroblastoma development (83). However, overexpression of Mycn in mouse sympathoadrenal progenitors is insufficient for tumor formation in nude mice but leads to enhanced neural differentiation (91). Increased expression of Mycn in mouse neural crest cells results in neuroblastoma-like tumors, suggesting that premature exposure of neural crest cells to high Mycn levels may be important in the development of neuroblastoma (92). Our scRNA-seq data reveal an expression of *MYCN* only in single cells of the human developing adrenal medulla not restricted to a specific cell type (Figure 3F).

A possible time-dependent co-expression of HIFs and MYCs during neural crest and adrenal development may indicate a possible interaction of the two signaling pathways during these embryological processes, but precise data are unfortunately not available in this regard. The role of the cell-specific expression of the

respective isoforms of HIF and MYC and how exactly they are related to each other remains unclear. Furthermore, most studies in this context rely on Xenopus, mouse and chicken embryo studies. Transferability to humans needs to be clarified. Nevertheless, data indicate that the embryogenic origin of the cells and the associated differentiation status influenced by HIF and MYC may contribute decisively to the phenotype of adrenal neoplasms.

5 HIF and MYC signaling in catecholamine-producing neoplasms of the neural crest: neuroblastoma, pheochromocytoma and paraganglioma

Pediatric neuroblastomas and PPGLs are characterized by a high degree of heterogeneity in disease presentation associated with distinct differentiation stages, affected by HIF and MYC signaling pathways.

Neuroblastoma is the most common solid neoplasm in childhood and its clinical course varies from spontaneous tumor regression to fatal malignant progression (Figure 4); even differentiation into benign forms (ganglioneuroma or ganglionneuroblastoma) are described (93). Malignant expansion in neuroblastoma appears manly triggered by *MYCN* amplification or/and *ALK* and *ATRX* mutations (94). Additional factors that influence prognosis include the location of the primary tumor and the age of the patient. For example, adrenal neuroblastomas are more frequently *MYCN*-amplified and are often in tumor stage 4 (International Neuroblastoma Staging System Committee (INSS) system), in which the primary tumor has spread to distant organs, compared with extra-adrenal neuroblastomas (95). Children with stage 4 neuroblastoma who are younger than 1 year have a significantly better prognosis than older children at stage 4 (96).

Epigenetic and transcriptional profiling in neuroblastoma cell lines identified two distinct neuroblastoma cell identities: the undifferentiated mesenchymal-type (MES) and adrenergic-type (ADRN; to be strictly distinguished from an adrenergic phenotype in PPGLs) that can interconvert and reflect cells from different stages of differentiation (Figure 4) (97). Patient samples allowed a further subclassification of the adrenergic group linked to distinct clinical outcomes into *MYCN*-amplified, *MYCN* non-amplified high-risk and *MYCN* non-amplified low-risk signatures (98). High MYCN/c-MYC target gene expression is a hallmark of malignant neuroblastoma progression (99). Elevated expression of a subset of MYCN/c-MYC target genes identifies a patient subtype with poor overall survival, independent of established risk markers such as *MYCN* amplification, disease stage, and age at diagnosis (99). Malignant progression, therapy resistance and disease relapse in neuroblastoma are associated with an undifferentiated phenotype, which is linked to the broad activation/repression of MYCN/c-MYC target genes (97, 98). Some results suggest that the phenotype and thus the clinical outcome strongly depend on the precise ratio of MAX to MYCN (100). In addition, hypoxia - in this case in particular the expression of HIF2α - is described to block

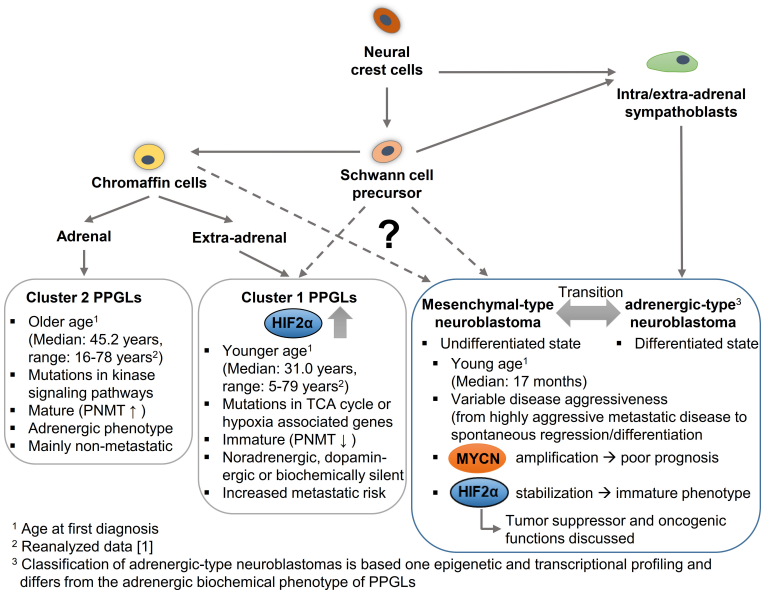

FIGURE 4
Hypothetical model to explain the cellular origin leading to different phenotypic features in pheochromocytomas/paragangliomas (PPGLs) and neuroblastomas in dependence of HIF2α and MYCN. PPGLs and neuroblastomas originate from neural crest cells; while mature neuroblastomas arise from a sympathoblast lineage, mature cluster 2 PPGLs originate form a chromaffin cell lineage. Both lineages arise via Schwann cell precursors that may give rise to more immature cluster 1 PPGLs and mesenchymal-type neuroblastomas. Increased expression and stabilization of HIF2α is associated with an immature phenotype and enhanced aggressiveness in PPGLs, while for HIF2α in neuroblastoma both an oncogenic and a tumor suppressor function are discussed. MYCN amplification correlates with a worse prognosis in neuroblastomas. PNMT, Phenylethanolamine N-methyltransferase; TCA cycle, Tricarboxylic acid cycle.

differentiation and potentially also trigger dedifferentiation in neuroblastoma cells, which may contribute to a more aggressive tumor behavior (2, 101, 102). However, the role of this mechanism in *MYCN*-amplified neuroblastoma is not yet fully understood and there are also data attributing a tumor suppressor function to HIF2α in neuroblastoma (5–7). Westerlund and colleagues showed that *EPAS1* expression correlates with features of low-risk neuroblastoma (6). For clear-cell carcinomas it has been shown that HIF2α sensitizes to ferroptosis, an iron-dependent form of cell death (103). *MYCN*-amplified neuroblastoma cells are highly sensitive to ferroptosis (104), which also suggests a rather tumor-suppressive role of HIF2α in neuroblastoma. Further studies are needed to finally clarify the role of HIF2 α in neuroblastoma. The complex regulation of both signaling pathways in neuroblastoma and the resulting consequences for differentiation and metastasis, suggests a close association of the MYC and HIF signaling pathways in neuroblastomas.

Neuroblastomas occur in many of the same regions as PPGLs. New insights into the putative cellular origins of both entities came from comparative snRNA-seq analyses of human embryonal/fetal adrenal glands and neuroblastoma/PPGLs (12, 18, 105). Whereas neuroblastomas showed prominent transcriptional similarity with early normal neuroblast populations, PPGLs were transcriptionally more similar to normal cells with chromaffin cell-like morphology (Figure 4) (18, 105). Regulatory super-enhancer elements involved in neuroblastoma-specific chromosomal rearrangements were predominantly active in early neuroblasts of the developing adrenal glands, indicating neuroblastoma-driving events may have occurred predominantly in early neuroblast populations and not in SCP or chromaffin cell-like populations (18). The different cellular origin may also explain why PPGLs are diagnosed at older ages (median: 41.8 years; range: 5.5-83.2 years (1)) and pediatric cases are comparatively rare. Nevertheless, taking into account the low growth rate of PPGLs (volume doubling time of 5-7 years (106, 107)), it seems reasonable that tumorigenesis may be initiated during embryogenesis in at least some of these tumors (discussed later in more detail). Increased expression and stabilization of HIF2α in a specific subset of PPGLs (pseudohypoxic PPGLs) is characterized by a more immature phenotype, enhanced disease aggressiveness and onset at younger age (1, 62).

A large proportion of PPGLs are heritable due to germline pathogenic variants (PVs) in one of the described PPGL susceptibility genes (e.g. PVs in: *FH*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *MDH2*, *VHL*, *HIF2α*, *EGLN1/2*, *MAX*, *TMEM127*, *NF1*, *RET*). Hereditary PPGLs are diagnosed at younger age and differ with respect to their plasma and urinary catecholamine/metanephrine profiles from sporadic PPGLs (108). Diagnosis of PPGLs largely depends on the biochemical assessment of catecholamine excess by measurement of plasma or urinary metanephrines. Due to slow growth, neglected consideration of PPGL in childhood and the immature or even non-functional catecholamine phenotype of pseudohypoxic PPGLs, the prevalence of PPGL in pediatrics may be underestimated. We recently reviewed the biochemical diagnosis of pediatric catecholamine-producing tumors and discuss in this context also the diagnosis of neuroblastoma (109).

Based on their transcriptional profile, PPGLs are divided into two main cluster groups (110, 111). Cluster 1 PPGLs bear PVs encoding two groups of genes, that either lead to a direct stabilization of HIF2α (cluster 1B, including mutations in *VHL*, *HIF2α*, *EGLN1/2*) or encode enzymes of the tricarboxylic acid (TCA) cycle (cluster 1A) that indirectly affect HIF2α stabilization (62). In contrast to the pseudohypoxic cluster 1 PPGLs, which are diagnosed earlier in life (median: 31.1 years; range: 5.5-79.3 years (1)), cluster 2 PPGLs (median: 45.2 years; range: 16.0-78-4 years) are characterized by an activation of kinase signaling pathways. The immature catecholamine phenotype of cluster 1 PPGLs is characterized by the absence of glucocorticoid-induced expression of PNMT, the rate-limiting enzyme that converts norepinephrine to epinephrine. In contrast to epinephrine-producing cluster 2 PPGLs that occur almost exclusively in the adrenal, cluster 1 PPGLs tend to metastasize more frequently and occur at intra- and extra-adrenal locations. In contrast to neuroblastomas, extra-adrenal tumor location in PPGL patients is associated with higher disease aggressiveness and shorter disease-specific survival (112). In addition, two other PPGL clusters were described based on mRNA expression analysis (111, 113). The WNT altered cluster is characterized by increased expression of genes in the WNT signaling pathway, while the cortical admixture cluster show expression of adrenal cortex markers (111).

Considering that 96% of pediatric PPGLs belong to the pseudohypoxic cluster (34% cluster 1A, 66% cluster 1B) (114), it becomes obvious that somatic mutations in cluster 1 genes likely occur during embryogenesis, while tumorigenesis of cluster 2 PPGLs may be initiated later in life. The HIF2α-mediated, pseudohypoxic PPGLs are often multifocal tumors and/or metastatic, which indicate somatic mutations even before the settlement of migratory neural crest progenitors at different locations (Figure 4). Moreover, paragangliomas with somatic gain-of-function mutations in *HIF2α* are associated with mosaicism and identical mutations in multiple tumors arising from postzygotic mutations at early stages of embryogenesis (115, 116). Remarkably, *HIF2α* mutations appear to be restricted to PPGLs, whereas they are mostly absent in tumor samples of other tumor entities included in The Cancer Genome Atlas (TCGA) and Genomics Evidence Neoplasia Information Exchange (GENIE) databases (117).

In PPGLs increased expression and stabilization of HIF2α is associated with a more immature/less differentiated phenotype that is accompanied by increased disease aggressiveness (1, 118). PPGLs with enhanced expression/stabilization of HIF2α do not express PNMT, whereas HIF2α expression in neuroblastomas marks a subpopulation of immature neural crest-like cells (2, 119). The induced expression of HIF2α target genes and the associated pseudohypoxic environment may provide cells of cluster 1 PPGLs with a selection advantage that favors tumorigenesis in these cells. This hypothesis is supported by patients with PPGL that occurred as a complication of cyanotic congenital heart disease (CCHD).

These patients suffer from chronic hypoxia and frequently carry somatic PVs in *HIF2α* (120–122). PVs in *HIF2α* or already an increased HIF2α expression during neural crest may provide a survival benefit for neural crest derived cells. The organ of Zuckerkandl, the largest source of extra-adrenal chromaffin cells in mammals, disappears postnatally by a glucocorticoid-mediated mechanism of autophagy (123). An impairment of this mechanism during embryogenesis or immediately after birth may initiate premature paraganglioma formation. Unlike in neuroblastoma, where spontaneous but also drug-induced transitions from different stages of differentiation are observed (97), in the case of PPGLs this hypothesis would tend to suggest that the differentiation status is largely determined by the cellular origin and the underlying mutation (124). The slow growth and comparatively low aggressiveness of PPGLs, indicating a comparable low cellular flexibility, would potentially further support this hypothesis.

In hereditary PPGL patients, multifocal and bilateral tumors are associated with an earlier onset of the disease than in patients with solitary PPGLs (108). This might be explained by the initiation (second hit) of these tumors from a single tumor stem cell already during neural crest cell migration (Knudson two-hit hypothesis (125)). This would imply that in some patients with germline PVs the second chromosomal hit already occurs during embryogenesis, before the neural crest cells migrate to their paraganglial or adrenal localization.

Germline PVs in *MAX* are associated with bilateral and multifocal PPGLs (67%) (126, 127) and the occurrence of both neuroblastomas and PPGLs (128, 129), which highlights the importance of the MYC/MAX convergence point in these catecholamine-producing neoplasms. Furthermore, it suggests that the chromosomal second hit can occur early enough in embryogenesis to allow differentiation of the migrating neural crest cells into both, neuroblasts and chromaffin cells. Although tumors with *MAX* loss-of-function mutations classically belong to cluster 2 PPGLs, data on catecholamines suggest a biochemical phenotype intermediate between the established epinephrine-producing phenotype of cluster 2 PPGLs and a norepinephrine-producing phenotype of cluster 1 PPGL (22, 118, 127). PPGLs with *MAX* mutations belong to a specific molecular subgroup defined as cortical admixture subtype, which overexpress both PPGL markers and adrenal cortex markers (111). The intermediate phenotype of *MAX*-mutated PPGLs suggests that a fully functional MYC/MAX complex is required to facilitate differentiation. Whether c-Myc and MYCN assign different roles by the manifestation of the different phenotypic feature remain unclear. However, it would be reasonable to assume that they exert different functions, given their role in neural crest cell differentiation and migration.

A better understanding of the underlying cellular and molecular mechanisms also opens up for new therapeutic approaches to treat these catecholamine-producing neoplasms of the neural crest. In addition to the involvement of HIF2α in tumorigenesis of PPGLs, we and others also demonstrated the involvement of HIF2α in PPGL metastasis (1, 62, 130, 131), as reflected by the increased metastasis rate of pseudohypoxic cluster 1 PPGLs compared with cluster 2 PPGLs (1). Through the development of selective small-molecule inhibitors targeting HIF2α/ARNT dimerization, potentially suitable drugs are available, which are of particular interest for the more aggressive pseudohypoxic PPGLs and neuroblastomas. Nevertheless, some preclinical PPGL and neuroblastoma models show a lack of efficiency of these inhibitors (1, 63, 132), which is also in line with some clinical data from other HIF2α-dependent tumor entities that show resistance towards the available HIF2α inhibitors (133, 134). This partial lack of efficiency in HIF2α-dependent tumors may provide preliminary evidence that mechanisms independent of ARNT/HIF2α dimerization, for instance through interactions with the MYC/MAX complex (27, 34, 39), are involved in tumorigenesis and metastasis of these tumors. The ARNT-independent mechanisms of HIF2α may offer alternative therapeutic approaches for some patients who exhibit resistance to HIF2α inhibitors or may even be more efficient.

Direct therapeutic targeting of MYC/MYCN has been a challenge for decades considering its "undrugability" protein structure, but a least some small molecules are available that inhibit dimerization of MYC/MAX (135). However, this approach has shown only limited effectiveness (136). This may indicate other factors that influence the complex and thus MYC-target gene expression. For example, HIF2a expression in neuroblastomas is also associated with increased aggressiveness and a more undifferentiated phenotype. Similar to observations in PPGLs, ARNT-dependent inhibition of HIF2α by PT2385 (HIF2α inhibitor) is not sufficient to regulate HIF2α downstream target genes in neuroblastoma (63), which also indicates a ARNT-independent mechanisms of HIF2α in neuroblastoma (see discussion above). This further underlines the potential importance of the HIF-MYC/MAX interaction in these tumors.

6 Conclusion

Emerging single-cell methods have provide further insight in neural crest-derived cell linages, particularly in the adrenal gland, while epigenetic and transcriptomic data have increased our knowledge of tumorigenesis and progression of adrenal neoplasms. A tight regulation of HIF and MYC signaling pathways during neural crest development appears to be essential, among other things, for the complete and correct formation of the adrenal medulla and the sympathetic and parasympathetic ganglia. Dysregulation in either pathway may cause the emergence of neuroblastomas or pheochromocytomas, associated with an undifferentiated and more aggressive phenotype. Especially in pheochromocytomas/paragangliomas, increased expression and stabilization of HIF2α causes these tumors to appear at a younger age. An improved understanding of the cellular origin and pathogenesis of these pediatric adrenal neoplasms will contribute to a more efficient subtype classification and allow more precise and effective treatment of these young patients; in particular, the HIF-MYC/MAX interaction should be further considered.

Author contributions

NB carefully reviewed the literature and wrote the first version of the manuscript. FW and GE provided conceptual input, contributed to the editing, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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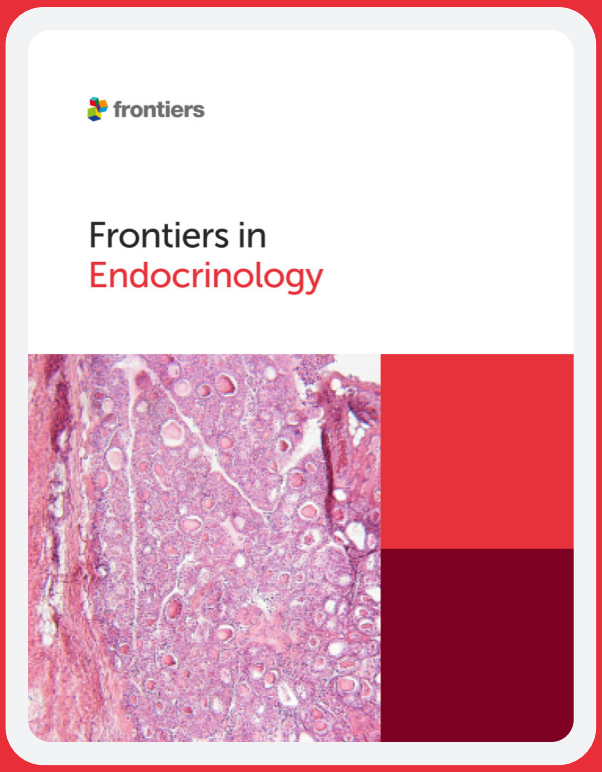

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