UNIVERSITÀ
DEGLI STUDI
FIRENZE

DOTTORATO DI RICERCA IN SCIENZE BIOMEDICHE

CICLO XXXIV

COORDINATORE Prof. Fabrizio Chiti

Genetic diagnostic yield of rare endocrine diseases through Next-Generation Sequencing: our-7-year- experience based on targeted gene panels

Settore Scientifico Disciplinare MED/13

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Anni 2018/2021

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1. Summary

Currently, NGS-based genetic testing has become an indispensable component of the comprehensive diagnostic workup in rare endocrine diseases. For this reason, our clinical genetics laboratory has designed a custom NGS panel including all the known candidate and susceptibility genes for Congenital Hypogonadotropic Hypogonadism (CHH) and Pheochromocytoma/Paraganglioma (Pheo/PGL/HNPGL), respectively. In addition, the *VHL* mutational screening has been developed to diagnose the von Hippel-Lindau syndrome (VHL) in suspected cases. This study aimed to evaluate the diagnostic yield basedon genetic testing in the above-mentioned rare endocrine diseases. After the NGS sequencing, a comprehensive bioinformatic analysis of each variant was performed in order to evaluate their pathogenicity and genotype-phenotype correlations were examined.

The first part of this thesis focused on CHH, which is a congenital disorder covering a widespectrum of signs/symptoms, from classical forms with absent puberty, including Kallmannsyndrome and other syndromic conditions, to milder forms with Adult-Onset Hypogonadism (AOH). To date more than 40 candidate genes have been identified in approximately 40-50% of cases with a genetic diagnosis of CHH. We have enrolled 26 affected patients, of whom 12 showed the classic forms of disease whereas 14 were AOH. The total genetic diagnostic yield of our NGS panel, including 34 candidate genes with strong/definitive clinical evidence, was 23%, with the highest diagnostic rate in classic forms (42%) and the lowest one in the AOH (7%). The majority of mutated genes are well-knowncausative genes, with the exception of *DMXL2*, for which only a few familial cases have beendescribed so far. Interestingly, the unique case of AOH with a clear genetic diagnosis carriedan affected *DMXL2* allele, which is responsible for a mild reproductive

phenotype, according to the literature. Our finding contributes to elucidate the role of *DMXL2* gene in the aetiology of CHH.

The second part of this thesis focused on rare endocrine tumours. Among them, Pheo/PGL/HNPGL arise from neural crest cells and affect adrenal gland (Pheo) or extra-adrenal sites (PGL/HNPGL). More than 20 susceptibility genes have been reported to predispose to Pheo/PGL/HNPGL, with a diagnostic yield of approximately 30%. On the other hand, the VHL syndrome is a monogenic disorder arising from germline mutations in the *VHL* gene and involving multiple organs. All these conditions follow an autosomal mode of inheritance, with an incomplete and age-dependent penetrance related to the specific gene defect. We have enrolled 95 patients affected by Pheo/PGL/HNPGL and 8 suspected VHL cases. Concerning the former, our NGS panel containing 15 susceptibility genes revealed a genetic diagnostic yield of 20%, with the highest diagnostic rate in PGLs (33%) and the lowest one in Pheos (14%). The majority of mutated genes belongs to the *SDHx* family, in line with previous data. Interestingly, one Pheo patient carrying two germline defects in the *SDHD* and *VHL* genes received a diagnosis of VHL syndrome, thanks to the gene panel results. Regarding patients with suspected VHL, we diagnosed a "true" VHL syndrome in 25% of cases.

This study highlights the power of genetic testing as a diagnostic tool with relevant implications in genetic counselling and clinical management of the mutation carriers. Based on our results, CHH patients should be well-characterized prior to genetic testing, as the presence of a disease-causing genotype is almost exclusively found in classic forms. Hence, the genotype of AOH patients seems to be different from those with CHH. In case of rare endocrine tumours, we confirmed that genetic testing is relevant for correct and early diagnosis, leading to a better prognosis and to an appropriate treatment, of both the patient and her/his family members through an active surveillance.

2. Introduction

2.1. Introduction to rare endocrine diseases

Rare diseases are conditions which affect a small number of individuals compared to the general population, and there is no universal consensus about the definition of their frequency. In the United States, a rare disease is defined as a condition that affects fewer than 200,000 people (https://www.fda.gov/industry/designating-orphan-product-drugs-and-biological-products/orphan-drug-act-relevant-excerpts). The European Union defines a disease as rare if it affects fewer than 1 out of 2000 people within the general population (EC/141/2000). There are between 5000 and 8000 rare diseases, most of them with a known genetic basis (www.orpha.net). Among these, according to the National Institutes of Health, more than 175 distinct rare diseases affect the endocrine system. The symptoms related to these diseases may seem vague or overlapping with those of other complex diseases, which makes challenging differential diagnosis. In this context, rare endocrine diseases should be managed by experienced endocrinologists, who can solve the puzzle, recognizing the disease-pattern in a host of odd or unique symptoms. However, it is a challenge to diagnose these rare conditions by only assessing clinical features, and that is why many patients and their families undergo a long-lasting diagnostic odyssey. In this regard, a major breakthrough has been made thanks to the increasing availability and cost-effectiveness for clinical use of high throughput genetic tests, i.e. Next-Generation Sequencing (NGS)-based analyses. In fact, in the clinical endocrine practice, genetic testing is primarily requested to confirm a suspected clinical and endocrine diagnosis, especially when clinical features are ambiguous. Additionally, it also contributes to the identification of pre-symptomatic individuals. Thereby, their

risk to develop an inherited endocrine disorder can be predicted, and preventive measures might be taken. In addition, the knowledge on an inherited genetic variant is the basis to advice the patients´ family in respect of family planning and prenatal testing. Finally, the knowledge of the genetic cause underlying a certain endocrine disorder allows to understand its pathophysiology and thereby to develop and apply a personalized therapy. For all these reasons, a clinical and research collaboration between an expertise team, especially including endocrinologists and geneticists, is essential for diagnosis, management and surveillance of patients and their relatives.

Over the years, the laboratory of genetics of rare endocrine diseases at the AOU Careggi Hospital, where I have collaborated during my PhD, has developed NGS-based gene panels for the hypothalamus-pituitary axis dysfunction and for certain types of rare endocrine tumours. Concerning the first category, in 2018 we have introduced a targeted gene panel for Congenital Hypogonadotropic Hypogonadism (CHH) into the diagnostic setting. Concerning the rare endocrine tumours, Pheocromocytoma/Paraganglioma (Pheo/PGL), and von Hippel-Lindau (VHL) syndrome they have been studied since 2016 in the above- mentioned laboratory by performing multiple-gene panel and single-gene sequencing, respectively.

In this part of the thesis, the above-mentioned diseases will be introduced through the description of their epidemiology, clinical presentation, genetic bases and genetic counselling.

2.2. Congenital Hypogonadotropic Hypogonadism (CHH)

CHH is a rare endocrine disease affecting approximately 1 out of 4000 births (Mitchell et al.,2011), with a male to female ratio of approximately 4 to 1 (Seminara et al., 1998; Mitchell et al., 2011). It is caused by the deficient production, secretion or action of the Gonadotropin- Releasing Hormone (GnRH), which is the master hormone regulating the reproductive axis(Kim, 2015).

During embryonic development, the neuroendocrine GnRH neurons originate outside the central nervous system in the olfactory placode from both the neural crest and ectodermal progenitors, and then migrate into the brain in close association with growing axons of olfactory and/or terminal nerves (Schwanzel-Fukuda et al., 1989; Teixeira et al., 2010; Forniet al., 2011; Forni et al., 2014). Once in the hypothalamus, GnRH neurons detach from theiraxonal guides, disperse further into the brain parenchyma and stop migrating. At birth, post-migratory GnRH neurons have reached their final destination in the brain and are embedded in a complex neuronal network contributing to their proper maintenance and functionality. The activation of kisspeptin, which is the main regulator of GnRH neuron activation and hormone secretion, results in the onset of puberty. Hence, the GnRH from neuron's axon terminals into the hypophyseal portal vasculature occurs (Bianco et al., 2009). GnRH acts via the GnRH receptor, which is expressed on the gonadotropic cells in the anterior pituitary gland. This action regulates both synthesis and release of gonadotropins, such as Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH), which control gonadal maturation and adult reproductive physiology via the hypothalamic–pituitary– gonadal (HPG) axis.

The failed/incomplete embryonic migration of GnRH neurons generally results in the KS phenotype. KS is found in about 50% of patients affected by CHH. In case of deficient/altered secretion and/or action of GnRH, the phenotype is normosmic CHH (nCHH).

2.2.1. Clinical presentation

CHH is a clinically heterogeneous condition covering a wide spectrum of symptoms, where the typical clinical features are incomplete or absent puberty and infertility (Bonomi et al., 2018). CHH can manifest itself with anosmia/hyposmia (KS) or as a normosmic form (nCHH). In addition, non-reproductive features can be recognized in CHH patients, such as midline facial defects (cleft lip or palate), unilateral renal agenesis, hearing loss, synkinesia,dental agenesis and short metacarpals (Young et al., 2019).

In the neonatal period, the lack of HPG axis activation might provide early diagnostic signsfor identifying CHH in male infants (Evain-Brion et al., 1982; Grumbach et al., 2005; Kaplanet al., 2010; Malin et al., 2000). In these cases, cryptorchidism and micropenis can be signs ofGnRH deficiency, although these features may vary during infancy, whereas no specific clinical signs of CHH are present in female neonates. The signs and symptoms of the diseasearise in adolescents, where the failure to activate the HPG axis leads to absent puberty (**Figure 1**). In contrast to constitutional delay of growth and puberty (CDGP) characterizedby an initiated and eventually spontaneously completed puberty, in the majority of CHH patients, puberty never occurs (absent puberty) or, less commonly, it is initiated then arrested (adult-onset CHH) (Pitteloud et al., 2002; Shaw et al., 2011; Young, 2012). Adolescents with CHH exhibit steady linear growth and thus lack the so-called growth spurt (van Buuren and Ooms, 2009; Lawaetz et al., 2015; Hero et al., 2015). Given the delayed epiphyseal closure, these individuals often have eunuchoid proportions. The mostcommon complaints in male adolescents with CHH include absent and/or minimal virilization and low libido. Among female adolescents, lack of breast development and/or primary amenorrhoea by the age of 15 years are the typical disturbances. Most patients are diagnosed late in adolescence or early in adulthood, when they access to the clinic for evaluation of infertility or even for osteoporotic fractures. It is worth noticing that the clinical heterogeneity of CHH makes differentiation

from CDGP difficult (Palmert and Dunkel, 2012; Lawaetz et al., 2015). The clinical manifestations that argue in favour of CHH since they rarely seen in CDGP, are micropenis and/or cryptorchidism in males, a poor senseof smell, which suggests KS, and other non-reproductive defects, mainly including sensorineural deafness, cleft lip, bimanual synkinesia, etc. (see above). In addition to these features, associated congenital phenotypes are also very useful as they indicate a syndromicform of CHH. In fact, it should be considered that, CHH can occur as a part of complex genetic syndromes, as in case of CHARGE syndrome, Gordon Holmes syndrome (GHS) and hypomyelinating leukodystrophy type 8 (HLD8). CHARGE is an abbreviation for several common features of the syndrome, i.e. coloboma, heart defects, choanal atresia, growth retardation, genital abnormalities and ear malformations with/without deafness (Hall, 1979;Hittner et al., 1979). On the other hand, GHS and HLD8 are neurological disorders, characterized by cerebellar ataxia/atrophy and eventually by nCHH.

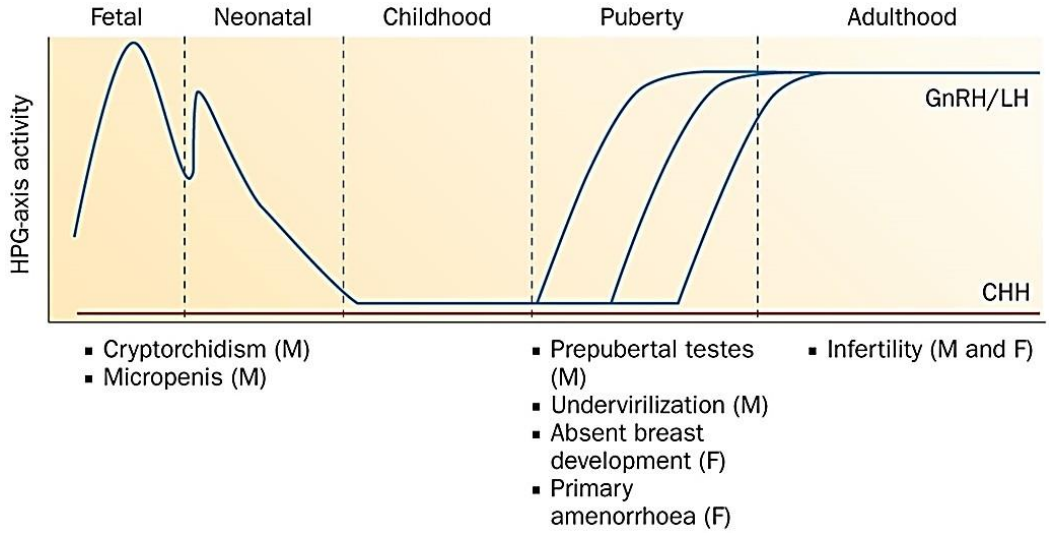

Figure 1: Activity of the HPG axis across the lifespan (from Boehm et al., 2015).

2.2.2. Genetics of CHH

CHH is heterogeneous not only clinically but also genetically. To date, more than 40 genes with variable expressivity, penetrance and inheritance have been identified as potential cause of the disease (**Table 1**) (Butz et al., 2021; Cangiano et al., 2021). These genes can take part in the following pathways: i) GnRH neuron migration / axon guidance; ii) GnRH neuron and gonadotropic cells differentiation and fate specification; iii) GnRH neuron activation and networking; iv) secretion and action of GnRH; v) gonadotropin secretion or action (Cangiano et al., 2021).

The higher prevalence of rare variants in CHH patients concerns the *FGFR1, CHD7, PROKR2, SEMA3A, ANOS1* and *GNRHR* genes (Table 1; see review Cangiano et al., 2021). Currently, through the sequencing of a targeted gene panel using NGS, a genetic diagnosis is possible in about 45-50% of cases, and, it is expected that novel CHH-associated genes will be discovered by WES analysis in the near future (Cioppi et al., 2021).

From a genetic point of view, this disease shows two peculiar features: 1) distinguishing between KS and nCHH is often difficult because mutations in genes involved in GnRH neuron migration / axon guidance might result in both forms of disease (Boehm et al., 2015).In fact, with the exception of certain genes purely associated with KS or with nCHH, some others (e.g. *FGFR1* and *PROKR2*) can be involved in both clinical manifestations, even within the same kindred (Cangiano et al., 2021). 2) We must consider that CHH is no longerviewed as a Mendelian monogenic disease, since rare variants in two (digenicity) or more (oligogenicity) candidate genes have been found in the same patient, supporting a digenic/oligogenic inheritance in about 20% of cases (Boehm et al., 2015). Although the oligogenic basis of CHH makes the genotype-phenotype correlation even more complex, itmay explain the variable penetrance of the same pathogenic variant within the same familymembers (Cangiano et al., 2021; Butz et al., 2021).

It is important to note that some of these genes can be associated with other additional clinical features underlying a certain syndrome, hence a correct diagnosis could be improved by the identified gene defect(s). For instance, in *CHD7* mutations carriers, a severe CHH phenotype, including micropenis and cryptorchidism could be observed in a clinical context belonging to the CHARGE syndrome. Mutations in *OTUD4, RNF216* and *PNPLA6* also cause the GHS, characterized by neurological and reproductive defects. In addition, the*POLR3*-related leukodystrophy, in particular HLD8, could be associated to other clinical features, including oligodontia and/or hypogonadotropic hypogonadism.

In addition, most of these CHH-candidate genes can be responsible for other syndromic conditions which do not include CHH. This is the case of *PROKR2* gene, which is also associated with septo-optic dysplasia (SOD) and Morning Glory syndrome. In addition to *PROKR2*, other candidate genes cause SOD, including *FGF8, SOX2, HESX1, SOX3* and *OTX2* (Boehm et al., 2015; Cangiano et al., 2021). Besides CHH, *FGFR1* is responsible both for Hartsfield syndrome, characterized by the triad of holoprosencephaly, ectrodactyly,

and cleft/lip palate, and for split-hand/foot malformation (Simonis et al., 2013), whereas *FGF17* has been rarely associated with the Dandy-Walker syndrome (Zanni et al., 2011). *SOX10* is well-known to cause the Waardenburg syndrome, characterized by pigmentary abnormalities of the hair, skin, and eyes, by congenital sensorineural hearing loss and by the absence of "dystopia canthorum" (see references in Boehm et al., 2015). Individuals carrying *SOX10* mutations may have also neurologic abnormalities, including mental impairment, myelination defects and ataxia. In case of *NR0B1* mutation, the Congenital Adrenal Hypoplasia (CAH) phenotype could be observed (Esden-Tempska et al., 2012) while the *DMXL2* gene has been associated to polyendocrine deficiencies and polyneuropathies (Tata et al., 2014).

2.2.3. Genetic counselling in CHH

As stated above, many gene defects are associated with other non-reproductive developmental abnormalities, hence the clinical management of CHH patients has to be carefully conducted, possibly in collaboration with other medical specialties. In fact, there may not always be a clearly distinction between CHH with non-reproductive defects and CHH as part of a complex syndrome (Cangiano et al., 2020). In this context, particular attention should be paid to the carriers of *CHD7* mutations, given their causal effect both onCHH and CHARGE syndrome. In the majority of cases (~80%) pathogenic or likely pathogenic *CHD7* variants are associated with CHARGE features (Xu et al., 2018), althoughit is not always possible to differentiate the mutations resulting in CHH from those causing CHARGE syndrome. In addition, a clinical re-evaluation of patients and their relatives should be recommended in case of mutations in the *RNF216*, *OTUD4*, *PNPLA6* genes and in the *POLR3A*/*POLR3B* genes, in order to exclude any neurological

impairment underlying the GHS and the HLD8, respectively (Seminara et al. 2002; Margolin et al. 2013;Shi et al. 2014).

Concerning the reproductive issue, in the majority of CHH patients, fertility can be induced by using hormonal therapies (Boehm et al., 2015), thus mutations can be transmitted either through spontaneous pregnancy or through Assisted Reproductive Techniques (ART). Overall, the complexity of this disease makes predicting the exact health consequences for the offspring difficult; however, Preimplantation Genetic Testing for monogenic conditions(PGT-M) or prenatal diagnosis should be offered to couples, mainly for syndromic cases characterized by severe clinical features.

To note, the traditional view of CHH as a lifelong disease has been changed following the observation of spontaneous remission cases in about 10-20% of patients affected by CHH (Dwyer et al., 2016). Thus, all clinicians must be aware that a periodic suspension of the substitutive testosterone therapy should be advised in order to verify the "reversibility".

Table 1: List of CHH candidate genes with the description of mode of inheritance and phenotypes.

Gene	OMIM number	Locus	Inheritance	Prevalence in CHH patients	CHH phenotype	Additional phenotypes	Syndromic-associated phenotypes
AMH	600957	19p13.3	AD	Very low	KS/nCHH	Unknown	Unknown
AMHR2	600956	12q13.13	AD	Very low	KS/nCHH	Unknown	Unknown
ANOS1	300836	Xp22.31	XLR	4.5%	KS	Hearing loss, synk., RA	Unknown
AXL	109135	19q13.2	AR/oligo	4.85%	nCHH	Unknown	Unknown
CCDC141	616031	2q31.2	AR/di-oligo	Very low	nCHH	Unknown	Unknown
CHD7	608892	8q12.2	AD/AR/di-oligo	8.1%	KS/nCHH	Hearing loss	CHARGE sdr
DDC	120470	18q21.2	AD/di-oligo	Very low	KS/nCHH	Cleft-lip palate, synk.	Unknown
DMXL2	612186	15q21.2	AD	2.3%	nCHH	Hearing loss	Polyendocrine-polyneuropathy
DUSP6	602748	12q21.33	Oligo	1.3%	KS/nCHH	Hearing loss	Unknown
FEZF1	613301	7q31.32	AR	Very low	KS	Unknown	Unknown
FGF17	603725	8p21.3	Oligo	1.1%	KS/nCHH	Unknown	Unknown
FGF8	600483	10q24.32	Oligo	1.2%	KS/nCHH	Cleft-lip palate, synk.	SOD
FGFR1	136350	8p11.23	AD/AR/di-oligo	8.9-11.4%	KS/nCHH	Cleft-lip palate, synk.	Hartsfield sdr
FLRT3	604808	20p12.1	Oligo	0.8%	KS/nCHH	Cleft-lip palate	Unknown
FSHB	136530	11p14.1	AR	Very low	nCHH	Unknown	Unknown
GATA2	37295	3q21.3	AD	Very low	nCHH	Unknown	Unknown
GLCE	612134	15q23	Unknown	Very low	KS/nCHH	Unknown	Unknown
GLI2	165230	2q14.2	AD	Very low	nCHH	Cleft-lip palate	Culler–Jones sdr
GNRH1	152760	8p21.2	AR/di-oligo	1.5%	nCHH	Unknown	Unknown
GNRHR	138850	4q13.2	AR/di-oligo	4.4%	nCHH	Unknown	Unknown
HESX1	601802	3p14.3	AD/AR	1.4%	KS/nCHH	Unknown	SOD

HS6ST1	604846	2q14.3	Oligo	Very low	KS/nCHH	Unknown	Unknown
IGSF10	617351	3q25.1	AD	Very low	nCHH	Unknown	Unknown
IL17RD	606807	3p14.3	Oligo	2.1%	KS/nCHH	Hearing loss	Unknown
KISS1	603286	1q32.1	AR	Very low	nCHH	Unknown	Unknown
KISS1R	604161	19p13.3	AR	1.6%	nCHH	Unknown	Unknown
KLB	611135	4p14	AD	4%	KS/nCHH	Cleft lip, synk. RA	Unknown
LEP	164160	7q32.1	AR	Very low	nCHH	Unknown	Obesity
LEPR	601007	1p31.3	AR	Very low	nCHH	Unknown	
LHB	152780	19q13.33	AR	Very low	nCHH	Unknown	Unknown
LHX3	600577	9q34.3	AR	Very low	nCHH	Hearing loss	Unknown
LHX4	602146	1q25.2	AD	Very low	nCHH	Unknown	Unknown
NDNF	616506	4q27	AD	Very low	KS	Unknown	Unknown
NR0B1	300473	Xp21.2	XLR	Very low	nCHH	Unknown	CAH
NSMF	608137	9q34.3	AR/di-oligo	1.0%	KS/nCHH	Unknown	Unknown
NTN1	601614	17p13.1	AD/di-oligo	Very low	KS/nCHH	Cleft-lip palate, synk.	Unknown
OTUD4	611744	4q31.21	AR/di-oligo	Very low	Unknown	Unknown	GHS
OTX2	600037	14q22.3	AD	Very low	nCHH	Unknown	SOD
PCSK1	162150	5q15	AR	Very low	nCHH	Unknown	Obesity
PITX2	60154	4q25	AD	Very low	nCHH	Unknown	Axenfeld–Rieger sdr
PLXNA1	601055	3q21.3	AR/di-oligo	Very low	KS/nCHH	Unknown	Unknown
PNPLA6	603197	19p13.2	AR	1.1%	nCHH	Unknown	GHS, Boucher–Neuhauser
POLR3A	614258	10q22.3	AR	1.1%	nCHH	Unknown	Hypomy.leukodyst, WR
POLR3B	614366	12q23.3	AR	1.1%	nCHH	Unknown	Hypomy.leukodyst, 4H

PROK2	607002	3p13	AR/di-oligo	2.4%	KS/nCHH	Synkinesia	Unknown
PROKR2	607123	20p12.3	AR/di-oligo	5.6%	KS/nCHH	Synkinesia	SOD
PROP1	601538	5q35.3	AR	Very low	nCHH	Unknown	Unknown
RNF216	609948	7p22.1	AR/di-oligo	Very low	nCHH	Unknown	GHS
SEMA3A	603961	7p12.1	AD/di-oligo	5.3%	KS/nCHH	Unknown	Unknown
SEMA3E	608166	7q21.11	Oligo	Very low	KS/nCHH	Unknown	CHARGE sdr
SEMA7A	607961	15q24.1	Oligo	4%	KS/nCHH	Unknown	Unknown
SMCHD1	614982	18p11.32	AD	Very low	KS	Unknown	Bosma-arhinia microphthalmia
SOX10	602229	22q13.1	AD	4.4%	KS	Hearing loss	Waardenburg sdr
SOX2	184429	3q26.33	AR	2.3%	nCHH	Hearing loss	SOD
SOX3	313430	Xq27.1	XLR	Very low	nCHH	Hearing loss	SOD
SPRY4	607984	5q31.3	Oligo	3.2%	KS/nCHH	Unknown	Unknown
STUB1	607207	16p13.3	AR	Very low	nCHH	Unknown	GHS
TAC3	162330	12q13.3	AR	0.9%	KS/nCHH	Unknown	Unknown
TACR3	162332	4q24	AR	1.5%	nCHH	Unknown	Unknown
TBX3	601621	12q24.21	AD	Very low	nCHH	Cleft-lip palate	Ulnar–mammary
TUBB3	602661	16q24.3	AD	Very low	KS	Cleft-lip palate	Moebius sdr
WDR11	606417	10q26.12	AD/di-oligo	Very low	KS/nCHH	Hearing loss	Unknown

AD: autosomal dominant; AR: autosomal recessive; CAH: congenital adrenal hypoplasia; GHS: Gordon Holmes syndrome; KS: Kallmann syndrome; nCHH: normosmic congenital hypogonadotropic hypogonadism; oligo: oligogenicity; RA: renal agenesis; synk: synkinesia; SOD: septo-optic dysplasia; sdr: syndrome; WR: Wiedemann–Rautenstrauch ; XLR: *X-linked* recessive.

2.3. Pheocromocytoma/Paraganglioma (Pheo/PGL)

Pheo/PGL are neuroendocrine tumours arising from neural crest cells, with an annual incidence of approximately 1 per 100 000 (Berends et al., 2018). In the 2017 World Health Organization (WHO) classification, Pheo is an adrenal tumour and PGL is an extra-adrenaltumour. Only anatomical location is used to distinguish between them, since the two tumour types cannot be differentiated on the basis of histologic findings (Tischler et al., 2017; Neumann et al., 2018). Pheos arise from the adrenal medulla' chromaffin cells, which are homologous to sympathetic neurons, usually producing and secreting the catecholamines, i.e. dopamine, norepinephrine (noradrenaline), and epinephrine (adrenaline). PGLs derive from paraganglia, which are seen in close association with components of the sympathetic and parasympathetic nervous system (Lack, 2007; Tischler et al., 1998; Oudijk et al., 2016; Hayashi et al., 2014). PGLs can arise in any location where paraganglia normally reside (**Figure 2**) (Asa et al., 2018). Sympathetic PGLs arise from the sympathetic ganglia in torax, abdomen and pelvis (DeLellis et al., 2004). In the head and neck region, PGLs have a parasympathetic origin (HNPGLs) (Williams, 2017). Approximately 80–85% of chromaffin cell tumours are Pheos, and 15–20% are PGLs (Lenders et al., 2005; Whalen et al., 1992; Pacak et al., 2001). HNPGLs represent only 0.6% ofall head and neck tumours and 0.03% of all tumours (LeeJH et al., 2002). Almost all Pheos and sympathetic PGLs are biochemically active and secrete catecholamines or their metabolites, while HNPGLs do not produce significant amounts of catecholamines (Chen et al., 2010; McNicol, 2010; Welander et al., 2011). These tumours are characteristically well-vascularized and typically benign; nonetheless, roughly 10–15% may metastasize to the lungs, bone, liver, and lymph nodes (Boedeker, et al., 2011).

Genetics and developments of diagnostic procedures, i.e. biochemical testing and specific imaging studies, helps improving the diagnostic rate.

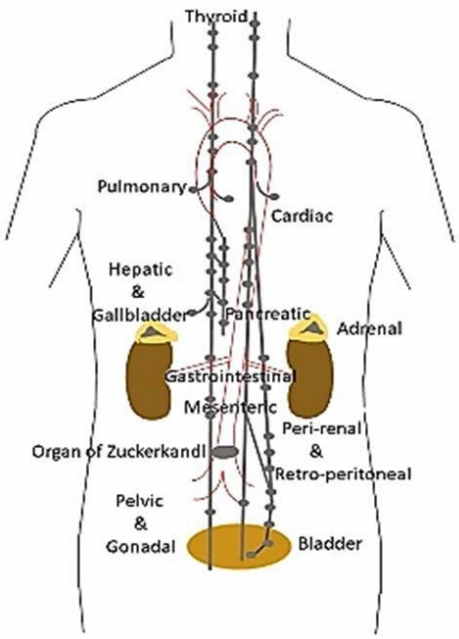

Figure 2: Location of paraganglia. This figureillustrates the location of normal paraganglia in the neck, thorax and abdomen. Locations in the head are not shown (from Asa et al., 2018).

2.3.1. Clinical presentation

The symptoms underlying Pheo/PGL are mainly due to the hypersecretion of catecholamines and can mimic more than 30 medical disorders, hence its name of "the greatmimic" (Young, 2016).

As stated above, pheocromocytoma and sympathetic paraganglioma (PPGL) often secrete catecholamines resulting in a variable clinical presentation (Manger, 2009). The diverse and variable signs and symptoms of PPGL are related to: i) the pattern of catecholamine release (continuous/sporadic); ii) the amount of catecholamine release (determined mainly by the size of the tumour); iii) the type of catecholamine released (dopamine, noradrenaline or adrenaline). Paroxysmal symptoms with a high variety of severity and frequency are a typical feature, presumably due to the sporadic secretory activity. Such symptoms may occur spontaneously or may be provoked by triggers,

including exercise, abdominal pressure, large meals, drugs (such as glucocorticosteroids, antidepressants, some anaesthetics, etc.), stress, alcohol and food, e.g. tyramine in some cheeses (Manger, 2009; Reisch et al., 2006; Lenders et al., 2014; Pacak, 2007; Eisenhofer et al., 2007). In about 80% ofpatients affected by PPGL an increase in blood pressure caused by the action of tumour catecholamines on the adrenergic receptors has been well-documented (Canu et al., 2019). This endocrine hypertension may occur as continuous or intermittent, with or without sudden spikes, associated or not with other symptoms of adrenergic activations, such as palpitations, sweating, trembling and anxiety (Canu et al., 2019). A recent meta-analysis showed that headache, palpitations and sweating occurred in 60%, 59%, 52% of patients, respectively, whereas other symptoms occurred at a much lower frequency (Soltani et al., 2015). It is worth noting that in some instances, the hypertensive crises can be very severe, with consequent cardiovascular complications, i.e. myocardial ischemia, arrhythmias, hearth failure, cerebral haemorrhage and sudden death. Diabetes and prediabetes representother signs of the hyperadrenergic state. In addition, PPGL patients are more often characterized by lower BMI compared to those without PPGL (Manger, 2009; Reisch et al.,2006; Lenders et al., 2014; Pacak, 2007).

On the other hand, almost all HNPGL cases do not produce significant amounts of catecholamines, thus the presence of catecholamine excess-related symptoms rarely occurs.They may present with a series of symptoms related to a temporal bone or cervical mass causing compression or infiltration of the adjacent structures, leading to hearing loss, pulsatile tinnitus, dysphagia, and cranial nerve palsies (Taieb et al., 2014).
Of note, many Pheos/PGLs are unidentified during life and are diagnosed only during autopsies, meaning that the clinical diagnosis is missed in a substantial number of patients (McNeil et al., 2000; Lo et al., 2000). The main reason behind this phenomenon is the absenceof specific clinical symptoms or the lack of biochemical evidence. However, it

is worth mentioning that these rare tumours can be lethal if left undiagnosed. Thus, rapid recognition is relevant. In the last few years, these undiagnosed cases are increasingly being discovered on the basis of family and germline mutation testing.

2.3.2. Genetics of Pheo/PGL

These diseases have the highest known hereditability rate among all human tumours. Up to 30% of affected patients carry germline mutations in one of at least 20 known susceptibility genes (**Table 2**), and this number is expected to increase in the near future, thanks to the discovery of novel disease-causing genes through the widespread diffusion of Whole Exome Sequencing (WES) in the clinical setting.

The germline genetic profiling of Pheo/PGLs has shown the occurrence of two main clusters due to the activation of two different pathogenic pathways (Burnichon et al., 2011): i) pseudo-hypoxia cluster, including tumours mainly related to *VHL* or *SDHx* mutations; ii) cluster of kinase receptor signalling and protein translation pathway, including tumours mainly linked to *NF1* and *RET* mutations. The two different pathogenic pathways cause different phenotypes in terms of secretory pattern and biochemistry (Eisenhofer et al., 2001).

Among the most frequently mutated genes in patients affected by Pheo/PGL are *SDHB*, *SDHD*, *VHL*, *RET* and *NF1*. Notably, for each disease-susceptibility gene, a mutated allele is sufficient to predispose to the disease, showing an autosomal dominant pattern of inheritance. All the detailed gene information is listed in **Table 2**. It is important to note that some of these genes (*NF1*, *VHL*, *RET*, *SDHx*) are associated with overlapping syndromic conditions where Pheo can represent one of the clinical features underlying the syndrome. By contrast, patients with mutations of *TMEM127* and *MAX* genes

generally have only Pheo/PGL. It should be noted that a minority of *SDHx* mutation carriers have also gastrointestinal stromal tumours (GISTs), renal-cell carcinomas (RCC), or pituitary adenomas (Neumann et al., 2019).

The clinical features of the overlapping syndromes (**Table 2**) have been known for more than 100 years, starting with neurofibromatosis type 1, von-Hippel-Lindau disease, MEN-2 and Carney-Stratakis syndrome. A brief description of the above-mentioned syndromes andtheir gene defects are reported below.

Table 2: List of Pheo/PGLs candidate genes with the indication of mode of inheritance and associated phenotypes.

Gene	OMIM number	Locus	Inheritance	Associated-syndrome	Other non-chromaffin tumours
DNMT3A	602769	2p23.3	AD	Tatton-Brown-Rahman syndrome, Heyn-Sproul-Jackson syndrome	Unknown
EGLN1	606425	1q42.2	AD	Familial Erythrocytosis	Unknown
EGLN2	606424	19q13.2	AD	Unknown	Unknown
EPAS1	603349	2p21	AD	Familial Erythrocytosis	Unknown
FH	150800	1q43	AD	Leiomyomatosis and RCC syndrome	Unknown
IDH1	147700	2q34	AD	Unknown	Unknown
KIF1B	605995	1p36.22	AD	Charcot-Marie-Tooth 2A1	Unknown
MAX	154950	14q23.3	AD	Unknown	Rarely also RCC
MDH2	154100	7q11.23	AD	Unknown	Unknown
NF1	613113	17q11.2	AD	NF1 syndrome	Cutaneous neurofibromas, malignant peripheral-nerve-sheath tumours, breast cancer
RET	164761	10q11.21	AD	MEN-2 syndrome	Medullary thyroid carcinoma, hyperparathyroidism
SDHA	600857	5p15.33	AD	PGL5	Rarely also pituitary adenoma, GIST, RCC
SDHAF2	613019	11q12.2	AD/ maternal imprinting	PGL2	Unknown
SDHB	185470	1p36.13	AD	PGL4	Rarely also pituitary adenoma, GIST, RCC
SDHC	602413	1q23.3	AD	PGL3	Rarely also pituitary adenoma, GIST, RCC
SDHD	602690	11q23.1	AD/ maternal imprinting	PGL1	Rarely also pituitary adenoma, GIST, RCC
SLC25A11	604165	17p13.2	AD	PGL6	Unknown
TMEM127	613403	2q11.2	AD	Unknown	Unknown
VHL	608537	3p25.3	AD	VHL syndrome	Retinal and CNS hemangioblastomas, RCC, pancreatic neuroendocrine tumours, ELST

AD: autosomal dominant; CNS: central nervous system; ELST: endolymphatic sac tumour; GIST:gastrointestinal stromal tumour; RCC: renal cell carcinoma.

2.3.2.1. Neurofibromatosis type 1

Heterozygous pathogenic mutations of the *NF1* gene are well-known causes of neurofibromatosis type 1 (OMIM # 162200). This condition is characterized by neurofibromas, café au lait spots, axillary freckling, iris hamartomas (Lisch nodules), bone abnormalities, gliomas of the central nervous system, macrocephaly, and cognitive deficits.Pheos and PGLs are present in 1 to 3% of affected patients (Gutmann et al., 1997; Bausch etal., 2007; Gruber et al., 2017).

2.3.2.2. Von Hippel-Lindau disease

The *VHL* gene is responsible for the dominantly inherited von Hippel-Lindau disease (OMIM #193300). This condition is considered as a familial cancer syndrome, predisposing to a variety of malignant and benign neoplasms, including Pheo/PGL. The occurrence of Pheo/PGL is common in these patients, and it may be part of the patient's initial presentation, even in early infancy. In these cases, mutations in codons 98, 161 and 167 of the VHL protein are often found (Neumann et al., 1991; Bender et al., 2001; Neumann et al., 2018). A distinction between different types of *VHL* mutations and the related phenotype(s) has to be made: i) missense *VHL* mutations predict von Hippel–Lindau disease type 2; ii) loss of function *VHL* mutations are often associated with RCC and in rare cases with pheocromocytoma, resulting in von Hippel–Lindau disease type 1 (Neumann et al., 2019). Detailed clinical and genetic features of this condition will be discussed apart in the next chapter.

2.3.2.3. MEN-2

The multiple endocrine neoplasia type II (MEN-2; OMIM #162300) is caused by heterozygous mutations in the *RET* oncogene. It can be divided into two main subtypes: i) type 2A, which is characterized by medullary thyroid carcinoma (MTC), Pheos and parathyroid hyperplasia; ii) type 2B, which is characterized by aggressive MTC, Pheos, mucosal neuromas. Most MEN-2B-affected individuals may also present characteristic physical features, including full lips, thickened eyelids, high-arched palate, marfanoid habitus, skeletal anomalies and gastrointestinal problems (Neumann et a., 2007; Pick et al., 2012). The p. Met918Thr variant of *RET* seems to be the most commonly identified variant in patients affected by MEN-2B. It would be rare for these patients to first present with Pheo, since the typical features of this syndrome are manifested relatively early, even in infancy, such as early-onset MTC, ganglioneuromas (typically involving the tongue, lips, and eyelids), skeletal deformities, joint laxity and intestinal ganglioneuromatosis (Neumann et a., 2007; Pick et al., 2012).

2.3.2.4. Carney-Stratakis syndrome

The autosomal dominant Carney-Stratakis syndrome is associated with *SDHx* mutations and characterized by the association of Pheos, PGLs, or both with GIST and pituitary adenoma (Settas et al., 2018; Xekouki et al., 2019).

2.3.3. Genetic counselling in Pheo/PGL

After the detection of the mutation, the identified gene defect guides the tailoring of imaging studies and subsequent medical management (Neumann et al., 2019). For instance, imaging studies for hemangioblastomas of the eyes and central nervous system and for tumours of the ears, kidneys, and pancreas are performed if *VHL* is mutated. Once the pathogenic *RET* mutation is identified in a patient with pheocromocytoma, clinicians must be aware that virtually all mutation carriers will have MTC, whereas 20% of patients with MEN-2A, but not MEN-2B, will have hyperparathyroidism. In case of *SDHx*, *MAX*, or *TMEM127* mutation carrier, the screening for other chromaffin tumours or for rare kidney cancers, pituitary adenomas and GISTs is routinely carried out

The results of gene testing need to be considered also in the pre-surgery planning. In fact, *RET* germline mutations are often associated with bilateral adrenal tumours, which can occur metachronously, with intervals of more than a decade, thus the preservation of sufficient adrenal cortex is vital (Castinetti et al., 2014; Neumann et al., 2019). Minimally invasive surgery performed by expert surgeons is a crucial step in these cases. An organized, long-term postoperative care program is essential for patients with germline mutations and should be in the hands of endocrinologists. The goals of this program consist of surveillance for and management of associated nonchromaffin tumours, including: i) MTC and primary hyperparathyroidism in patients with MEN-2; ii) hemangioblastomas of the retina, cerebellum, and spine, along with RCC and pancreatic neuroendocrine tumours, in patients with VHL disease; iii) peripheral-nerve-sheath tumours and breast cancer in patients with neurofibromatosis 1 (NF1 carriers); iv) GISTs, RCCs, and pituitary tumours in patients with *SDHx* and *MAX* mutations.

In the context of genetic counselling, a genetic testing should be offered to all first-degree relatives of a mutation carrier. Testing of relatives results in a 50% likelihood of

identifying mutation carriers, who are typically asymptomatic. Clinical surveillance and management related to the identified gene defect should be offered to these asymptomatic mutation (Lenders et al., 2014). The age at onset and gene-specific age-related penetrance guide the screening for pheocromocytoma, paraganglioma, and nonchromaffin tumours in order to identify these neoplasms at an early stage, when they are resectable. It is important that both patients and clinicians understand the concept of "disease-penetrance", as the disease does not develop in all mutation carriers. In fact, all susceptibility genes for Pheo/PGL show an incomplete and age-dependent penetrance. For example, the mean penetrance of Pheo/PGL is 50% by the age of 44 years in *RET* mutation carriers, while it is 50% at the age of 52 years in carriers of *VHL* mutations (Castinetti et al., 2014; Bender et al., 2001). On the other hand, the *SDHx* mutations penetrance depends on the type of affected gene. In case of *SDHA* mutation carriers, the mutation penetrance is 39% by the age of 40 years in the index cases, whereas it is only 10% at the age of 70 years with a lifetime disease- penetrance of 1.7% in the relatives. The *SDHB* mutation penetrance is similar in probands and relatives, accounting for 22% by the age of 60 years, whereas disease penetrance is 8% for *SDHC* and 43% for *SDHD* by the age of 60 years (Benn et al., 2018; Andrews et al., 2018). Penetrance estimations are currently ongoing for *MAX, TMEM127, SDHAF2* and for the newer genes (Neumann et al., 2019).

2.4 Von Hippel-Lindau syndrome (VHL)

VHL is an autosomal dominant disease affecting several organ systems and leading to the growth of cysts and/or tumours. The estimated prevalence ranges from 1 per 30 000 to 1 per 50 000, and males and females are equally affected (Mikhail and Singh, 2021). Two differenttypes of VHL syndrome have been reported, depending on the occurrence of Pheos amongwith the other cystic lesions/tumours forming part of this syndrome. VHL type 1 is characterized by a very low risk for Pheo, whereas VHL type 2 develop Pheo in 10-20% of cases. In this latter syndrome type, Pheo appears earlier in patients compared to sporadic cases, and the manifestation is often bilateral, but malignant cases are rare (Chen et al., 1995;Eisenhofer et al., 2001).

Hemangioblastoma, which are central nervous system's vessel-related tumours, are the most frequent feature in VHL syndrome, developing in 60-80% of VHL patients, generally around the age of 30 years. The most frequently affected regions are the spinal cord (50%), the cerebellum (37%) and the brainstem (10%). Angiomas of the retina occur in 50% of cases,and these benign tumours are often recurrent, bilateral and multiplex.

RCC is the second most common lesion in VHL, occurring approximately in 24%-70% of patients. RCC is often bilateral, multiplex and rapidly progressing tumour with a potentialto form metastases early to the regional lymph nodes, liver and brain.

Pancreatic lesions (usually cysts) arise in 25% of VHL cases, but autopsy confirms this numberis around 70%. Pancreatic tumours also appear in 5-10% of patients, and they represent significant morbidity and mortality (Mikhail and Singh, 2021).

2.4.1. Clinical presentation

The clinical presentation is influenced by the type of tumours, by their size and location, byassociated edema and by the presence of cysts as well. Hemangioblastomas of the spinal cord can cause hyperesthesia and pain, whereas tumours of the cerebellum are accompaniedby headaches, dizziness, nausea, imbalance and ataxia. Brainstem tumours are associated with hyperesthesia, headaches, dysphagia and hyperreflexia. Although the hemangioblastomas are slowly growing benign tumours, the subsequent increased pressurein the central nervous system represents the major cause of VHL-associated morbidity andmortality.

In case of retinal angiomas, retinal detachment and blindness regularly develop in patients who are not subjected to a definitive therapy, e.g. laser coagulation and cryotherapy.

Pheos may be asymptomatic, but they may also cause a wide spectrum of symptoms as reported in the previous paragraph.

In case of pancreas lesions, clinical symptoms are related to the size of the cyst, and abdominal discomfort or pain can be common, while pancreatitis seems to be rare. Pancreatic tumours related to VHL are usually asymptomatic, but in some instances they may result in abdominal pain, jaundice, pancreatitis, or even gastrointestinal bleeding (Hammel et al., 2000).

Haematuria and pain might be present if RCC occurs, but this carcinoma is usually silent for years, and such symptoms appear in advanced stages only.
VHL patients with endolymphatic sac tumours, which occur in the inner ear, may present with tinnitus, vertigo or hearing loss.

2.4.2. Genetics of VHL

Germline mutations of the *VHL* tumour suppressor gene are responsible for the VHL syndrome. This gene encodes a protein which forms a stable ubiquitin-ligase complex withother proteins, such as RBX1, Cullin-2, Elongin-B and C. Under normoxic condition, this complex mediates the function of RNA polymerase II and participates in the ubiquitin-mediated degradation of hypoxia-inducible factor 1-alpha (HIF1-A). Under hypoxic condition or when *VHL* is mutated, the lack of HIF1 degradation leads to the consequent dimerization of HIF1-A and HIF1-B proteins, which translocate to the nucleus promoting the transcription of target growth factors genes, i.e. *VEGF, EPO, PDGF, LDH, GLUT1* **(Figure3).** This mechanism is called pseudo-hypoxia, and it has also been described in tumours associating with mutations in *SDHx* genes (see previous paragraph). In addition, VHL participates in two other cellular pathways regarding: i) regulatory functions of the mitochondria and the cell cycle; ii) organization of microtubules and formation of cilium (Kaelin, 2002).

Generally, loss of function *VHL* mutations are associated with the type 1 syndrome, while missense mutations with the type 2 (Neumann et al., 2019). Furthermore, mutations which disrupt the VHL-HIF1A protein interaction more often cause RCC, whereas mutations affecting other protein domains lead to Pheos (Mikhail and Singh, 2021). Of note, those missense mutations more commonly found in Pheos are located at the surface of the proteinrather than in the deeper regions (Ong et al., 2007).

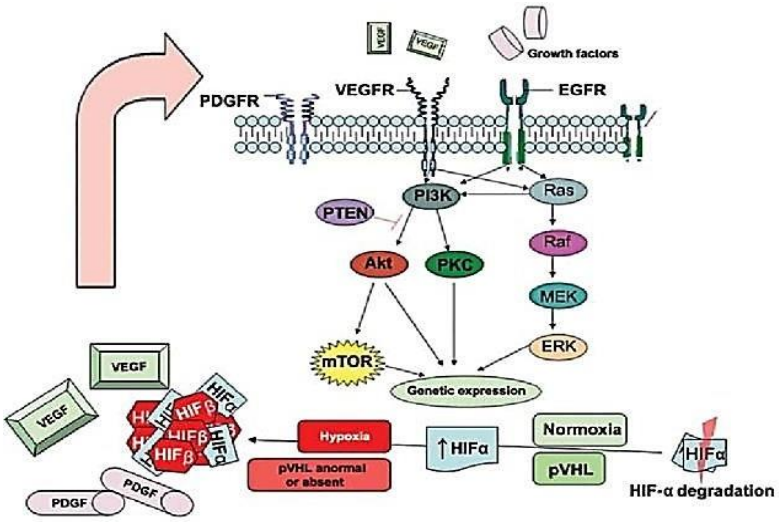

Figure 3: Molecular scheme of mechanism of action of von Hippel-Lindau protein (VHL). In conditions of hypoxia or *VHL* gene inactivation and consequently, absence of codified protein (pVHL) there is no HIF degradation, resulting in accumulation of complex HIF1-A and HIF1-B in nucleus to promote oncogenesis, through activation of target genes (from Heldwein et al., 2009).

2.4.3. Genetic counselling in VHL

The identification of one *VHL* mutation leads to a lifelong monitoring by a multi-professional healthcare team, which involves various clinicians collaborating and communicating with oncologists to choose the best possible patient's management. In fact,annual evaluation for hearing problems, neurological symptoms and blood pressure monitoring is recommended in VHL patients and in their first-degree relatives. In addition,consultations with an ophthalmologist should take place yearly. A MRI of the brain and entire spine is recommended every two years starting at age of 16 to screen for central nervous system hemangioblastomas. An abdominal ultrasound for visceral lesions is also advised every 1-2 years. Annual blood or urinary fractioned metanephrines are used to screen for Pheos starting at age of 5 (both for index case and relatives).

Family members of VHL patients need to be screened for the identified gene defect and, if the mutation is present, active surveillance in order to detect VHL-complications in an early stage, i.e. neurological defects, hearing and vision loss and renal impairment, is mandatory.

It is important to consider that, VHL is an autosomal dominant disease, hence there is 50% chance of an offspring to inherit the mutation from their parents. Given the severity of this syndrome, PGT-M or prenatal diagnosis should be offered to couples according to the Italian legislation.

2.5. NGS-based genetic tests and their application in endocrine diseases

In recent years, high throughput genetic tests, i.e. NGS, have become increasingly availablefor clinical use at reasonable costs, and significant progress has been achieved regarding thediagnostic yield in human genetic diseases (Chong et al., 2015; Shendure et al., 2017). Currently, NGS-based genetic testing has become an indispensable component of the comprehensive diagnostic workup in paediatric endocrinology, and increasingly also as part of adult endocrine diagnostics, in addition to the common biochemical laboratory analyses. In fact, germline genetic alterations not only cause congenital disorders, such as CHH, but also play a major role in tumour development, as we can see for Pheo/PGLs, VHLand the other overlapping endocrine diseases.

In endocrine disorders presenting with characteristic phenotypic expression and caused bypathogenic variants in only one gene, like VHL syndrome and MEN1, single gene testing isstill the best strategy. However, in the majority of rare endocrine disorders, the presence of multiple susceptibility/candidate genes as well as similar/overlapping phenotypes has driven the need for the parallel analysis of several genes by targeted NGS. In this case, different genes can be analysed within the same diagnostic run and assessment pipeline, leading to an improvement of the diagnostic yield (Eggermann et al., 2020). Concerning CHH, the yield of genetic testing is already over 40%, while 30% of germline mutations cannow be detected in Pheo/PGLs.

In addition, other NGS approaches addressing the whole exome (WES) can be considered, taking into consideration their advantages/disadvantages in a diagnostic context. These methods cover thousands of unselected genes potentially leading to the discovery of new genetic causes. Especially trios would seem to be a promising approach in order to find the *de novo* mutation. On the other hand, for a diagnostic purpose,

bioinformatics pipelines needto become further automated to facilitate the interpretation of such data (Eggermann et al.,2020), as the attribution of a pathogenic role to a novel promising candidate gene represents the major challenge. Another challenge in the diagnostic use of WES is the handling of incidental findings, i.e. genetic alterations associated with conditions or diseases unrelatedto the patient's present condition, for which the patient management and genetic counselling become very difficult. For all these reasons, targeted NGS panels are consideredas the first choice of testing to perform in the diagnostic field.

Nevertheless, it is worth noting that the main challenge in NGS –based methods, includingtargeted gene panels, is the interpretation of Variants of Unknown Significance (VUSs). Hence, family screening, appropriate tools and expertise are needed for correct interpretation of these findings in the clinical practice.

3. Aims

The general objective of this thesis was to investigate the diagnostic yield of genetic testing in CHH, Pheo/PGL/HNPGL and suspected VHL cases. For this purpose, a targeted NGS gene panel was designed in order to sequence candidate and susceptibility genes for CHH and Pheo/PGL/HNPGL/VHL, respectively, in affected patients. In addition, a number of secondary objectives have been addressed in function of the type of disease.

3.1. Secondary objectives in CHH disease

Concerning CHH disease, we aimed to evaluate:

- the most frequently mutated genes in our cohort, including 26 affected patients.
- the genetic diagnostic yield in the two main CHH clinical phenotypes, i.e. classic forms ofCHH with or without other congenital anomalies and milder forms of Adult- Onset CHH.

- the genotype-phenotype correlation, in order to attribute a clinical value to the identified variant(s).

3.2. Secondary objectives in rare endocrine tumours

3.2.1. Secondary objectives in Pheo/PGL/HNPGL

Concerning Pheo/PGL, we aimed to evaluate:

- the most frequently mutated genes in our cohort, including 95 affected patients.

- the clinical differences between the mutated cohort and the "wild type" one in terms of: i)age of disease-onset; ii) presence of multiple lesions; iii) onset of metastasis.
- the genotype of relatives by performing family segregation studies, in order to detect healthy mutation carriers who could develop the disease.

- the genotype-phenotype correlations.
- the *VHL* mutation carriers without a diagnosis of VHL syndrome, in order to follow themup for the occurrence of the clinical signs underlying this syndrome.

3.2.2. Secondary objectives in VHL

Concerning VHL, we aimed to evaluate:

- the percentage of "true" VHL cases in our cohort of suspected cases (n=8).
- the genotype-phenotype correlations, in order to establish the type of *VHL* mutations leading to either disease 1 or disease 2 of this complex syndrome.

4. Materials and Methods

4.1. Study population

A total of 129 subjects gave informed consent for the present study and was enrolled. Among these, Pheos (n=57), mediastinal/abdominal PGLs named as "PGLs" (n=9), head andneck PGLs named as "HNPGLs" (n=29), suspected VHL (n=8) and CHH (n=26) are included(**Table 3**).

Considering Pheos/HNPGLs/PGLs and suspected VHL cases, 24-hour urinary fractionated metanephrines and normetanephrines were evaluated. Considering CHH patients, blood samples were drawn in the morning, after an overnight fast, for the determination of total testosterone (assay sensitivity: 0.35 nmol/l), LH (assay sensitivity: 0.2 IU/l) and FSH (assay sensitivity: 0.2 IU/l) by electrochemiluminescent method (Modular Roche, Milan, Italy). Possible brain abnormalities (including atrophy or absence of olfactory bulbs and abnormalities of the hypothalamus–pituitary region) have been investigated evaluating previous recent Magnetic Resonance Imaging (MRI). All available MRIs have been performed with contrast media by qualified radiologic institutes or hospital radiology divisions. In addition, olfactory dysfunction has been previously assessed using the Smell Identification Test (UPSIT), a qualitative suprathreshold olfaction test (Koenigkam-Santos et al., 2011).

After obtaining signed informed consent, genomic DNA of each patient was extracted by the QIAsynfony CDN kit (Qiagen). DNA quality and quantity were measured by the Qubitds assay on the Qubit 2.0 fluorimeter (Thermo Fisher Scientific).

Table 3: Number of recruited subjects divided according to their clinical condition and gender.

		PGLs			
	Pheos	**Mediastinal/ Abdominal (PGL)**	**Head and Neck (HNPGLs)**	**Suspected VHL**	**CHH**
Female	**34**	**6**	**22**	**4**	**11**
Male	**24**	**3**	**7**	**4**	**15**
Total	**57**	**9**	**29**	**8**	**26**
			38		

4.2. Targeted NGS gene panel desing

In 2016, a panel of 13 Pheo/PGL susceptibility genes was designed using the online Sure Design software (Agilent Technologies). After two years, a unique panel of 49 genes was designed by using the same technology, in order to analyse both the CHH candidate genes(n=34) and an increased number of major susceptibility genes for rare endocrine tumours (n=15 total) (**Table 4**).

Table 4: List of genes with their canonical isoforms, flanking regions and coding exons analysed byusing either the former or the latter gene panel.

Gene panel (1 or 2)*	***Project***	***Gene***	***Isoform***	***Flanking regions (n)***	***Coding exons (n)***
1 and 2	Pheo/PGL	*EGLN1*	NM_022051	20	5
1 and 2	Pheo/PGL	*EPAS1*	NM_001430	20	16
1 and 2	Pheo/PGL	*FH*	NM_000143	20	10
1 and 2	Pheo/PGL	*KIF1B*	NM_015074	20	46
1 and 2	Pheo/PGL	*MAX*	NM_002382	20	5
1 and 2	Pheo/PGL	*RET*	NM_020975	20	20
1 and 2	Pheo/PGL	*SDHA*	NM_004168	20	15

1 and 2	Pheo/PGL	*SDHAF2*	NM_017841	20	4
1 and 2	Pheo/PGL	*SDHB*	NM_003000	20	8
1 and 2	Pheo/PGL	*SDHC*	NM_003001	20	6
1 and 2	Pheo/PGL	*SDHD*	NM_03002	20	4
1 and 2	Pheo/PGL	*TMEM127*	NM_017849	20	3
1 and 2	Pheo/PGL	*VHL*	NM_000551	20	3
2	Pheo/PGL	*MDH2*	NM_005918	20	9
2	Pheo/PGL	*SLC25A11*	NM_003562	20	8
2	CHH	*ANOS1*	NM_000216	20	14
2	CHH	*AXL*	NM_001278599	20	14
2	CHH	*CHD7*	NM_017780	20	37
2	CHH	*DMXL2*	NM_015263	20	43
2	CHH	*DUSP6*	NM_001946	20	3
2	CHH	*FEZF1*	NM_001024613	20	4
2	CHH	*FGF17*	NM_003867	20	5
2	CHH	*FGF8*	NM_006119	20	5
2	CHH	*FGFR1*	NM_001174063	20	17
2	CHH	*FLRT3*	NM_013281	20	1
2	CHH	*GNRH1*	NM_001083111	20	3
2	CHH	*GNRHR*	NM_000406	20	3
2	CHH	*HESX1*	NM_003865	20	4
2	CHH	*HS6ST1*	NM_004807	20	2
2	CHH	*IL17RD*	NM_017563	20	13
2	CHH	*KISS1*	NM_002256	20	2
2	CHH	*KISS1R*	NM_032551	20	5
2	CHH	*LEP*	NM_000230	20	2
2	CHH	*LEPR*	NM_002303	20	18
2	CHH	*NR0B1*	NM_000475	20	2
2	CHH	*NSMF*	NM_015537	20	15
2	CHH	*OTUD4*	NM_017493	20	6
2	CHH	PCSK1	NM_000439	20	14
2	CHH	*PNPLA6*	NM_006702	20	33
2	CHH	*PROK2*	NM_001126128	20	4
2	CHH	*PROKR2*	NM_144773	20	2
2	CHH	*SEMA3A*	NM_006080	20	17
2	CHH	*SEMA3E*	NM_012431	20	17
2	CHH	*SEMA7A*	NM_003612	20	14
2	CHH	*SOX10*	NM_006941	20	3
2	CHH	*SPRY4*	NM_001127496	20	1
2	CHH	*TAC3*	NM_013251	20	5
2	CHH	*TACR3*	NM_001059	20	5
2	CHH	*WDR11*	NM_018117	20	29

*1= former gene panel containing 13 Pheo/PGL susceptibility genes; 2=latter gene panel including atotal of 49 genes divided into 34 CHH candidate genes and 15 Pheo/PGL susceptibility genes.

4.3. Targeted NGS gene panel sequencing

DNA libraries indexed with oligonucleotides compatible with Agilent's sequencing chemistry were prepared and captured using the HaloPlex Target Enrichment System for Illumina sequencing (Figure 4). Only the coding regions and the flanking sequences (±20 nucleotides) were included for the enrichment and library preparation. The enriched libraries were quantified with Qubit High Sensitivity (Life Technologies, Foster City, CA, USA) and Bioanalyzer 2100 (Agilent technologies, Santa Clara, California) (Figure 5). The sequencing was performed in paired-end on the MiSeq instrument (Illumina, San Diego, California) to generate 250 nucleotide-long sequences with an average coverage depth of at least 30X. Quality of reads was checked through FastQC (v.0.11.9) (https://www.bioinformatics.babraham.ac.uk/). Reads were analysed with an Illumina pipeline based on Burrows–Wheeler Aligner (BWA) for sequence alignment on GRCh37 (hg19) reference on Broad Institute Genome Analysis Tool kit (GATK) for genotyping and on Annotate Variation (ANNOVAR) for variant annotation.

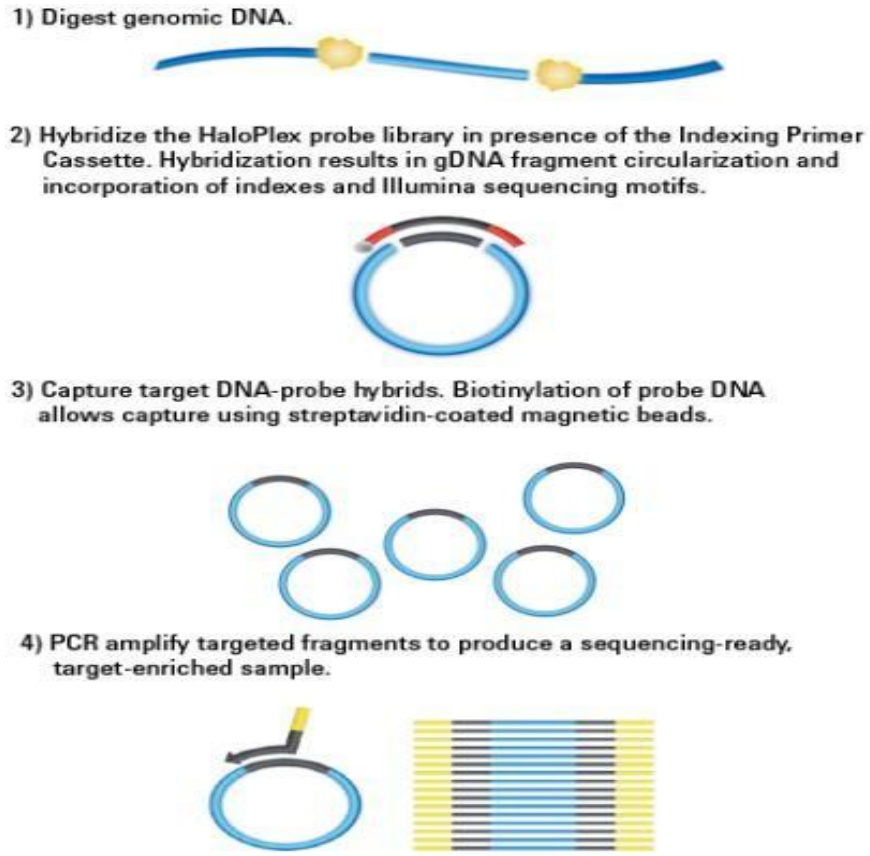

Figure 4: Overall HaloPlex target-enriched sequencing sample preparation workflow.

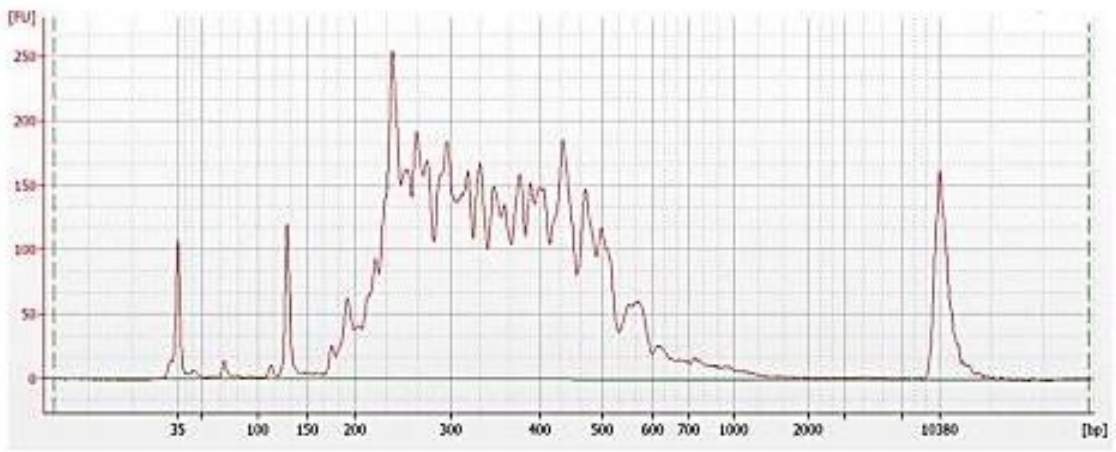

Figure 5: Validation of enrichment by Agilent 2100 Bioanalyzer analysis.

4.4. Variant filtering

A standard variant filtering was applied to all samples as illustrated in **Figure 6**. Briefly, we selected missense variants, stop gains/losses, frameshift insertions/deletions and splicing variants, filtering out common polymorphisms (≥1% in the general population), after consulting the genome aggregation database gnomAD (http://gnomad-old.broadinstitute.org/). The obtained data was further filtered according to their clinical significance based on ClinVar and InterVar databases. Only those variants reported as "pathogenic" or "likely pathogenic" or as of unknown significance/conflicting interpretation of pathogenicity were prioritized. For this latter category, RENOVO algorithm (https://bioserver.ieo.it/shiny/app/renovo) was used in order to reclassify their as into pathogenic/likely pathogenic or benign. Pathogenicity of the prioritized variants was also manually checked using Varsome (https://varsome.com/), which allowed us to confirmwhether they were already reported in the literature or not. In some instances, PRALINE database, a multiple sequence alignment tool, was used to assess amino acid residues conservation across species.

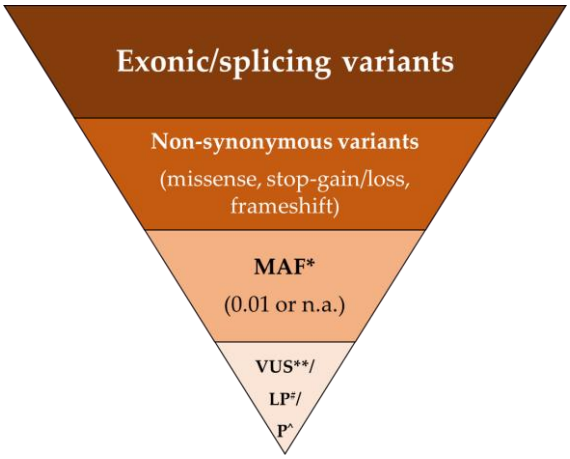

Figure 6: Schematic illustration of standard variant filtering. *MAF: Minor Allele Frequency according to the frequencies reported both in all populations and in the Non-Finnish European

oneby using the GnomAD database. **VUS: variant of unkown significance or variants with conflicting interpretation of pathogenicity according to ClinVar and Intervar predictions were reclassify as (likely) pathogenic or benign by using RENOVO algorithm, where feasible. #LP: likely pathogenic according to ClinVar and Intervar predictions. ^P: pathogenic according to ClinVar and Intervar predictions.

4.5. Variants validation with Sanger sequencing

To confirm the prioritized variants, we performed Sanger sequencing. Standard PCR was performed in a 25-µl reaction volume by using the HotStarTaq Plus Master Mix PCR protocol (QIAGEN), and the following thermal cycler program, according to the manufacturer's instructions: 5-minutes initial heat activation step at 95°, followed by 35 cycles consisting of denaturing at 94° for 1 minute, 1 minute at annealing temperature and elongation at 72° for 1 minute, and a final 10-minutes extension step at 72°C. Presence of amplification products was verified through electrophoresis on a 2% agarose gel. PCR products purification was performed by using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) in a 6-µl reaction volume, through a 15-minutes initial incubation at 37°C, followed by a 15-minutes incubation at 80°C. Sequencing reactions were performed using the The BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies) in a 20-µl reaction volume by using the following protocol: 1-minute initial denaturation step at 95°C and 25 cycles of 10-seconds denaturation at 95°C, 5-seconds annealing at 58°C, and 2-minutes extension at 60°C. Capillary electrophoresis was performed on the 4-capillary SeqStudio™ Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA).

4.6. Multiplex ligation-dependent probe amplification (MLPA)

In order to detect clinically relevant deletions, i.e. copy-number variants (CNVs), in the *ANOS1, SHDx* and *VHL* genes, we performed the MLPA analysis.

4.6.1. Detection of CNVs in the *ANOS1* gene

The SALSA MLPA Probemix P132 Kallmann-1 assay (MRC-Holland) was used for the detection of deletions or duplications in the *ANOS1* gene, which is associated with KS. The SALSA MLPA Probemix P132 Kallmann-1 contains 32 MLPA probes with amplification products between 136 and 400 nucleotides. This includes 16 probes for the *ANOS1* gene and4 flanking probes that detect locations on both sides of the *ANOS1* gene. In addition, 12 reference probes are included detecting locations on the X-chromosome. The Coffalyser.Netsoftware was used for the data analysis in combination with the appropriate lot-specific MLPA Coffalyser sheet.

The standard deviation of each individual probe over all the reference samples should be ≤0.10 and the dosage quotient (DQ) of each individual reference probe in the patient samplesshould be between 0.80 and 1.20. When these criteria are fulfilled, the cut-off values for theDQ of the probes which can be used to interpret MLPA results are reported in **Table 5**.

Table 5: Interpretation of results: **a**) dosage quotient values indicating a normal copy number status/deletion/duplication of the *ANSO1* gene in male samples; **b**) dosage quotient values indicating a normal copy number status/deletion/duplication of the *ANSO1* gene in female samples.

a

Copy Number status: Male samples	Dosage quotient
Normal	0.80 < DQ < 1.20
Deletion	DQ = 0
Duplication	1.65 < DQ < 2.25
Ambiguous copy number	All other values

b

Copy Number status: Female samples	Dosage quotient
Normal	0.80 < DQ < 1.20
Homozygous deletion	DQ = 0
Heterozygous deletion	0.40 < DQ < 0.65
Heterozygous duplication	1.30 < DQ < 1.65
Heterozygous triplication/Homozygous duplication	1.75 < DQ < 2.15
Ambiguous copy number	All other values

4.6.2. Detection of CNVs in the *SDHx* genes

The SALSA MLPA Probemix P226 SDH assay (MRC-Holland) was used for the detection ofdeletions or duplications in the *SDHB*, *SDHC*, *SDHD*, *SDHAF1*, and *SDHAF2* genes, whichare the main causes of hereditary Pheo/PGLs. The SALSA MLPA Probemix P226-D1 SDH contains 45 MLPA probes with amplification products between 130 and 494 nucleotides. This includes 9 probes for the *SDHB* gene, 10 probes for *SDHC*, 7 probes for *SDHD*, 2 probesfor *SDHAF1* and 4 probes for *SDHAF2*. In addition, 13 reference probes detecting autosomalchromosomal locations are included. The Coffalyser.Net software was used for the data analysis in combination with the appropriate lot-specific MLPA Coffalyser sheet.

The expected results for the probes detecting autosomal sequences are allele copy

numbersof 2 (normal), 1 (heterozygous deletion), or 3 (heterozygous duplication). The standard deviation of each individual probe over all the reference samples should be ≤0.10 and the dosage quotient (DQ) of each individual reference probe in the patient samples should be between 0.80 and 1.20. When these criteria are fulfilled, the cut-off values for the DQ of the probes which can be used to interpret MLPA results for autosomal chromosomes or pseudo-autosomal regions are reported in **Table 6**.

Table 6: Interpretation of results based on dosage quotient values indicating normal copy number status/deletion/duplication of the *SDHx* and *VHL* genes.

Copy number status	Dosage quotient
Normal	0.80 < DQ < 1.20
Homozygous deletion	DQ = 0
Heterozygous deletion	0.40 < DQ < 0.65
Heterozygous duplication	1.30 < DQ < 1.65
Heterozygous triplication/Homozygous duplication	1.75 < DQ < 2.15
Ambiguous copy number	All other values

4.6.3. Detection of CNVs in the VHL gene

The SALSA MLPA probemix P016 VHL assay (MRC-Holland) was used for the detection ofdeletions in the human *VHL* gene, in order to confirm a clinical diagnosis of Von Hippel- Lindau syndrome. The SALSA MLPA Probemix P016-C2 VHL contains 29 MLPA probes with amplification products between 166 and 427 nucleotides. This includes 9 probes for the*VHL* gene (two or more probes for each exon), 6 probes for genes located close to *VHL* (*FANCD2, BRK1/C3orf10/HSPC300, IRAK2* and *GHRL*), 2 probes on 3p which are further telomeric or centromeric from *VHL*, and 12 reference probes detecting sequences on other chromosomes. The Coffalyser.Net software was used for the data

analysis in combination with the appropriate lot-specific MLPA Coffalyser sheet.

The expected results for *VHL*-specific MLPA probes are allele copy numbers of 2 (normal), 0 (homozygous deletion), 1 (heterozygous deletion), 3 (heterozygous duplication), and 4 (heterozygous triplication/homozygous duplication). The standard deviation of each individual probe over all the reference samples should be ≤0.10 and the dosage quotient (DQ) of each individual reference probe in the patient samples should be between 0.80 and 1.20. When these criteria are fulfilled, the cut-off values for the DQ of the probes which can be used to interpret MLPA results for autosomal chromosomes or pseudo-autosomalregions are reported in **Table 6**.

4.7. Mutational screening of the *VHL* gene

In suspected cases of VHL syndrome, the three coding exons (**Figure 7**) of the *VHL* gene were sequenced by Sanger method. For each coding exon, standard PCR was performed in a 25-µl reaction volume by using the HotStarTaq Plus Master Mix PCR protocol (QIAGEN) with primer sequences, annealing temperatures and sizes of the amplified reported in **Table 7**. The protocol used is already been described in the 3.5 paragraph (see above).

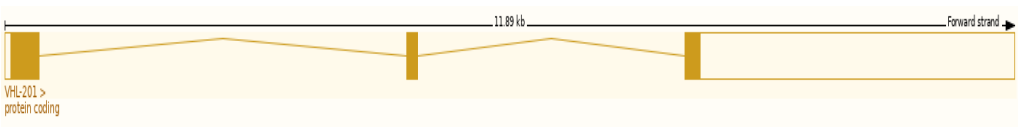

Figure 7: Schematic representation of the *VHL* gene encompassing 11,89Kb. Each orange rectangle represents an exon, whereas the lines connecting them are the introns (source: Ensembl Database (https://www.ensembl.org/index.html).

Table 7: Primer sequences of the *VHL* gene, with their annealing temperature and product size.

Forward	Reverse	Ta	Size	Product name
CGTTCCATCCTCTACCGAG	CTGCTGGGTCGGGCCTAA	60	502	Exon 1
GCCACCGGTGTGGCTCTT	ATAACGTGCCTGACATCAGGC	60	277	Exon 2
CCATCAGTAGTACAGGTAGTTG	GGAACCAGTCCTGTATCTAGA	60	358	Exon 3

Ta: annealing temperature.

4.8. Family segregation studies

When DNA from relatives of mutated patients was available, we have performed family segregation studies (n=12 total families). To this purpose, sequence analysis of the identifiedvariants in the index cases was conducted in the family members by Sanger sequencing, inorder to: i) assess the clinical significance of the variants identified in CHH patients; ii) detecthealthy mutation carriers in case of rare endocrine tumours pedigrees.

4.9. Statistical analysis

SPSS software (version 25.0 Chicago, IL, USA) was used for analysing clinical data of rare endocrine tumours cohort. The age of disease-onset was expressed as mean±SD, whereas the occurrence of multiple lesions and/or metastases were considered as categorical parameters ad expressed as percentages. Comparison of age at disease-onset between "wildtype" and mutated groups was performed with unpaired two-sided Student's t tests. Relative risk and 95% confidence interval were calculated for the association of categorical parameters, and chi-squared test was used for comparisons, using Fisher's exact test. A p- value ≤ 0.05 was considered statistically significant for each test.

5. Results

5.1. Congenital Hypogonadotropic Hypogondism cohort

5.1.1. Clinical characterization

Among the 26 CHH patients (15 males and 11 females), 8 individuals (4 males and 4 females)were affected by Kallmann syndrome (KS), whereas 18 individuals (11 males and 7 females) presented with normal sense of smell, i.e. normosmic hypogonadotropic hypogonadism (nCHH). All available clinical data of each patient, including hormone values at the diagnosis and magnetic resonance imaging (MRI) results, are reported in **Table 8a** and **8b,** according to their gender.

Given the wide clinical heterogeneity underlying the CHH disorder, we divided our study population according to the following main clinical phenotypes:

1) **Classic form of isolated CHH**, characterized by delayed or absent puberty in males and primary amenorrhea in females, which was called as "CHH1".
2) **Classic form of CHH with additional features,** i.e. hypo/anosmia (KS), cleft/lip palate, ear and eye malformations, hypocortisolism, which was considered as syndromic CHH andcalled as "CHH2".
3) **Milder forms of CHH,** characterized by post-pubertal hypotestosteronemia in males and secondary amenorrhea in females, which was called as Adult-Onset CHH or "AOH".

In our cohort, the majority of patients were AOH accounting for 54% of cases, whereas

CHH1 and CHH2 cases accounted for 8% and 38% of cases, respectively (**Figure 8**).

Table 8: Clinical data of our CHH cohort, including their CHH phenotype, sense of smell, hormonallevels at the diagnosis and magnetic resonance imaging data. The table is divided according to the patient's gender: **a)** clinical data of male patients; **b)** clinical data of female patients.

a)

atientcode	*Clinical phenotype (CHH1*; CHH2**; AOH***)*	*Sense of smell (nCHH/KS)*	*Hormone values at the diagnosis*			*MRI*
			LH (U/L)	*FSH (U/L)*	*T (nmol/L)*	
CHH 16	CHH1	nCHH	0.59	2.42	1.4	Negative
CHH 23	CHH1	nCHH	0.4	0.5	3.3	Negative
CHH 7	CHH2	KS	n.a.	n.a.	n.a.	Inner ear malformations
CHH 9	CHH2	nCHH	0.2	0.3	n.a.	Negative
CHH 10	CHH2	KS	0.9	1.3	2.0	Olfactory bulbs absence
CHH 11	CHH2	KS	<0.5	0.43	2.1	Negative
CHH 17	CHH2	nCHH	<0.1	<0.1	1.04	Negative
CHH 26	CHH2	KS	n.a.	n.a.	n.a.	Negative
CHH 1	AOH	nCHH	2.64	2.76	5.3	n.a.
CHH 3	AOH	nCHH	n.a.	n.a.	n.a.	n.a.
CHH 4	AOH	nCHH	1.49	4.09	9.1	Negative
CHH 5	AOH	nCHH	1.6	1.1	9	l emptySella
CHH 8	AOH	nCHH	0.83	1.44	7.3	Sellar floorcongenital anomaly
CHH 15	AOH	nCHH	0.00	0.00	1.04	Negative
CHH 20	AOH	nCHH	<0.3	<0.3	0.3	Negative

b)

atient code	*Clinical phenotype (CHH1*; CHH2**; AOH***)*	*Sense of smell (nCHH/KS)*	*Hormone values at the diagnosis*		*MRI*
			LH (U/L)	*FSH (U/L)*	
CHH 6	CHH2	KS	0.18	0.7	Mesencephalic MAV#
CHH 12	CHH2	KS	0.52	1.08	Olfactory bulbs absence
CHH 14	CHH2	KS	n.a.	n.a.	Empty sella

CHH 21	CHH2	KS	2	2.9	Negative
CHH 2	AOH	nCHH	12.6	5.3	Negative
CHH 13	AOH	nCHH	2.3	8.2	Negative
CHH 18	AOH	nCHH	2.8	6.7	n.a.
CHH 19	AOH	nCHH	0.72	3.39	Negative
CHH 22	AOH	nCHH	n.a.	n.a.	n.a.
CHH 24	AOH	nCHH	0.52	3.41	Negative
CHH 25	AOH	nCHH	n.a.	n.a.	n.a.

CHH1*: isolated congenital hypogonadotropic hypogonadism; CHH2**: congenital hypogonadotropic hypogonadism with other congenital anomalies; AOH***: adult-onset hypogonadism, i.e. post-pubertal hypotestosteronemia in males and secondary amenorrhea in females; KS: Kallmann syndrome; nCHH: normosmic CHH; MAV#: arteriovenous malformations; n.a.: not available.

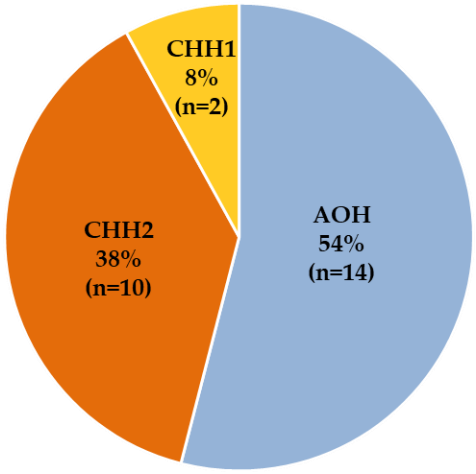

Figure 8: Percentages of our CHH cohort, divided according to the above-mentioned clinical phenotypes, i.e. adult-onset CHH (AOH), isolated CHH (CHH1) and syndromic CHH (CHH2).

Among the syndromic CHH patients (n=10) (**Table 9**), 6 subjects (2 males and 4 females) exhibited both olfactory and reproductive defects without additional congenital features, i.e. KS patients. Besides these, one male patient (CHH 11) affected by KS also presented withcleft lip. In addition, one KS male patient (CHH 7) showed the clinical signs of CHARGE syndrome, i.e. congenital hypogonadotropic hypogonadism, opacity of the left lens, hearingloss and inner ear malformations. Among the normosmic patients

presenting with CHH2 (2 males), one index case (CHH 17) showed retinal detachment and blindness, suggesting adiagnosis of CHARGE syndrome. The remaining one (CHH 9), a 29-years-old man, also exhibited hypocortisolism and diabetes mellitus and the diagnosis of this syndromic form is still under definition.

Table 9: List of congenital anomalies in CHH2 patients.

Patient code	*Gender (F/M)*	*Others non-reproductive phenotypes*	*Overlapping syndrome*
CHH 6	F	Hyposmia	KS
CHH 7	M	Anosmia, opacity of the left lens, hearing loss, inner ear malformations	CHARGE syndrome
CHH 9	M	Hypocortisolism, diabetes mellitus, lumbar and femoral osteoporosis	To be defined
CHH 10	M	Hyposmia	KS
CHH 11	M	Anosmia, cleft lip, femoral and vertebral osteoporosis	KS
CHH 12	F	Anosmia	KS
CHH 14	F	Anosmia	KS
CHH 17	M	Retinal detachment and blindness	Likely CHARGE syndrome
CHH 21	F	Hyposmia	KS
CHH 26	M	Hyposmia	KS

KS: Kallmann syndrome.

5.1.2. Genetic findings

Here, we report both the targeted NGS gene panel results and the MLPA analysis for the *ANOS1* gene.

5.1.2.1. Targeted NGS gene panel results

The targeted NGS gene panel contains 34 candidate genes of CHH (see Materials and Methods).

After bioinformatics filtering, a total of 10 patients carried at least one variant in the analysed genes, for a total of 12 VUS/likely pathogenic/pathogenic variants in 7 genes (**Figure 9**). Each of these variants (n=12) has been validated with Sanger sequencing. The overall results are illustred in Figure 9.

Among identified variants, splicing (n=2), stop gain (n=3), frameshift deletions (n=2) and missense variants (n=5) were reported (**Table 10**). Three of them were previously reported in different studies, whilst the remaining ones were novel (**Table 10**). Based on the literature data and Varsome verdict, we have a total of 7 pathogenic and 2 likely pathogenic variants,beside 3 variants of unknown significance (**Table 10**).

The most frequently mutated gene was the *FGFR1* (n= 3/12 variants), accounting for 25%, followed by *CHD7* and *DMXL2* genes (n=2/12 variants for each one), whose gene defects represent the 17% of variants identified in our cohort. In one patient (CHH 11), 2 different *PROK2* variants have been identified. In the other affected genes (*AXL, GNRHR* and *SEMA3A*) only single variants have been reported, accounting each one for 8% of the totality (**Figure 10).**

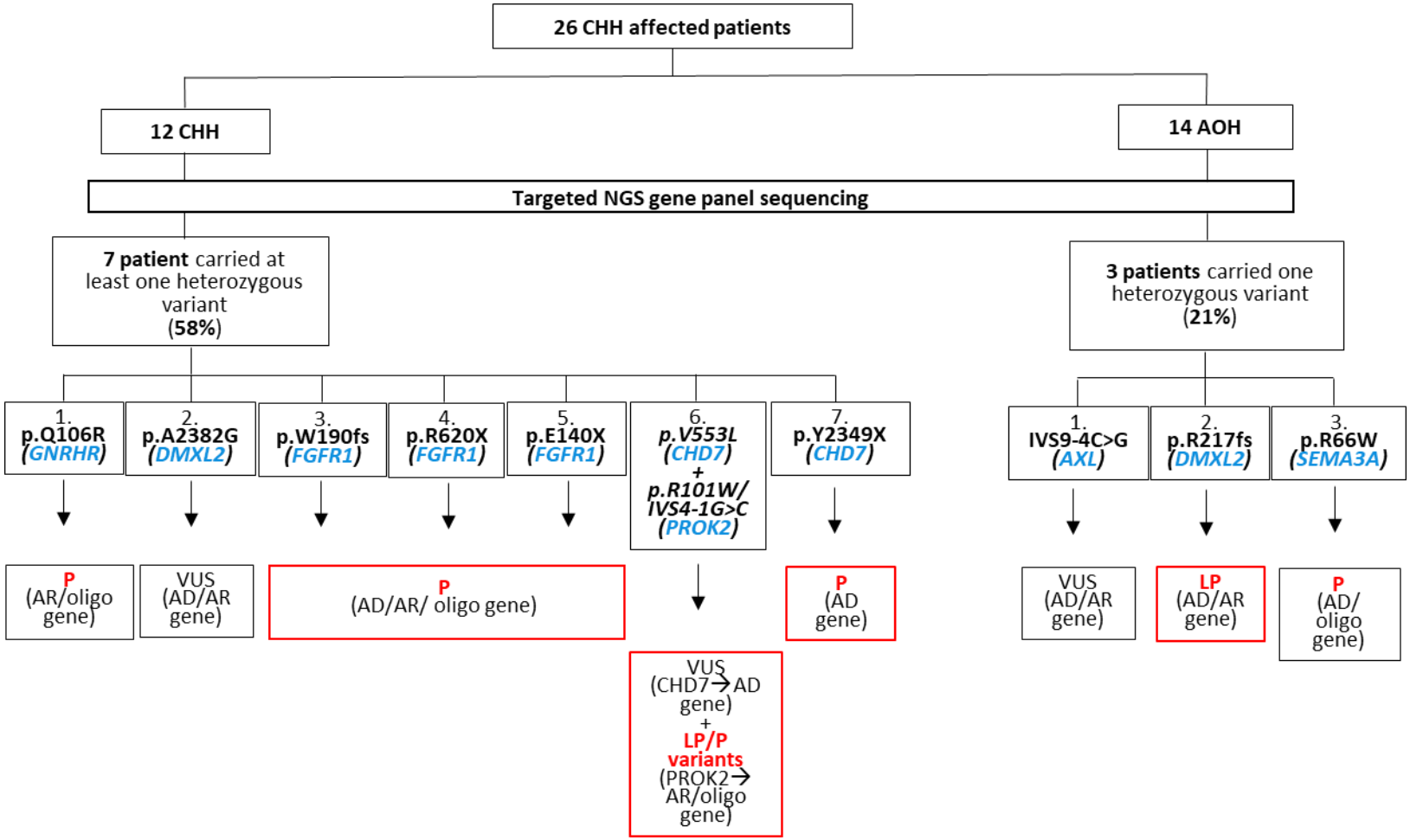

Figure 9: Flow chart illustrating the targeted NGS gene panel sequencing results of 26 CHH patients. Affected genes are represented in blue. Rectangles highlighted in red show those variants which can explain the phenotype of CHH (see paragraph 5.1.4). Oligo: oligogenic; VUS: variants of unknown significance; LP: likely pathogenic; P: pathogenic.

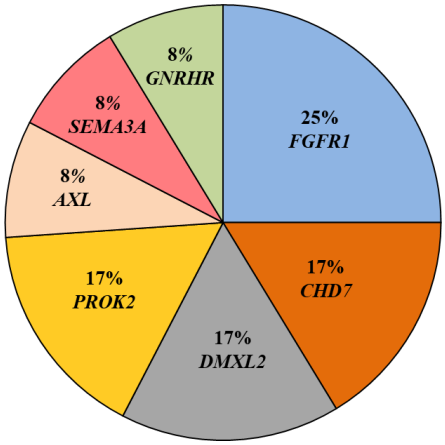

Figure 10: Distribution of gene variants identified in our CHH cohort.

The percentage of mutated genes based on the number of mutation carriers (n=10) is illustrated in **Figure 11**. 3/10 patients were carriers of a *FGFR1* variant, representing the 30%of the mutated cohort. Concerning *CHD7* and *DMXL2*, 2/10 patients carried variants in thesegenes accounting for 20% of cases. Other gene defects (*AXL, GNRHR, PROK2* and *SEMA3A*)were each reported in 10% of patients (1/10).

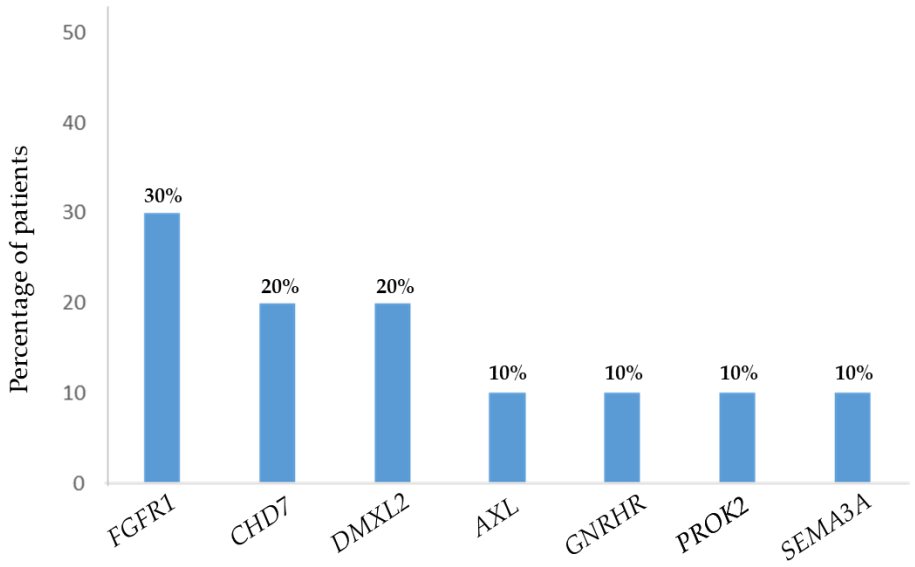

Figure 11: Distribution of mutated genes in CHH mutation carriers.

Table 10: List of gene variants identified in our CHH cohort, reporting the type of variant, status, MAF, Varsome verdict, carrier code and datafrom published studies.

Gene	*Inheritance*	*Variant*	*Type of variant*	*Status*	*MAF*	*Varsome verdict*	*Carrier code*	*Literature*
AXL	AR/oligo	NM_021913.5:c.1135-4C>G	Splicing	Het	0.0000177	VUS	CHH 8	Not reported
CHD7	AD	NM_017780:exon2:c.G1657C:p.V553L	Missense	Het	n.a.	VUS	CHH 11	Not reported
		NM_017780:exon33:c.C7047A:p.Y2349X	Stop Gain	Het	n.a.	P	CHH 7	Not reported
DMXL2	AD/AR	NM_015263:exon7:c.650_653del:p.R217fs	Frameshift deletion	Het	n.a.	P	CHH 2	Not reported
		NM_015263:exon28:c.C7145G:p.A2382G	Missense	Het	n.a.	VUS	CHH 9	Not reported
FGFR1	AD	NM_001174063:exon4:c.G418T:p.E140X	Stop Gain	Het	n.a.	P	CHH 14	Not reported
		NM_001174063:exon5:c.570delG:p.W190fs	Frameshift deletion	Het	n.a.	P	CHH 10	Not reported
		NM_001174063:exon14:c.C1858T:p.R620X	Stop Gain	Het	n.a.	P	CHH 12	Disease-causing *(Dodè et al., 2003; Pitteloud et al., 2006)*
GNRHR	AR/oligo	NM_000406:exon1:c.A317G:p.Q106R	Missense	Het	0.00418	P	CHH 6	Pathogenic *(de Roux et al., 1997)*
PROK2	AR/oligo	NM_001126128.2:c.286-1G>C	Splicing	Het	n.a.	P	CHH 11	Not reported
		NM_001126128:exon4:c.C301T:p.R101W	Missense	Het	0.0000704	LP	CHH 11	Not reported
SEMA3A	AD/oligo	NM_006080:exon2:c.C196T:p.R66W	Missense	Het	0.0000289	LP	CHH 18	Synergistic effect genes *(Hanchateet al., 2012)*

AD: autosomal dominant; AR: autosomal recessive; Het: heterozygous; LP: Likely Pathogenic; MAF: Minor Allele Frequency; n.a.: not available; oligo: oligogenicity; VUS: Variant of Uncertain Significance; P: Pathogenic.

5.1.2.1.1. Function and expression pattern of mutated genes

Here we report the function and expression data of each mutated gene, after consulting GeneCards and specific expression databases, such as Human Protein Atlas and Human Proteome Map.

- AXL

This gene encodes a receptor tyrosine kinase that transduces signals from the extracellular matrix into the cytoplasm by binding growth factor GAS6, thus regulating many physiological processes including cell survival, cell proliferation, migration and differentiation. This gene seems to be quite ubiquitous, with a high mRNA and protein expression profiles in testis, urinary bladder and skeletal muscle.

- CHD7

This gene encodes a protein that contains several helicase family domains and that is a probable transcription regulator, maybe involved in the 45S precursor rRNA production. Itshows a high expression in brain tissues, especially in cerebellum, and a low expression in other tissues.

- DMXL2

This gene encodes a protein with 12 WD domains, which are involved in many functions including participation in signal transduction pathways, such as Notch signalling pathway.It seems to be a ubiquitous gene, with a high protein expression in adult frontal

cortex.

- FGFR1

This gene encodes a tyrosine-protein kinase that acts as cell-surface receptor for fibroblast growth factors and plays an essential role in the regulation of embryonic development, cellproliferation, differentiation and migration. It shows a ubiquitous expression profile.

- GNRHR

This gene encodes a seven-transmembrane G-protein coupled receptor, which is expressedon the surface of pituitary gonadotrope cells. Following binding of GnRH, the activation ofthe receptor causes the release of gonadotropic luteinizing hormone (LH) and follicle stimulating hormone (FSH). This gene shows a pituitary gland-specific expression.

- PROK2

This gene encodes a protein expressed in the superchiasmatic nucleus (SCN) circadian clock.The secreted form of the encoded protein serves as a chemoattractant for neuronal precursorcells in the olfactory bulb. The *PROK2* mRNA expression profile is enhanced in testis, blood,and bone marrow, whereas the protein seems to be monocytes-specific.

- ***SEMA3A***

This gene is a member of the semaphorin family and encodes a protein, that is crucial for the normal neuronal pattern development. In fact, the secreted protein can function as either a chemorepulsive agent, inhibiting axonal outgrowth, or as a chemoattractive agent, stimulating the growth of apical dendrites. This gene shows a ubiquitous expression profile,with a high mRNA expression levels in brain, gastrointestinal tract and muscle tissue.

5.1.2.2. *ANOS1* MLPA analysis

ANOS1 is mapping to an *X-linked* locus predisposing to submicroscopic chromosomal deletions. Given that this gene is associated with KS, only patients presenting with hyposmia/anosmia (n=8) were subjected to this analysis. All the analysed patients were found to be wild type. The assay output of one wild type patient is reported in **Figure 12**.

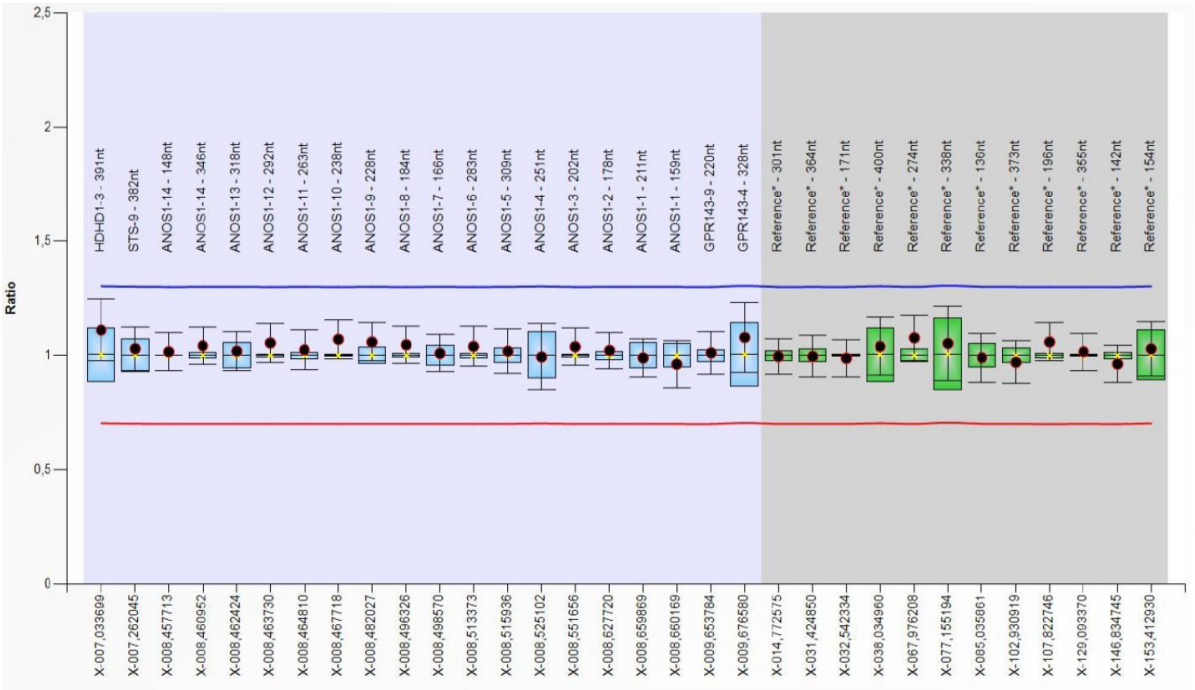

Figure 12: Ratio between each *ANOS1* probe (light grey) and the reference ones (dark grey). Normal range is considered as 0.7-1.3.

5.1.3. Family segregation studies

Family members were available only for 3 index cases (CHH 7, CHH 12 and CHH 18) in order to assess the variants segregation in these families (Figure 13). The 3 variants in *CHD7, SEMA3* and *FGFR1* genes were predicted as pathogenic.

As reported in Figure 13a, the novel stop gain variant in the *CHD7* gene (p.Tyr2349X) carried by the CHH 7 patient was de novo, as her parents did not carry this variant. Concerning the CHH 18 pedigree, the known likely pathogenic missense variant in the *SEMA3A* gene (p.Arg66Trp) was inherited from the father, who had no symptoms of

CHH (Figure 13b). Finally, the pathogenic stop gain *FGFR1* variant (p.Arg620X) carried by the CHH 12 patient was transmitted from the mother, who presented with CHH and normalsense of smell (**Figure 13c**).

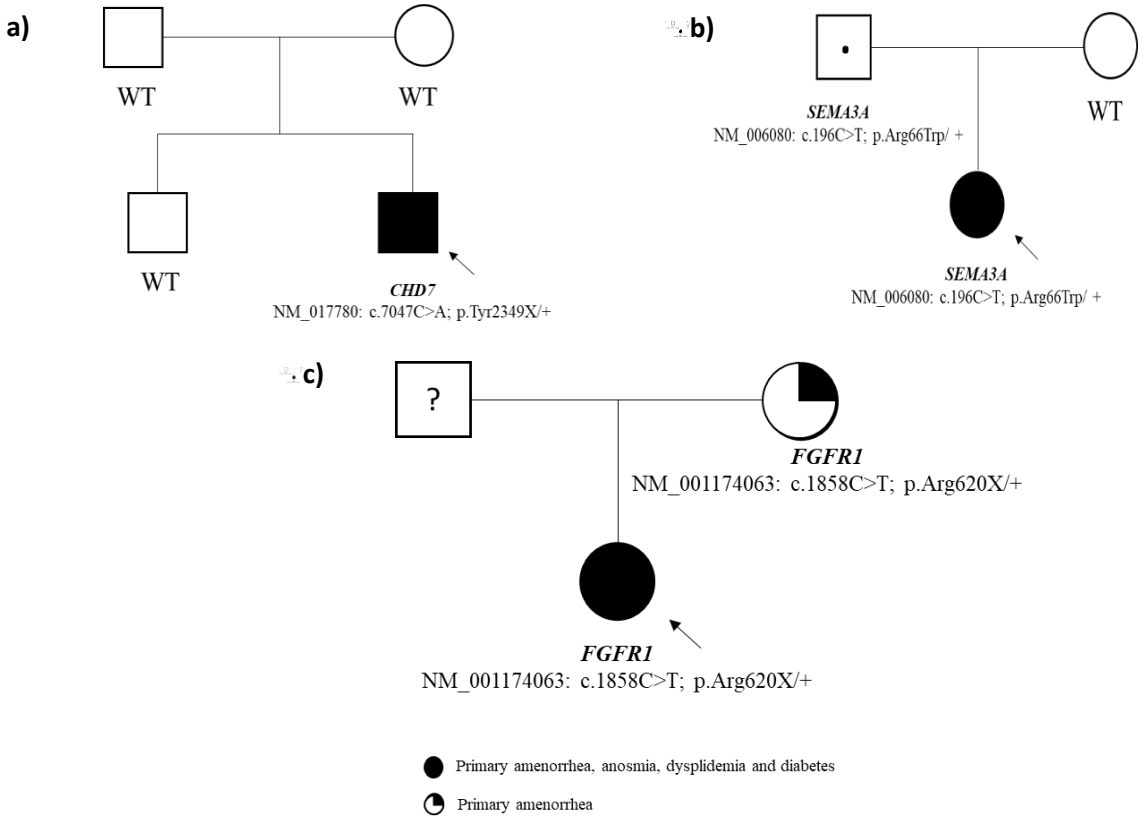

Figure 13: Segregation analysis in 3 families of mutation carriers: a) Pedigree of CHH 7 index case, carrying the *CHD7* variant (p.Tyr2349X); b) Pedigree of CHH 18 index case, carrying the *SEMA3A* variant (p.Arg66Trp); c) Pedigree of CHH 12 index case, carrying the *FGFR1* variant (p.Arg620X). The probands are indicated by an arrow.

5.1.4. Genotype-phenotype correlations among CHH mutated patients

Concerning the female mutation carriers (n=5) (**Table 11a**), 3 were affected by KS, whilst

2 presented with the adult-onset form of disease. Among the KS patients, CHH 12 and CHH14 received the genetic diagnosis of the disease, being carriers of heterozygous pathogenic variants (p. Arg620X and p.Glu140X, respectively) in the *FGFR1* gene, which shows a wideinheritance pattern including the autosomal dominant one. Notably, CHH 12 inherited thevariant (p.Arg620X) from her mother (**Figure 13c**), who shares the CHH phenotype with thedaughter, except for the impaired sense of smell. The remaining KS patient carried the heterozygous pathogenic hot spot (p.Gln106Arg) in the *GNRHR* gene, which shows an autosomal recessive or oligogenic mode of inheritance. Hence, her genetic diagnosis is still unknown. Among the AOH patients, in only one case the genetic cause can be established, i.e. the heterozygous loss of function variant (p.Arg217fs) in the *DMXL2* gene with an AD/AR mode of inheritance, identified in the CHH 2 index case. The remaining AOH indexcase (CHH 18) was found to be an heterozygous carrier of the pathogenic variant (p.Arg66Trp) in the *SEMA3A* gene which shows an autosomal dominant or oligogenic modeof inheritance. The family segregation study of this variant (**Figure 13b**) allowed us to rule out a dominant mode of inheritance of *SEMA3A* gene, since the father, from whom it was inherited, did not show any symptoms associated to CHH.

Concerning the male mutation carriers (n=5) (**Table 11b**), three were affected by KS, one showed the normosmic form of CHH and another one presented with the AOH. Among theKS patients, CHH 7 and CHH 10 received a clear genetic diagnosis of the disease. The CHH 7 presented with CHARGE syndrome, characterized by hearing loss, inner ear malformation and a severe form of CHH with extremely small testicles. His phenotype can be attributed to the *de novo* heterozygous pathogenic variant (p.Y2349X) in the autosomal dominant *CHD7* gene (**Figure 13a**). The CHH 10 patient presenting with olfactory and reproductive defects, including a reduced bilateral testicular volume (10 ml), was found to be a heterozygous carrier of a loss of function *FGFR1* variant (p.Trp190fs). The remaining KS patient (CHH 11) with cleft lip and small testicles

represents a unique example of oligogeniciy in our cohort, as carrier of three variants in two different candidate genes, i.e. *CHD7* and *PROK2*. The missense variant in the *CHD7* gene (p.Val553Leu) is of unknown significance, whereas the two identified variant in the autosomal recessive *PROK2* gene (p.Arg101Trp and c.286-1G>C) are predicted to be likely pathogenic/pathogenic. Although DNA from family members was not available for variants phasing, we were able to confirmthat the two *PROK2* variants are *in trans* by using the Integrative Genomics Viewer (IGV) graphic report, thanks to their close proximity on DNA (c.286-1G>C and c.301C>T). The proband CHH 9, affected by a severe form of nCHH, also presented with hypocortisolism and diabetes mellitus. He was a heterozygous carrier of a missense variant in the *DMXL2* gene (p.Ala2382Gly). However, this variant was predicted to be of uncertain significance, hence, he did not receive a genetic diagnosis. This also applies to CHH 8 proband, showingthe adult-onset form of disease and carrying a heterozygous variant of unknwon significance (c.1135-4C>G) in the *AXL* gene with an AD/oligo mode of inheritance.

Overall, the genetic diagnostic yield of our gene panel in the total CHH cohort (n=26) was 26% (n=6/26). According to the main clinical phenotypes of CHH, i.e. classic and milder forms, we have found a genetic diagnostic yield of 41.6% (n=5/12) and 7% (1/14) in KS/overlapping syndrome and AOH cases, respectively (**Figure 9, Table 11**).

Table 11: Genotype-phenotype correlations among CHH mutation carriers, divided according to their gender in **a)** female patients and **b)** male patients.

a)

Patient code	*KS/ nCHH/ AOH*	*Additional features (overlapping syndrome)*	*Heterozygous Genotype*	*Inheritance*	*Variant significance*	*Family segregation*	*Genetic diagnosis*
CHH 6	KS	Not reported	***GNRHR*** (NM_000406:exon1:c.A317G:p.Q106R)	AR/oligo	Pathogenic	n.a.	No
CHH 12	KS	Not reported	***FGFR1*** (NM_001174063:exon14:c.C1858T:p.R620X)	AD/AR/oligo	Pathogenic	Yes	Yes
CHH 14	KS	Not reported	***FGFR1*** (NM_001174063:exon4:c.G418T:p.E140X)	AD/AR/oligo	Pathogenic	n.a.	Yes
CHH 2	AOH	Not reported	***DMXL2*** (NM_015263:exon7:c.650_653del:p.R217fs)*	AD/AR	Likely pathogenic	n.a.	Yes
CHH 18	AOH	Not reported	***SEMA3A*** (NM_006080:exon2:c.C196T:p.R66W)	AD/oligo	Pathogenic	Yes (the healthy father sharedthe same genotype)	No (but it could act asa genetic modifier)

b)

Patient code	*KS/ nCHH/ AOH*	*Testicular volume at diagnosis*	*Additional features (overlapping syndrome)*	*Heterozygous Genotype*	*Inheritance*	*Variant significance*	*Family segregation*	*Genetic diagnosis*
CHH 7	KS	4 ml (bilateral)	Hearing loss and inner ear malformation (CHARGE syndrome)	***CHD7*** (NM_017780:exon33:c.C7047A:p.Y2349X)	AD	Pathogenic	No	Yes
CHH 10	KS	10 ml (bilateral)	Not reported	***FGFR1*** (NM_001174063:exon5:c.570delG:p.W190fs)	AD/AR/oligo	Pathogenic	n.a.	Yes
CHH 11	KS	4 ml (left), 5 ml (right)	Cleft lip	***CHD7*** (NM_017780:exon2:c.G1657C:p.V553L)	AD	VUS	n.a.	Yes (compound heterozygous *PROK2* Variants)
				PROK2 (NM_001126128:exon4:c.C301T:p.R101W)	AR/oligo	Likely pathogenic		
				PROK2 (NM_001126128.2:c.286-1G>C)	AR/oligo	Pathogenic		
CHH 9	nCHH	3 ml (bilateral)	Hypocortisolism and diabetes mellitus	***DMXL2*** (NM_015263:exon28:c.C7145G:p.A2382G)*	AD/AR	VUS	n.a.	No
CHH 8	AOH	12 ml (bilateral)	Not reported	***AXL*** (NM_021913.5:c.1135-4C>G)	AD/AR	VUS	n.a.	No

*These variants were found to be highly conserved among species by using PRALINE software. AD: autosomal dominant; AOH: adult-onset hypogonadism; AR: autosomal recessive; KS: Kallmann syndrome; n.a.: not available; nCHH: normosmic hypogonadotropic hypogonadism; VUS: variant of unknown significance.

5.2. Rare endocrine tumours (Pheo/PGL/HNPGL/VHL) cohort

5.2.1. Clinical characterization

Among the 103 subjects affected by these types of rare endocrine tumours, 57 individuals (23 males and 34 females) were affected by pheochromocytomas (Pheos), 29 individuals (7 males and 22 females) were affected by head and neck paragangliomas (HNPGLs), 9 individuals (3 males and 6 females) showed mediastinal/abdominal paragangliomas (PGLs)and 8 individuals (4 males and 4 females) were suspected von Hippel-Lindau syndrome cases (VHLs). In our cohort, the majority of patients were Pheos accounting for 55% of cases,followed by HNPGLs (28%), whereas PGLs and VHLs subjects accounted for 9% and 8% ofcases, respectively. (Figure 14).

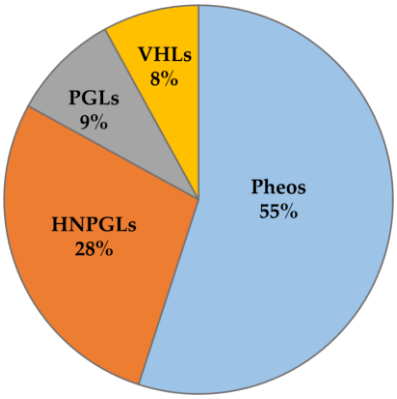

Figure 14: Distribution of the types of rare endocrine tumours in the study population. HNPGLs: Head and Neck Paragangliomas, PGLs: Paragangliomas; Pheos: Pheochromocytomas; VHLs: von Hippel-Lindau syndrome cases.

With the exception of gender, clinical data, such as age at disease-onset, clinical symptoms at the diagnosis, presence of multiple lesions and/or metastases were not available for all analysed patients (**Table 12**).

A female to male ratio of 1.5-3:1 was reported for Pheos/PGLs/HNPGLs, whereas in VHL syndrome males and females appeared to be equally affected (1:1) (**Table 12**).

Among the entire cohort with available age at disease-onset (n=81 patients), the mean age was 54.6 (± 15.5) years old, with a lower average in suspected VHL (37 years old, ± 28.6) anda higher one in Pheos (57.4 years old, ± 14.6) (**Table 12**).

Concerning the entire cohort with full clinical data (n=78), 41% of cases manifested symptoms, including hypertension, mass effect and HNPGL-related signs, allowing to diagnose their conditions **(Table 12**). Among symptomatic patients, the majority presented with PGL (66.6%), followed by VHL (50%), Pheos (38.6%) and HNPGLs (36.4%).

In addition, 16.7% and 12.8% were reported to have more than one lesion and distant metastases, respectively. VHL and PGL cases were found to be at higher risk of multiple lesions (33.3%), followed by HNPGLs (27%) and Pheos (7%). Concerning metastases, the involvement of bone, lymph nodes, pancreas and liver was reported in 50% of suspected VHL patients. Bone, lymph nodes, lung, liver and colon metastases were detected in 13.6%and 4.5% of Pheos and HNPGLs, respectively.

Table 12: Clinical data of patients affected by analysed rare endocrine tumours, according to the type of tumour.

	Gender (F:M)	Mean age at diagnosis in years (sd)	Symptomatic patients	Multiple lesions	Metastases
Pheos	1.5:1	57.4 (±14.6)	38.6% (n=17/44)	7% (n=3/44)	13.6% (n=6/44)
HNPGLs	3:1	52.5 (±15.3)	36.4% (n=8/22)	27% (n=6/22)	4.5% (n=1/22)
PGLs	2:1	50.4 (±12.0)	66.6% (n=4/6)	33.3% (n=2/6)	0% (n=0/6)
VHL	1:1	37 (±28.6)	50% (n=3/6)	33.3% (n=2/6)	50% (n=3/6)
Total	1.7:1	54.6 (±15.5)	41% (n=32/78)	16.7% (n=13/78)	12.8% (n=10/78)

Sd: standard deviation.

5.2.2. Genetic findings

Here we report: i) the targeted NGS gene panel results in 95 patients; ii) the MLPA analysis for the *SDHx* and *VHL* genes; iii) the mutational screening of *VHL* gene in selected patients(n=8) with suspected von Hippel-Lindau syndrome.

5.2.2.1. Targeted NGS gene panel results

The targeted NGS gene panel contains 15 susceptibility genes for Pheo/PGL/HNPGL (see Materials and Methods).

After bioinformatics filtering, a total of 20/95 patients carried at least one variant in the analysed genes, for a total of 19 VUS/likely pathogenic/pathogenic variants in 9 genes. Eachof these variants has been validated with Sanger sequencing.

Among the identified variants, 3 were found in more than one patient whereas 16 were

singleton (**Figure 15**). Among them, missense (n=7), stop gain (n=5), frameshift (n=4), splicing (n=2) and stop loss (n=1) variants have been identified, of which 11 were previouslyreported as disease-causing, whereas 8 were novel (**Table 13**). Based on the literature data and Varsome/RENOVO verdicts, we have a total of 16 pathogenic and 2 likely pathogenic variants, beside one variant of unknown significance (**Table 13**).

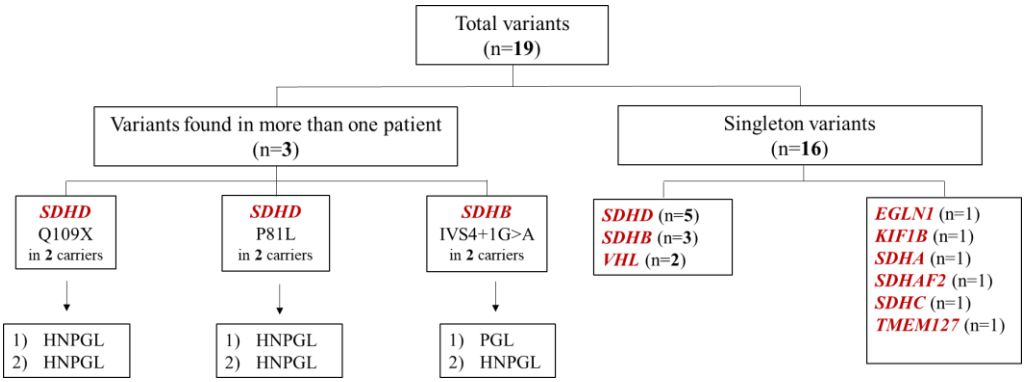

Figure 15: Flow-chart illustrating recurrent and singleton variants. Concerning the former ones, thetype of variant and the corresponding disease at clinical diagnosis of carriers are reported. Concerning the singleton variants (n=16), only affected genes with the number of carriers are shown.HNPGL: Head and Neck Paraganglioma; PGL: Paraganglioma.

The most frequently mutated gene was the *SDHD* gene (n= 7/19 variants), accounting for 36.8%, followed by *SDHB* (n=4/19 variants) and *VHL* (n=2/19) genes, whose defects representthe 21% and 10.5% of identified variants, respectively. In the other affected genes (*SDHA, SDHAF2, SDHC, KIF1B, EGLN1* and *TMEM127*) only 1/19 variant has been reported,accounting each one for 5.3% of the totality (**Figure 16**).

The percentage of mutated genes based on the number of mutation carriers (n=20) is illustrated in **Figure 17**. 9/20 patients were carriers of a *SDHD* variant, representing the 45%of the mutated cohort. Concerning *SDHB* and *VHL*, 5/20 and 2/20 patients carried variants in these genes accounting for 25% and 10% of cases, respectively. Other gene defects (*EGLN1, KIF1B, SDHA, SDHAF2, SDHC* and *TMEM127*) were each reported in 5% of patients (1/20).

Table 13: List of gene variants identified in our cohort affected by the four types of rare endocrine tumours, with type of variant, status, MAF, Varsome verdict, RENOVO verdict for VUSs, carrier code and literature data.

Gene	*Variant*	*Type of variant*	*Status*	*MAF**	*Varsome verdict*	*RENOVO verdict*	*Carrier code*	*Literature*
EGLN1	NM_022051: c.153G>A; p.Trp51X	Stop gain	Het	n.a.	P	-	FI877	Not reported
KIF1B	NM_015074:c.4052C>T; p.Pro1351Leu	Missense	Het	0.00000879	LP	n.a.	FI1111	Not reported
SDHA	NM_004168:c.955A>G;p.Ile319Leu	Missense	Het	n.a.	VUS	LP	FI1422	Not reported
SDHAF2	NM_017841:c.232G>A; p.Gly78Arg	Missense	Het	n.a.	P	-	FI1162	Reported
SDHB	NM_003000:c.423+1G>A; IVS4+1G>A	Splicing	Het	0.0000176	P	-	FI1219, FI1346	Reported
	NM_003000:c.541-2A>G, IVS5-2A>G	Splicing	Het	n.a.	P	-	FI1271	Reported
	NM_003000:c.572G>A; p.Cys191Tyr	Missense	Het	n.a.	P	-	F11119	Not reported
	NM_003000:c.591delC, p.Ser198Alafs*22	Frameshift	Het	n.a.	P	-	FI1139	Reported
SDHC	NM_003001:c.387G>A, p.Trp129X	Stop gain	Het	n.a.	P	-	FI1190	Not reported
SDHD	NM_003002:c.242C>T;p.Pro81Leu	Missense	Het	0.0000264	P	-	FI1234, FI1401	Reported
	NM_003002: c.275_278dupTGGA, p. Asp92fs*21	Frameshift	Het	n.a.	P	-	FI1174	Not reported
	NM_003002:c.325C>T; p.Gln109X	Stop gain	Het	n.a.	P	-	FI954, FI1075	Reported
	NM_003002:c.335C>T; c.218C>T; p.Thr112Ile	Missense	Het	0.00000399	VUS	P	FI1004	Reported
	NM_003002:c.337_340delGACT; p.Asp113Metfs*21	Frameshift	Het	n.a.	P	-	F1005	Reported
	NM_003002:c.470G>A;p.Tyr157X	Stop gain	Het	n.a.	P	-	FI1367	Not reported
	NM_003002:c.477_478delCT; p.Ter160Thrext*29	Stop loss	Het	n.a.	VUS	VUS	FI1426	Not reported
TMEM127	NM_017849:c.117_120delGTCT;p.Ile41ArgfsX39	Frameshift	Het	0.0000224	P	-	FI1316	Reported
VHL	NM_000551: c.154G>T, p.Glu52X	Stop gain	Het	0.0000123	P	-	FI1190	Reported
	NM_000551: c.337C>T, p.Arg113X	Stop gain	Het	n.a.	P	-	FI1306	Not reported
	NM_000551: c.524 A>G, p.Tyr175Cys	Missense	Het	n.a.	P	-	FI1132, FI1367	Reported

*MAF: Minor Allele Frequency in Non-Finnish European; Het: heterozygous; LP: Likely Pathogenic; n.a.: not available; VUS: variant of uncertain significance; P: pathogenic.

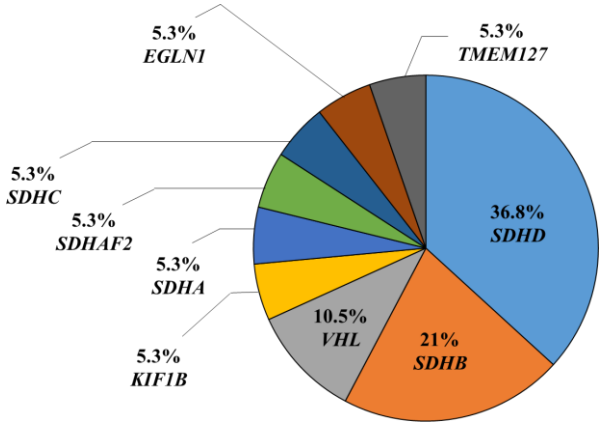

Figure 16: Distribution of gene variants identified in our cohort with Pheo/PGL/HNPGL.

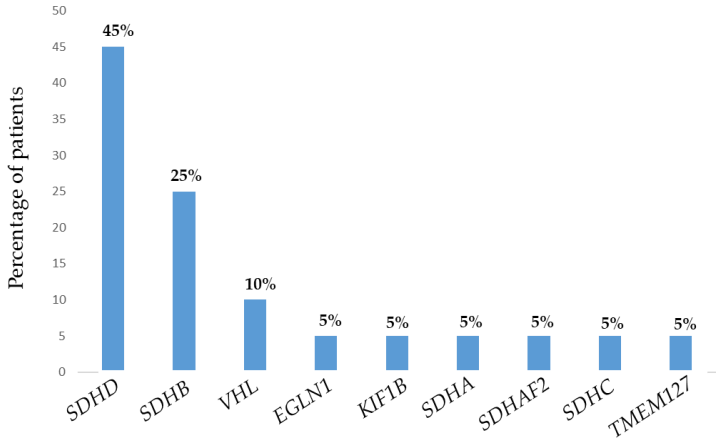

Figure 17: Distribution of mutated genes in mutation carriers affected Pheo/PGL/HNP

5.2.2.1.1. Function and expression pattern of mutated genes

Here we report the function and expression data of each mutated gene, after consulting GeneCards and specific expression databases, such as Human Protein Atlas and Human Proteome Map.

- EGLN1

This gene encodes a protein belonging to the pseudo-hypoxia cluster, which is involved in the post-translational formation of 4-hydroxyproline in hypoxia-inducible factor (HIF)

alpha. This protein functions as a cellular oxygen sensor, and under normal oxygen concentration, modification by prolyl hydroxylation is a key regulatory event that targets HIF subunits for proteasomal destruction via the von Hippel-Lindau ubiquitylation complex. This gene shows a mRNA tissue specificity for blood and skeletal muscle, whereasthe encoded protein seems to be ubiquitous.

- KIF1B

This gene encodes a motor protein belonging to the cluster of kinase receptor signalling and protein translation pathway, that is essential for anterograde transport of mitochondria andsynaptic vesicle precursors. Its mRNA expression profile is skeletal muscle-specific, whereas the KIF1B protein seems to be ubiquitous.

- SDHA

This gene belonging to the pseudo-hypoxia cluster encodes the major catalytic subunit of the succinate dehydrogenase (SDH), a complex of the mitochondrial respiratory chain responsible for transferring electrons from succinate to ubiquinone. It shows a mRNA tissue specificity for heart muscle and skeletal muscle, whereas the encoded protein appears to beubiquitous.

- SDHAF2

This gene belonging to the pseudo-hypoxia cluster encodes a mitochondrial protein with anessential role in the assembly of the SDH complex through the flavination of subunit A (SDHA). It shows a ubiquitous expression pattern.

- SDHB

This gene belonging to the pseudo-hypoxia cluster encodes the iron-sulfur subunit of the mitochondrial SDH complex. This subunit is highly conserved and contains three cysteine-rich clusters which may comprise the iron-sulfur centres of the enzyme. The *SDHB* gene shows ubiquitous expression levels.

- SDHC

This gene belonging to the pseudo-hypoxia cluster encodes one of two integral membrane proteins that anchor other subunits of the SDH complex to the inner mitochondrial membrane. It seems to be a ubiquitous gene, with a high protein expression in heart and liver.

- SDHD

This gene belonging to the pseudo-hypoxia cluster encodes the other integral membrane protein that anchor other subunits of the SDH complex to the inner mitochondrial membrane. It shows a ubiquitous expression pattern.

- TMEM127

This gene encodes a protein belonging to the cluster of kinase receptor signalling and protein translation pathway. The TMEM127 protein acts as a negative regulator of TOR signalling pathway mediated by mTORC1. It seems to be a ubiquitous gene, with a high protein expression in heart tissue.

- VHL

This gene encodes a protein belonging to the pseudo-hypoxia cluster, which is involved in the ubiquitination and degradation of HIF factor. It seems to be a ubiquitous gene, showinghigh protein expression levels in gallbladder and kidney.

5.2.2.2. *SDHx* and *VHL* genes MLPA analysis

A total of 95 patients affected by Pheo/PGL/HNPGL was subjected to the detection of CNVsin *SDHAF1*, *SDHAF2*, *SDHB*, *SDHC* and *SDHD* genes, since approximately 5-17% of pathogenic mutations in *SDHB*/*SDHC*/*SDHD* genes is attributed to large deletions/duplications, including founder mutations in *SDHB*, i.e. a Dutch founder deletionin exon 3 and a Spanish founder deletion in exon 1 (Bayley et al. 2005, 2009, Buffet et al. 2012).

All the analysed patients were found to be wild type. The assay output of one wild type patient is reported in **Figure 18**.

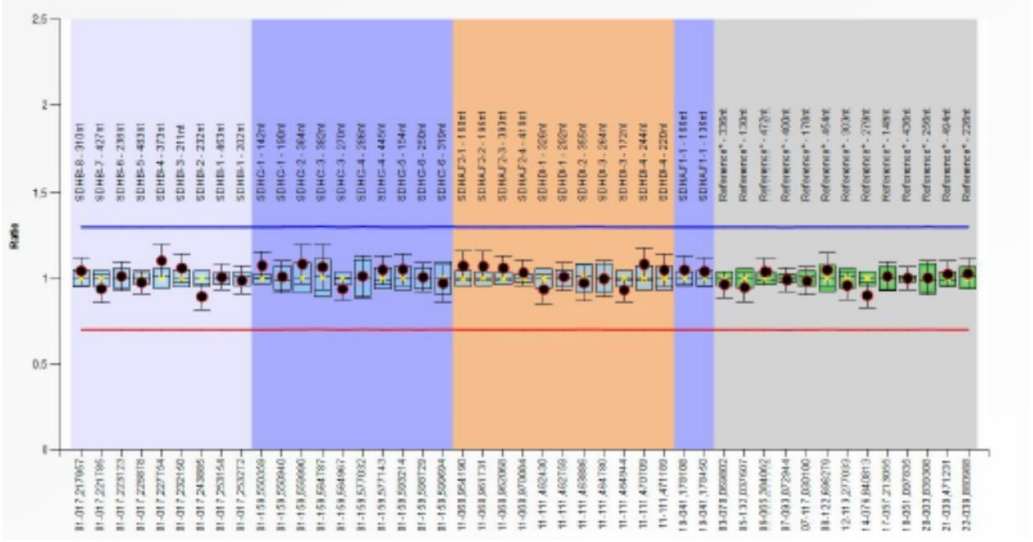

Figure 18: Ratio between each *SDHx* probe (light grey, indigo and orange) and the reference ones (dark grey). Normal range is considered as 0.8-1.2.

A total of 8 patients with suspected VHL syndrome was subjected to the detection of *VHL* CNVs, as 20-40% of reported *VHL* mutations are large intragenic deletions (0.5-250 kb) (Decker et al. 2014).

All the analysed patients were found to be wild type. The assay output of one wild type patient is reported in **Figure 19**.

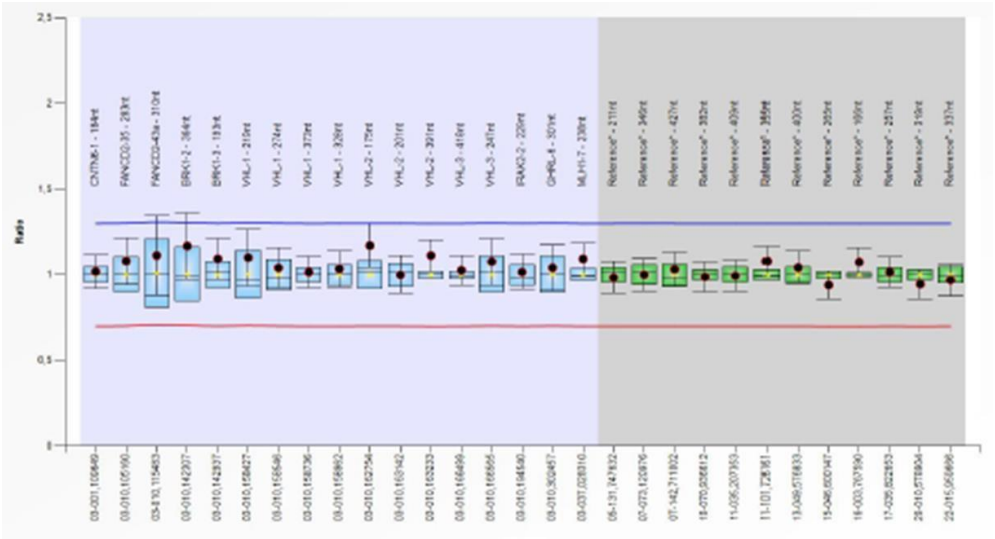

Figure 19: Ratio between each *VHL* probe (light grey) and the reference ones (dark grey). Normal range is considered as 0.8-1.2.

5.2.2.2.VHL mutation screening

Concerning patients with clinical suspect of VHL syndrome (n=8), the mutational screeningof *VHL* gene by performing Sanger sequencing has revealed the presence of 2 variants in two different patients (**Figure 20; Table13**). In particular, a novel stop gain variant (p.Arg113X) (**Figure 20a; Table 13**) predicted as pathogenic has been identified in a 14-yrs-old boy (FI1306). In addition, the known pathogenic p. Tyr175Cys variant (**Figure 20b; Table 13**) has been also detected in a 28-yrs-old woman (FI1132).

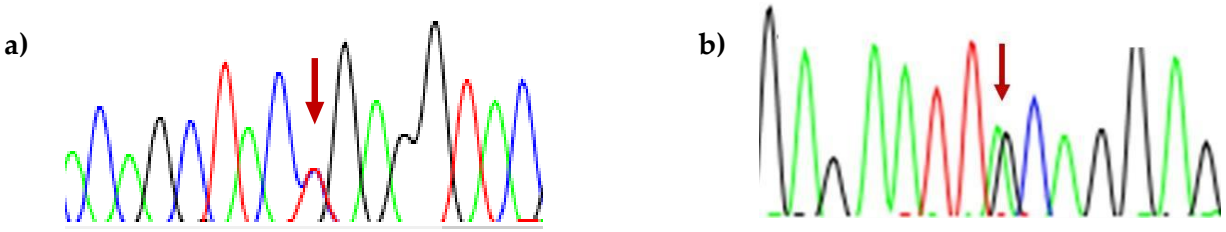

Figure 20: Sequence electropherograms of the two identified *VHL* variants. **a)** Sequence electropherogram of the stop gain variant (c.337C>T, p.Arg113X); **b)** Sequence electropherogram ofthe missense variant (c.524 A>G, p.Tyr175Cys). Each base substitution is indicated by a red arrow.

5.2.3. Clinical characterization of mutation carriers *versus* "wild type" subjects

5.2.3.1. Clinical presentation

Among the mutated cohort with available clinical information (n=16), 37.5% (n=6/16) presented with symptoms (**Figure 21**). All paragangliomas (n=2) and 1/5 pheochromocytoma showed hypertension, whilst a proportion of HNPGL cases (n=3/7)

manifested symptoms related to the mass effect. On the other hand, all of VHL patients (n=2) and more than half of HNPGLs were asymptomatic (incidentaloma cases). Interesting to note that, although the majority of Pheos (n=4/5) were incidentalomas, 3 of them were secreting tumours.

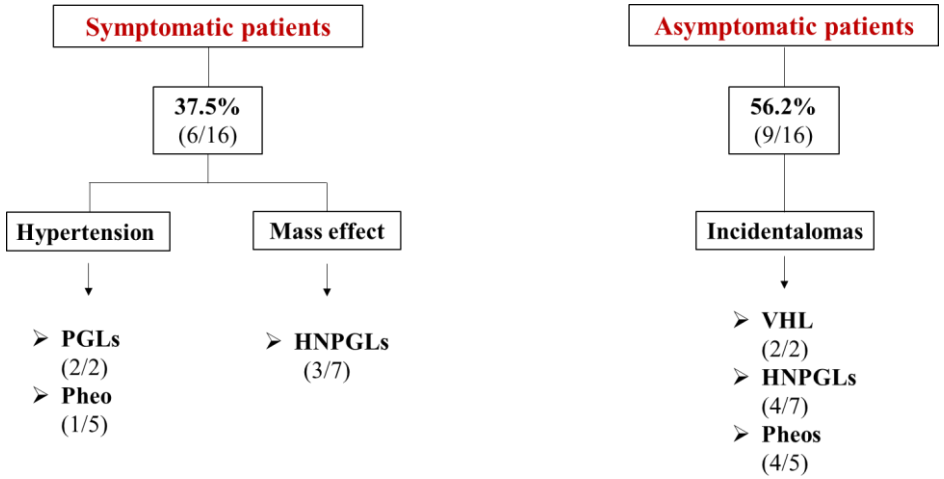

Figure 21: Flow-chart illustrating the proportion of symptomatic and asymptomatic mutated patients divided according to their phenotype.

Similarly, in the "wild type" cohort with available information based on clinical presentation (n=62), approximately 42% of patients (n=26/62) presented with hypertension or mass effect and 58% (n=36/62) was asymptomatic. This latter group included 66.6% of HNPGLs (n=10/15) and 59% of Pheos (n=23/39), respectively, beside 50% of PGL cases (n=2/4). Concerning VHL patients, 25% (n=1/4) manifested mass effect-related symptoms.

Overall, no significant differences on clinical presentation between mutated and "wild type" subjects have been observed.

Concerning Pheos with available urinary catecholamine levels (n=38), we have observed 80% (n=4/5) and 90% (n=30/33) of secreting tumours among mutated and "wild type" subjects, respectively. Thus, no significant differences on the presence of high urinary

catecholamine levels between the two groups was reported.

5.2.3.2. Mean age

Concerning age at disease-onset, available data accounted for 59 and 22 "wild type" and mutated patients, respectively.

The mean age of the mutation carriers (46.6 ± 18.4 years old±sd) was significantly lower (p=0.015) than that one of the "wild type" cohort (57.6 ± 13.2 years old±sd) (**Figure 22**).

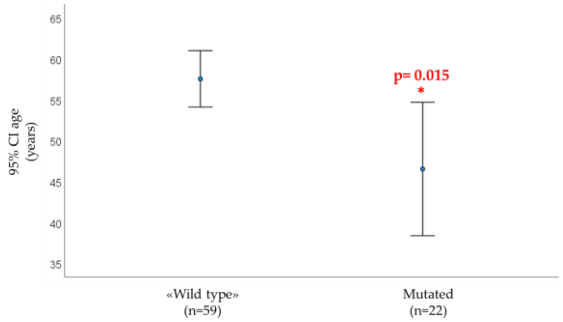

Figure 22: Comparison of the age at disease-onset between the "wild type" and the mutated patients.

5.2.3.3. Multiple lesions

Concerning the presence of multiple lesions, 16.7% of patients (n=13/78) presented multiplelesions. As reported in **Figure 23**, 9.7% (n=6/62) was "wild type", whereas 43.7% (n=7/16) carried one of the above-mentioned germline mutations in susceptibility genes. Among these latter, *VHL, SDHAF2, SDHB* and *SDHD* mutation carriers were found to present withmultiple lesions (**Table 14**).

The "wild type" and the mutated groups showed a significant difference in the

occurrenceof multiple lesions (p=0.001; **Figure 23**).

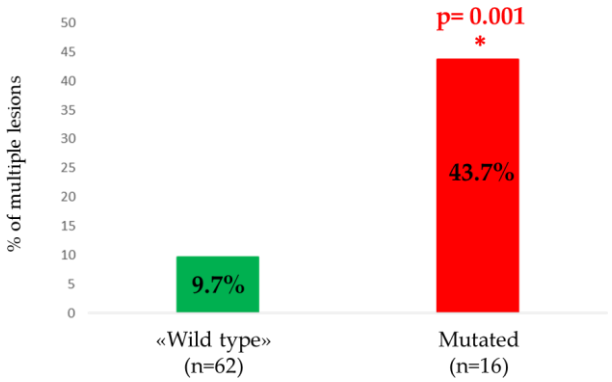

Figure 23: Comparison of the percentage of multiple lesions between the wild type and the mutatedpatients.

5.2.3.4. Metastases

Concerning the occurrence of metastases, 12.8% (n=10/78) of them manifested distal metastases. Among the mutated patients, 12.5% (n=2/16) reported bone or lymph node metastases and in the wild type one 12.9% (n=8/62) presented with metastases in different sites, including bone (n=3), liver (n=2), lymph nodes (n=1), pancreas/spleen (n=1) and colon(n=1).

Therefore, no differences concerning the occurrence of metastases between mutated and "wild type" subjects have been observed.

5.2.4. Family segregation studies

A total of 9 families of mutation carriers has been evaluated both to assess the variants segregation (**Figure 24**) and to identify pre-symptomatic individuals.

As reported in **Figure 24a**, the splicing variant in the *SDHB* gene (p.Tyr2349X) carried by the 31-yrs-old female patient (FI1271) with PGL was inherited from her mother aged 59 years, who did not manifest this condition. Also the *EGLN1* (p.Trp51X) and the *KIF1B* variants (p.Pro1351Leu) reported in two patients affected by Pheos, i.e. FI877 aged 16 yearsand FI1111 aged 46 years, respectively, were inherited from the healthy fathers (**Figure 24band 24c**). The stop gain variant in the *VHL* gene (p.Arg113X) , detected in a 14-yrs-old VHLboy (FI1306) was *de novo*, as his parents did not carry this variant (**Figure 24d**). Concerning the pedigree of the 57-yrs-old man (FI1119) affected by Pheo, the missense variant in the *SDHB* gene (p.Cys191Tyr) was transmitted to both sons, the older of whom has developed HNPGL (**Figure 24e**). The splicing variant in *SDHB* (c.423+1G>A) reported in a 61-yrs-old woman (FI1219) with PGL was also transmitted to one of her sons, who has developed the disease at the age of 29 years (**Figure 24f**). The same variant was detected in a 57-yrs-old female patient (FI1346) affected by HNPGL, who has inherited it from her mother free of disease at the age of 88 years, and then she has transmitted the mutated allele to her 30-yrs-old healthy son. None of the other carriers was affected (**Figure 24g**). Concerning the *SDHD*variant (p.Asp113Metfs21) identified in a 28-yrs-old man (FI1005) affected by HNPGL, alsohis father aged 57 years and his brother aged 24 years were found to carry this variant. Bothof them have developed the disease (**Figure 24h**). Finally, the pedigree of a 33-yrs-old male patient (FI1190) affected by HNPGL is reported (**Figure 24i**). This patient has inherited the *SDHC* variant (p.Trp129X) from the father and the *VHL* one (p.Glu52X) from the mother. Neither parents nor his brother who is carrying the *SDHC* variant developed.

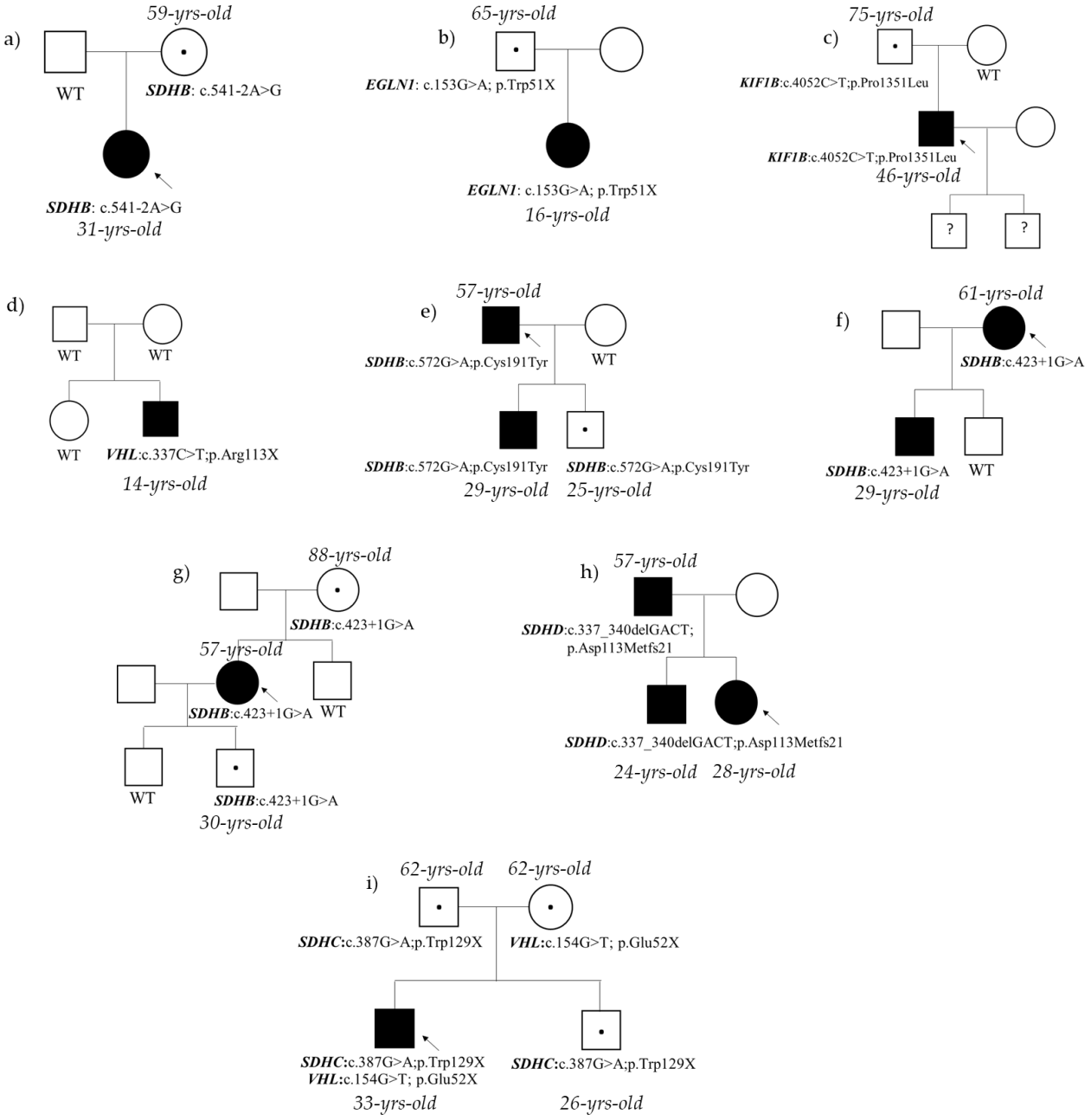

Figure 24: Segregation analysis in 9 families of mutation carriers: **a)** Pedigree of FI1271 index case, carrying the *SDHB* variant (c.541-2A>G); **b)** Pedigree of FI877 index case, carrying the *EGLN1* variant(p.Trp51X); **c)** Pedigree of FI1111 index case, carrying the *KIF1B* variant (p.Pro1351Leu); **d)** Pedigreeof FI1306 index case, carrying the *VHL* variant (p.Arg113X); **e)** Pedigree of FI1119 index case, carrying the *SDHB* variant (p.Cys191Tyr); **f)** Pedigree of FI1219 index case, carrying the *SDHB* variant (c.423+1G>A); **g)** Pedigree of FI1346 index case, carrying the *SDHB* variant (c.423+1G>A); **h)**Pedigree of FI1005 index case, carrying the *SDHD* variant (p.Asp113Metfs21); **i)**

Pedigree of FI1190 index case, carrying the *SDHC* (p.Trp129X) and the *VHL* (p.Glu52X) variants. The probands are indicated by an arrow. The quotation mark indicates an unknown genotype. Age of each mutation carrier is shown.

5.2.5. Genotype-phenotype correlations

It is worth noting that all these susceptibility genes show an autosomal dominant inheritance, since one germline mutation affecting one copy of a gene is sufficient to increasethe risk of developing tumors. Hence, Pheo/PGL/HNPGL and VHL syndrome can be considered as autosomal dominant conditions.

Among the mutation carriers (n=22), 21 patients were found to carry at least one germline disease-causing variant, whereas the FI1426 female patient affected by HNPGL was a carrierof a variant of uncertain significance (**Table 13**). Thus, this latter patient did not receive thegenetic diagnosis for her condition (**Table 14**).

Here we report the genotype-phenotype correlations of patients carrying one germline disease-causing variant, according to the type of tumour, i.e. Pheo, HNPGL, PGL and VHLsyndrome (**Table 14**).

5.2.5.1. Pheochromocytomas

Concerning Pheo patients with a genetic diagnosis (n=8), 3 were females and 5 were males.

Among female individuals, one 16-yrs-old girl (FI877) affected by a secreting form of tumour (Table 15) without multiple lesions was found to carry a novel stop gain variant

(p.Trp51X) in the *EGLN1* gene. This variant was inherited from the 65-yrs-old father, who has not yet developed the disease (Figure 24b). A 79-yrs-old woman (FI1422) presenting with elevated urinary catecholamines but without multiple lesions and metastases was carrier of a novel missense *SDHA* variant (p.Ile319Leu), which was predicted as likely pathogenic after using RENOVO tool. Another woman (FI1316) was a carrier of a known disease-causing variant (p.Ile41ArgfsX39) in the *TMEM127* gene.

Among male individuals, the FI1004 and FI1111 probands both presented secreting tumoursand bone and lymph node metastases, respectively. The former aged 35 years and carried a known missense variant (p.Thr112Ile) in the *SDHD* gene, which was considered as pathogenic. The latter aged 46 years and carried a likely pathogenic variant (p.Pro1351Leu)in the *KIF1B* gene, which was inherited from his father (**Figure 24c**). The other two patients,FI1119 and FI1174, aged 57 and 78 years, respectively, were found to carry novel variants inthe *SDHB* and *SDHD* genes, respectively. The FI1119 proband was a carrier of a missense variant (p.Cys191Tyr), which was transmitted to both sons (**Figure 24e**). Interestingly, onlythe oldest one (29-yrs-old) has already developed the disease, whilst the other son aged 25 years did not yet show this condition. The FI1174 index case presenting without multiple lesions and low urinary catecholamine levels carried a novel frameshift one (p. Asp92fs*21).Finally, a digenic case has been reported. In fact, a novel stop gain variant (p.Tyr157X) in the *SDHD* gene predicted as pathogenic and a known pathogenic *VHL* missense variant (p.Tyr175Cys) have been identified in the same patient (FI1367) only affected by Pheo.

5.2.5.2. Head and Neck Paragangliomas

Concerning HNPGL patients with a genetic diagnosis (n=8), 6 were females and 2 were males.

Among female individuals, two patients (FI954 and FI1075) carried the same mutation in the *SDHD* gene (p.Gln109X), which was previously reported as pathogenic. One of them (FI1075) aged 37 years showing multiple lesions and elevated urinary normetanephrine levels (575 mcg/die) (Table 15). No clinical data was available for the FI954 proband. The 28-yrs-old patient (FI1005) carried a known pathogenic *SDHD* variant (p.Asp113Metfs*21),which was inherited from the father, who also presenting with a HNPGL. As reported in Figure 24h, also her 24-yrs-old brother, who has inherited the mutated allele from the father, showed a HNPGL. This familial case represents an example of a clear genotype-phenotypecorrelation, i.e. all mutation carriers will almost likely develop the disease. One 53-yrs-old woman (FI11162) with multiple lesions, carried a known pathogenic missense variant (p.Gly78Arg) in the *SDHAF2* gene. The 57-yrs-old patient (FI1346) without multiple lesionswas found to carry a known splicing variant (IVS4+1G>A) in the *SDHB* gene. By performingfamilial segregation studies (**Figure 24g**), we have observed that also her 88-yrs-old motherand her son carried that variant, despite they were healthy, suggesting that IVS4+1G>A shows an incomplete penetrance. Finally, a 61-yrs-old patient with a non-secreting tumourand multiple lesions (FI1401) carried a known pathogenic missense variant (p.Pro81Leu) inthe *SDHD* gene , which was also identified in the FI1234 index case (see above).

Among male individuals, the 33-yrs-old patient (FI1190) with low catecholamines levels was a carrier of two variants in two different genes (*SDHC* and *VHL*), i.e. a digenic case. Hehas inherited the *SDHC* variant (p.Trp129X) from his healthy father and the *VHL* variant (p.Glu52X) from his healthy mother (**Figure 24i**). Also his 26-yrs-old brother carried the *SDHC* variant but he did not yet show the disease. The remaining male patient was the FI1234 proband presenting with high urinary normetaneprhine levels (441 mcg/die) (**Table15**) and multiple lesions. He was found to carry the known pathogenic missense variant (p.Pro81Leu) in the *SDHD* gene.

5.2.5.3. Mediastinal and Abdominal Paragangliomas

Concerning PGL patients with a genetic diagnosis (n=3), all of them were females. Interestingly, all these patients carried known pathogenic variants in the *SDHB* gene.
Both the 40-yrs-old FI1139 proband and the 61-yrs-old patient (FI1219) presented with low with low urinary catecholamine levels. The FI1139 proband with a single lesion carried a frameshift variant (p.Ser198Alafs*22). The FI1219 patient with multiple lesions carried the splicing IVS4+1G>A variant, which was transmitted to the 29-yrs-old affected son **(Figure 24f).** Finally, the FI1271 patient aged 31 years was found to carry the splicing IVS5-2A>G variant, which was inherited from the 59-yrs-old mother who did not yet show the disease (**Figure 24a**), confirming the incomplete penetrance of the *SDHB* variants (see FI1346 case).

5.2.5.4. Von Hippel-Lindau syndrome

Concerning VHL patients with a genetic diagnosis (n=2), one was a female and the other one was a male.

The female patient (FI1132) aged 28 years showing pheochromocytoma and pancreatic neuroendocrine neoplasia and was found to carry a known pathogenic *VHL* variant (p.Tyr175Cys). The male patient (FI1306) aged 14 years and was characterized by facial dysmorphism, hypospadias, unilateral renal agenesis and multiple retinal and cerebellar hemangioblastomas. He carried a *de novo* stop gain variant in the *VHL* gene (p.Arg113X) (**Figure 24d**), which has never been reported and it was predicted as pathogenic.

Overall, the genetic diagnostic yield of our gene panel in patients affected by Pheo/PGL/HNPGL was 20% (n=19/95) (**Figure 25**). According to the type of tumour, we

have found a genetic diagnostic yield of 14% (n=8/57) in Pheos, 33.3% (n=3/9) and 27.6% (n=8/29) in PGLs and HNPGLs, respectively (**Figure 25**).

In suspected VHL cases (n=8), a clinical diagnosis of VHL syndrome based on the presenceof a *VHL* gene defect was made in 25% of cases (n=2/8).

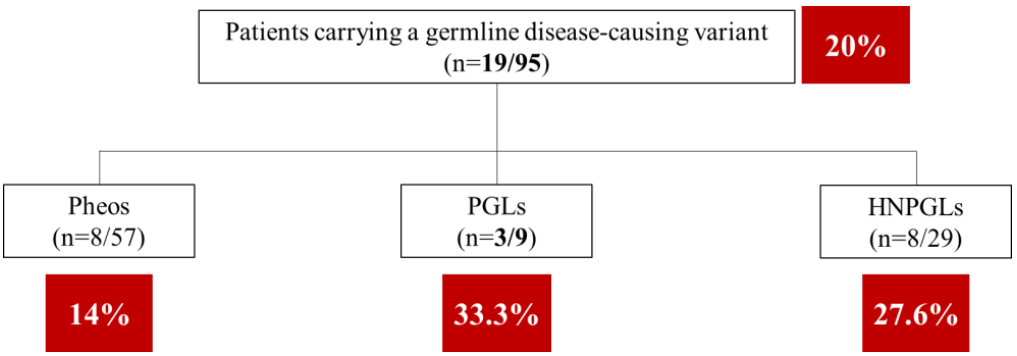

Figure 25: Schematic representation of the genetic diagnostic yield in pheochromocytomas and paragangliomas. Red rectangles represent the diagnostic rate based on gene panel.

Table 14: Genotype-phenotype correlations among Pheo/HNPGL/PGL/VHL mutation carriers.

Patient Code	*Gender*	*Age at disease-onset*	*Phenotype (Pheo; HNPGL; PGL; VHL)*	*Multiple lesions (Yes/No)*	*Metastases (Yes/No)*	*Elevated urinary catecholamines* (Yes/No)*	*Genotype*	*Variant significance*	*Family segregation*	*Genetic diagnosis*
FI877	F	16	Pheo	No	n.a.	Yes	*EGLN1* (p.Trp51X)	Pathogenic	Yes	Yes
FI1316	F	53	Pheo	n.a.	n.a.	n.a.	*TMEM127*(p.Ile41ArgfsX39)	Pathogenic	No	Yes
FI1422	F	79	Pheo	No	No	Yes	*SDHA* (p.Ile319Leu)	LP	No	Yes
FI1004	M	35	Pheo	No	Yes	Yes	*SDHD* (p.Thr112Ile)	Pathogenic	No	Yes
FI1111	M	46	Pheo	No	Yes	Yes	*KIF1B* (p.Pro1351Leu)	LP	Yes	Yes
FI1119	M	57	Pheo	n.a.	n.a.	n.a.	*SDHB* (p.Cys191Tyr)	Pathogenic	Yes	Yes
FI1174	M	78	Pheo	No	No	No	*SDHD* (p. Asp92fs*21)	Pathogenic	No	Yes
FI1367	M	67	Pheo	n.a.	n.a.	n.a.	*SDHD* (p.Tyr157X) *VHL* (p.Tyr175Cys)	Pathogenic/ Pathogenic	No	Yes
FI954	F	66	HNPGL	n.a.	n.a.	n.a.	*SDHD* (p.Gln109X)	Pathogenic	No	Yes
FI1005	F	28	HNPGL	n.a.	n.a.	n.a.	*SDHD* (p.Asp113Metfs*21)	Pathogenic	Yes	Yes
FI1075	F	37	HNPGL	Yes	No	n.a.	*SDHD* (p.Gln109X)	Pathogenic	No	Yes
FI1162	F	53	HNPGL	Yes	No	n.a.	*SDHAF2* (p.Gly78Arg)	Pathogenic	No	Yes
FI1346	F	57	HNPGL	No	No	n.a.	*SDHB* (IVS4+1G>A)	Pathogenic	Yes	Yes
FI1401	F	61	HNPGL	Yes	No	No	*SDHD* (p.Pro81Leu)	Pathogenic	No	Yes
FI1426	F	34	HNPGL	Yes	No	No	*SDHD* (p.Ter160Thrext*29)	VUS	No	No
FI1190	M	33	HNPGL	No	No	No	*SDHC* (p.Trp129X) *VHL* (p.Glu52X)	Pathogenic/ Pathogenic	Yes	Yes
FI1234	M	50	HNPGL	Yes	n.a.	Yes	*SDHD* (p.Pro81Leu)	Pathogenic	No	Yes
FI1139	F	40	PGL	No	No	No	*SDHB* (p.Ser198Alafs*22)	Pathogenic	No	Yes
FI1219	F	61	PGL	Yes	No	No	*SDHB* (IVS4+1G>A)	Pathogenic	Yes	Yes
FI1271	F	31	PGL	n.a.	n.a.	n.a	*SDHB* (IVS5-2A>G)	Pathogenic	Yes	Yes
FI1132	F	28	VHL	No	n.a.	n.a.	*VHL* (p.Tyr175Cys)	Pathogenic	No	Yes
FI1306	M	14	VHL	Yes	No	No	*VHL* (p.Arg113X)	Pathogenic	Yes	Yes

*We considered elevated urinary catecholamines if at least one of the two catecholamines exceeds the reference cut-off (see table 15). LP: likely pathogenic; n.a.: not available.

Table 15: Urinary catecholamine levels for 13 mutated patients with available data.

Patient code	Phenotype (Pheo; HNPGL; PGL; VHL)	Metanephrine (cut-off <320 mcg/die)	Normetanephrine (cut-off < 390 mcg/die)
FI877	Pheo	488	6512
FI1004	Pheo	326	5650
FI1111	Pheo	4033	5234
FI1174	Pheo	115	265
FI1422	Pheo	9940	2732
FI1075	HNPGL	100	575
FI1190	HNPGL	158	184
FI1234	HNPGL	125	441
FI1401	HNPGL	51	184
FI1426	HNPGL	139	172
FI1139	PGL	122,5	383
FI1219	PGL	15	39
FI1306	VHL	95	145

6. Discussion

Here we discuss the results of both Congenital Hypogonadotropic Hypogonadism and the four types of rare endocrine tumours. According to the ACMG and ACGS guidelines (Richards et al., 2015), we have considered a clear genetic diagnosis based on the followingcriteria: i) gene inheritance patterns, i.e. biallelic defects for AR genes, monoallelic defects for AD genes and multiple gene defects for those genes showing an oligogenic mode of inheritance; ii) type of identified variant, i.e. pathogenic and likely pathogenic variants wereconsidered of diagnostic value, whereas variants of uncertain significance were excluded.

6.1 Congenital Hypogonadotropic Hypogonadism

The CHH is a rare endocrine congenital disease, for which reveal genetic factors have beenidentified. It is worth noting that, this is a clinically and genetically heterogeneous condition. From a clinical point of view, it covers a wide spectrum of signs/symptoms, fromclassical forms (KS and nCHH) with/without other congenital features to mild forms with adult-onset (AOH). Classical forms are diagnosed at birth or due to pubertal delay, whereas mld forms can be recognized as post-pubertal CHH cases, i.e. hypotestosteronemia (Testosterone < 4-4.5 nmol/L) in males and secondary amenorrhea in females (Cangiano et al., 2021). From a geneticpoint of view, more than 40 genes with variable expressivity, penetrance and inheritance have been identified as candidate in the CHH aetiology (Cangiano et al., 2020; Butz et al., 2020). Beside monogenic forms,

oligogenicity, i.e. mutations in two or more candidate genesin the same patient, represents another mode of inheritance, further complicating the interpretation of the detected genetic variants and genetic counselling. With the advent of high-throughput NGS, several genes can be analysed at the same time, which is especially relevant in this disease considering its digenic/oligogenic nature.

In this study, we designed a custom NGS panel, containing 34 CHH candidate genes with clinical evidence up to 2018 (*ANOS1, AXL, CHD7, DMXL2, DUSP6, FEZF1, FGF17, FGF8, FGFR1, FLRT3, GNRH1, GNRHR, HESX1, HS6ST1, IL17RD, KISS1, KISS1R, LEP, LEPR, NR0B1, NSMF, OTUD4, PCSK1, PNPLA6, PROK2, PROKR2, SEMA3A, SEMA3E, SEMA7A,*
SOX10, SPRY4, TAC3, TACR3, WDR11), to identify the genetic cause of this complex diseasein our study population (n=26 affected patients).

Among our 26 CHH patients, a slightly male predominance (1.4:1) was reported, although a male to female ratio of approximately 4:1 has been reported in the literature (Seminara etal., 1998; Mitchell et al., 2011).

Classical forms of CHH were reported in 46% of cases, whereas 54% of patients were affected by AOH. Among the former, 38% of patients showed CHH with additional features, including KS syndrome (n=7), CHARGE syndrome (n=2) and one syndrome yet tobe defined (n=1), whilst 8% of cases showed isolated CHH (nCHH). Since a common geneticorigin of classical and milder forms of CHH has been reported (Cangiano et al., 2019), we decided to investigate the genetic diagnostic yield of our custom NGS panel in the entire cohort.

A total of 12 variants predicted as VUS/likely pathogenic/pathogenic has been filtered in, of which 3 were previously reported in literature, whereas 9 were novel. The most commonlyfound variants mapped to the *FGFR1* gene (25%), in line with previous eight studies basedon targeted NGS panels (Quaynor et al., 2016; Wang et al., 2017; Aoyama et

al., 2017; Cassatella et al., 2018; Zhou et al., 2018; Kim et al., 2019; Amato et al., 2019; Butz et al., 2021).The second most frequently mutated genes in our study were *CHD7, DMXL2* and *PROK2*, each accounting for 17%. Finally, single variants in the *AXL, GNRHR* and *SEMA3A* only represent 8% of the total identified variants.

Calculating the detection rate in our cohort, *FGFR1* variants were detected most commonly,in an average of 11.5% of all investigated patients, in accordance with the 11.4% of averagedetection rate previously reported (Butz et al., 2021). Both *CHD7* and *DMXL2* variants werefound in 7.7% of patients, while *PROK2, AXL, GNRHR* and *SEMA3A* in 3.8% of patients.

The detection rate of *CHD7* gene defects is similar to that one reported in the literature (8.1%, n=48/592) (Cangiano et al., 2021), whereas concerning *DMXL2*, rare variants have been found in only 2.3% by another study (Stamou et al., 2019). Except for *PROK2*, whose prevalence ofrare variants in CHH patients have been previously reported in 2.4%, *AXL, GNRHR* and *SEMA3A* gene defects are in the literature more frequently reported, with a 4.85%, 5.3% and4.4% of average detection rate, respectively (see references in Cangiano et al., 2021).

Overall, we have identified a total of 10/26 patients carrying at least one potential disease-causing variant. Among these, only one patient was found to carry three variants in two different genes, i.e. a digenic case. In the literature, oligogenicity was reported between 0%among Japanese populations (Aoyama et al., 2017) and 7-21% among Chinese and Caucasian ones (Quaynor et al. 2016; Wang et al., 2017; Zhou et al., 2018; Cassatella et al., 2018; Maione et al., 2018; Amato et al. 2019; Butz et al., 2021). In the present study, oligogenicity represents only the 3.8% of cases, and this percentage is likely to increase in the future with enlarging our cohort, especially the number of patients with classic CHH. Only 26% of cases (n=6/26) received the genetic diagnosis supporting the phenotype.

Although current guidelines state that the genetic cause of classic CHH can be found in almost 45% of cases (Boehm et al., 2015; Cangiano et al., 2021), a recent publication supportsour results, as the authors have identified a genetic diagnosis in only 13% of cases tested (n=5/38) (Butz et al., 2021). This is likely due to the wide spectrum of CHH phenotypes included in the study, i.e. KS, nCHH, AOH and delayed puberty. In particular, Butz and colleagues have clearly identified the genetic diagnosis in 5/30 patients, who were affected either by KS or nCHH. None AOH/delayed puberty case has been genetically diagnosed. Inline with these results, the majority of our patients with a genetic diagnosis were affected by a classical form of CHH (n=5/12; 42%), except for one female patient presenting with AOH (n=1/14; 7%). The presence of a relatively high proportion of AOH cases included in our study population influenced our genetic diagnostic rate (see paragraph 9.1.3.). In fact, the diagnostic yield of our custom NGS panel is different in classic *versus* milder phenotypes, reaching the highest percentage in CHH with additional congenital features.

Here we discuss in details the genetic findings in relationship with the clinical phenotypes.

6.1.1. Genetic diagnostic yield in the classic forms of CHH

A total of 12 patients showed classic forms of CHH, including 2 patients with isolated CHH,7 patients presenting with both reproductive and olfactory defect (KS), 2 patients affected by CHARGE syndrome and one case showing a complex undefined CHH phenotype consisting of nCHH, hypocortisolism and diabetes mellitus.

6.1.1.1. KS patients

Among 7 KS patients, 5 patients were found to be carriers of variants in *GNRHR, FGFR1, CHD7* and *PROK2* genes. According to the literature, the most frequently mutated genes inKS are usually *ANOS1, CHD7, FGFR1, PROK2, PROKR2,* and *SEMA3A* (Alkelai et al. 2017).However, in our current study, *ANOS1* defects have not been identified, neither point mutations nor copy number variants.

6.1.1.1.1. Patients with genetic diagnosis

- *FGFR1 mutation carriers*

The CHH 10 index case is a male patient showing absent puberty and hyposmia, with verylow hormone values but a bilateral testicular volume of 10 ml at the diagnosis. He was foundto be a heterozygous carrier of a *FGFR1* novel frameshift deletion (c.570delG; p.Trp190fs), which shifts the way the sequence is read, hence its pathogenic role. Although it is a novel variant, a stop gain variant at the same codon (p.Trp190X) in the extracellular domain has already been reported as *de novo* in one KS male patient with syndactyly and dental agenesis (Men et al., 2020), supporting the potential involvement of loss of function mutations at this amino acid residue (Trp^{190}) in the pathogenesis of the KS disease. The case described by Menand and colleagues (2020) showed a more severe phenotype than our CHH 10 proband, likely because he was found to carry an additional variant in the *IL17RD* gene, i.e. a digenic case.

The CHH 12 index case is a female patient showing primary amenorrhea and anosmia, withfamily history for impairment of pubertal development. In fact, both her mother and her maternal cousins presented with primary amenorrhea, one of which also showed

anosmia.The patient is a heterozygous carrier for a known pathogenic *FGFR1* variant (p.Arg620X), which was described in the literature as a cause of a wide spectrum of CHH phenotype, ranging from KS with cleft lip to nCHH (Dodè et al,. 2003; Pitteloud et al., 2006; Xu et al., 2007). The family segregation analysis of this variant showed that it was maternally inherited **(Figure 13c**), confirming its pathogenic role in the phenotypes of the maternal family members. As Xu and colleagues reported (2007), we have confirmed that this variantcan be responsible also for the isolated absent puberty observed in the mother, supporting the variable expressivity of this variant. We can also speculate that unrevealed variant(s) inadditional novel gene(s) may explain the more severe phenotype of the index case and her maternal cousin, who presenting anosmia besides primary amenorrhea.

The CHH 14 proband is a female patient showing primary amenorrhea, anosmia and emptysella, without a family history for CHH. She was a heterozygous carrier of a *FGFR1* stop gain variant (p.Glu140X), which has never been reported in literature. However, given boththe intrinsic pathogenic nature of the variant as loss of function and its *in silico* prediction asdamaging, we were able to consider it as the genetic cause of her clinical condition. No family members were available to perform the segregation studies.

Finally, the proband CHH 11 was a male patient affected by KS with cleft lip, who carried three variants in two different genes (*PROK2* and *CHD7*), i.e. an example of oligogenicity. In particular, he was compound heterozygous for two *PROK2* variants, i.e. one missense (p.p.Arg101Trp) and one splicing ones (c.286-1G>C), in addition to one heterozygous *CHD7* missense variant (p.Val553Leu). The missense *PROK2* variant has been already reported inone Maghrebian KS patient (Sarfati et al., 2013), whereas the other two identified variants were novel. It is worth noting that this patient did not respond to the gonadotropin therapy. This may be due to the impairment of the Prokineticin 2, suggesting the lack of a functional *PROK2* allele. In fact, by performing *in silico* mRNA expression analysis (https://www.proteinatlas.org/humanproteome/tissue), we can observe a PROK2 expression not only in the brain, but also in the testis, where it seems

to be involved in the physiological testicular angiogenic activity (Brouillette et al., 2014). In addition, an enrichment of these transcripts in seminiferous tubules has been detected, especially in primary spermatocytes. Thus, the presence of *PROK2* transcripts in spermatocytic cell, together with its angiogenic role in the testis, suggests a putative involvement of *PROK2* inthe proper functioning of spermatogenesis. To note that this patient presented another congenital features, such as cleft lip. Such an observation confirms the hypothesis that multiple gene defects could synergize to produce a more severe CHH phenotype (Dodè et al. 2006; Pitteloud et al., 2007).

6.1.1.1.2. Patient carrying a mutation without diagnostic value

- *GNRHR mutation carrier*

The proband CHH 6 is a female patient affected by primary amenorrhea and hyposmia, without family history for amenorrhea and reproductive defects. She was found to be a heterozygous carrier for the well-known pathogenic hot spot in the *GNRHR* gene (p.Gln106Arg), which was first described by de Roux and colleagues (1997). This mutation leads to a reduced GnRH-binding affinity and a decreased GnRH-stimulated signalling (deRoux et al., 1997; Leanos-Miranda et al., 2002; Cioppi and Riera-Escamilla et al., 2019), resulting in the normosmic form of CHH. Thus, this genotype cannot explain the olfactory phenotype of this patient. In addition, the presence of a *GNRHR* functional allele has a compensatory effect, avoiding the disease onset. Hence, we expect that this patient would be a carrier of additional pathogenic variant(s) in other not yet identified candidate genes, which would be the main responsible for her KS phenotype. The mild missense mutation in the GNRHR could be a modifier of the expressivity of the disease.

Overall, the genetic diagnostic yield of our custom NGS panel in the KS cohort is high, accounting for approximately 57% (n=4/7) of cases. For this reason, the genetic screening

ofcandidate CHH genes in selected cases, such as KS, is strongly recommended. The identification of the genetic cause in more than 50% of cases allows to optimize genetic counselling, especially when pregnancy is desired. In fact, in the majority of cases fertility can be induced by using hormonal therapies (Boehm et al., 2015) and there is a 50% chanceof transmitting the mutated allele to the offspring. This is especially relevant for those subjects carrying a mutation in a dominant gene, such as *FGFR1* and *CHD7*, since the change of having an affected child is 50%. However, even for mutations with a well-established Mendelian mode of inheritance, the prediction of transmission risk of disease features is complicated by phenotypic expression and variable disease severity that could be modifiedby different underlying genetic background. It should be noted that, a child who has inherited a mutation in a syndromic gene, such as *FGFR1* or *CHD7*, could manifest a more severe phenotype than the carrier parent. Overall, the complexity of this disease makes predicting the exact health consequences for the offspring difficult. However, PGT-M should be offered to couples, especially for *FGFR1* and *CHD7* mutation carriers, whose consequences in child could be very severe.

6.1.1.2. Overlapping syndromic cases

Among 3 patients in whom CHH occurs as part of a complex genetic syndrome, 2 patients were found to be carriers of variants in *CHD7* and *DMXL2*.

6.1.1.2.1. Patient with genetic diagnosis

- *CHD7 mutation carrier*

The male proband CHH 7 suffered from CHARGE syndrome, since he presented with absent puberty, opacity of the left lens, hearing loss and inner ear malformations. In

addition, he also showed anosmia. CHARGE syndrome is a genetic disorder due to dominant mutations of the *CHD7* gene in approximately 90% of cases, presenting highly variable phenotypes in carriers belonging to the same family (Janssen et al., 2012). Our proband was found to carry a novel *CHD7* heterozygous stop gain variant (p.Tyr2349X), which has proven to be *de novo* (**Figure 13a**). This variant was predicted to be pathogenic and the family segregation study confirmed the *in silico* results. As reported in literature, *CHD7* variants predicted to be pathogenic or likely pathogenic are usually found in patientswith CHARGE features or even undiagnosed CHARGE syndrome (Xu et al., 2018). In this case, CHARGE syndrome was clinically diagnosed, but appropriate genetic counselling based on the presence of the pathogenic *CHD7* variant is a challenge, as a high degree of variable expressivity is often observed in CHARGE-syndrome families (Lalani et al., 2006; Delahaye et al., 2007). The phenotypic differences in this disease could be due to the type ofvariant (missense *versus* loss of function) and the presence of genetic modifiers, such as mildmissense variants in other CHH genes, which could enhance the penetrance and expressivity of pathogenic *CHD7* variants.

6.1.1.2.2. Patient carrying a mutation without diagnostic value

- *DMXL2 mutation carrier*

The male proband CHH 9 was affected by partial anterior hypopituitarism, i.e. hypogonadism and hypocortisolism, and decompensated diabetes mellitus. His clinical diagnosis is still under definition. Our custom gene panel revealed the presence of a novel heterozygous missense variant in the *DMXL2* gene (p.Ala2382Gly), which lies within a highly conserved amino acid residue and was predicted as VUS. This gene was associated with syndromic CHH phenotype (Tata et al., 2014). In particular, a homozygous loss of function variant (p.1942_1946del) has been identified in three Senegalese brothers from a

consanguineous family, who showed a polyendocrine-polyneuropathy syndrome, with ataxia and dysarthria, characterized by mild CHH, central hypothyroidism, peripheral demyelinating neuropathy and diabetes mellitus (Tata et al. 2014). Interestingly, our patient comes from Nigeria. It has been demonstrated that heterozygous knockout mice showed delayed puberty, poor reproductive outcomes and abnormal glucose metabolism, as DMXL2 protein seems to be also involved in both constitutive and glucose-induced secretion of insulin by pancreatic beta cells (Tata et al., 2014). These findings in mice could explain the phenotype of our CHH 9 proband. However, we cannot consider the detected variant as diagnostic, since it was not predicted as pathogenic and family members were not available.

Overall, the genetic diagnostic yield of our custom NGS panel in more complex phenotypes is approximately 33% (n=1/3). Concerning CHARGE syndrome, the diagnostic rate based on *CHD7* testing is 50% (n=1/2), suggesting that the wild type *CHD7* patient could be a phenocopy of CHARGE syndrome.

6.1.2.Genetic diagnostic yield in the milder Adult-Onset forms of CHH

Among 14 AOH patients, 3 patients were found to be carriers of variants in *AXL*, *DMXL2* and *SEMA3A* genes.

6.1.2.1. Patient with genetic diagnosis

- *DMXL2 mutation carrier*

The proband CHH 2 was a female patient presenting with secondary amenorrhea. Genetic testing revealed the presence of a novel heterozygous missense variant in the

DMXL2 gene(p.Arg217fs), which is located in a highly conserved amino acid residue and it was predictedto be pathogenic. Given both its AD transmission and mild reproductive phenotypes associated with *Dmxl2* gene defects in mouse (Tata et al., 2014), we can state that this genotype is responsible for the AOH condition. To note, two dominant missense variants (p.Gln306His and p.Arg2416His) have been found to cosegregate with mild to severe non- syndromic hearing loss, suggesting the role of *DMXL2* in innear ear function (Chen et al., 2017; Wonkam-Tingang et al., 2021). However, the disease-causing *DMXL2* variant identified in the CHH 2 case (p.Arg217fs) was not associated with sensorineural hearing impairment, as the proband showed normal hearing. We could assume that different gene defects, i.e. frameshift *versus* missense variants, lead to different phenotypes, suggesting anincomplete penetrance and variable expressivity of this gene in the disease-onset.

6.1.2.2. Patients carrying mutations without diagnostic value

- *AXL mutation carrier*

The proband CHH 8 was a 36-years-old men, who reported normal pubertal development,starting by the age of 15 years, followed by the onset of hypogonadotropic hypogonadism at 21 years. He was referring to our clinic for AOH, severe oligoasthenoteratozoospermia and secondary hypothyroidism. The proband was found to be a heterozygous carrier of a novel *AXL* intronic variant 4 bp upstream from the start of exon 9 out of 20 (c.1135-4C>G), whose significance was predicted to be uncertain. The protein encoded by this gene is a member of the Tyro3-Axl-Mer (TAM) receptor tyrosine kinase subfamily, with a high expression profile in testis, which could explain the impairment of sperm production in thispatient. Prior studies showed that *Axl*/*Tyro3* null mice displayed developmental defects in GnRH neuron migration and survival (Pierce et al., 2008). Also in humans, rare heterozygous variants in this gene have been reported

in KS and nCHH patients, acting as dominant mutations (Salian-Mehta et al., 2014). Testing of the intronic *AXL* variant by the *in silico* tool Human Splicing Finder predicted no abnormal splicing. Thus, this variant alonecannot be sufficient to disrupt exon splicing.

- *SEMA3A mutation carrier*

The proband CHH 18 was a female AOH case, whose menarche occurred at the age of 14. After 3 years from pubertal development, she has experienced anorexia and stopped menstruating. Even when she gained weight during the anorexia recovery, menstrual cycles were absent. She was found to be a heterozygous carrier of a missense variant in the *SEMA3A* gene (p.Arg66Trp). This variant was previously identified in one KS male patient(Hanchate et al., 2012). Functional studies demonstrated that the variant results in the absence of the secreted protein (Hanchate et al., 2012). However, based on the seemingly normal reproductive phenotype of *Sema3a*$^{+/-}$ heterozygous mice (Schwarting et al., 2000; Cariboni et al., 2011), the authors suggest that monoallelic mutations in the *SEMA3A* gene are not sufficient to induce the KS phenotype (Hanchate et al., 2012). In support to this hypothesis, the father of our proband was found to share the genotype with the affected daughter, despite being healthy (**Figure 13b**). Neither the healthy father nor the affected daughter displayed KS. Therefore, the heterozygous *SEMA3A* variant (p.Arg66Trp) cannotbe considered as the genetic cause of CHH, but it could be influenced either by other mutantalleles or by environmental factors, such as anorexia, thus resulting in a milder CHH phenotype which characterizes the proband.

The only study focusing on AOH in the literature reports a frequency of rare variants in 39%of AOH cases (Cangiano et al., 2019). In our study, we found that 21.4% (n=3/14) of AOH patients carried rare variants in the analysed genes, but in only one case we were able to consider the variant as diagnostic. Overall, the genetic diagnostic yield of our custom NGSpanel in the AOH cohort is low, accounting for 7% (n=1/14) of cases. In fact, it has been recently reported that AOH subjects are frequently carriers of susceptibility

alleles with an impact on GnRH function that alone are not able to manifest as classic CHH (Cangiano et al., 2019). These genetic variants with a minor pathogenic impact may cumulate and interact with environmental factors to determine the phenotype, supporting a multifactorial origin of AOH (Cangiano et al., 2019). In this context, the identification of the genetic cause of disease in AOH cases is complex.

6.2 Rare endocrine tumours (Pheo/PGL/HNPGL/VHL)

Pheochromocytomas (Pheos) and paragangliomas are rare neural-crest derived tumours located in the adrenal medulla and extra-adrenal, respectively. Among extra-adrenal tumours, sympathetic paragangliomas (PGLs) arise from the paraganglia of the thorax, abdomen and pelvis, whereas the parasympathetic paragangliomas originate from the head and neck paraganglia, hence named as HNPGLs. Among all human neoplasia, Pheos/PGLs/HNPGLs show the highest frequency of hereditary forms of the disease, i.e. a single driver germline mutation is identified in at least 30% of cases (The NGS in PPGL Study Group et al., 2017). More than 15 autosomal dominant genes with age-dependent and incomplete penetrance have been implicated in the susceptibility to these tumours, which thus occur as genetically heterogeneous conditions (The NGS in PPGL Study Group et al., 2017). Among the 15 genes, *VHL* is involved in the aetiology of von Hippel-Lindau (VHL) syndrome. VHL is a monogenic disorder, with an autosomal dominant inheritance and a wide variability in penetrance and phenotype. From a clinical point of view, it is characterized by the development of tumours in several organs, most commonly hemangioblastoma of the central nervous system and retina. Other typical manifestations of VHL are renal cell carcinoma, endolymphatic sac tumors, islet-cell tumors of the pancreas and multiple pancreatic cysts, including pheochromocytoma (Maher and Kaelin, 1997). A wide variety of germline mutations has been identified, including exonic deletions (Franke et al., 2009), nonsense mutations and missense substitutions of the *VHL* gene (Dwyer and Tu, 2017).

Although all these diseases follow an autosomal mode of inheritance, it is important to underline that a single mutated allele of one of the susceptibility genes is not sufficient to develop the above-mentioned tumours. In fact, in case of a germline mutation, an additional somatic mutation in the same or in other susceptibility genes is needed to

determine the lossof heterozygosity or the dysregulation of multiple growth control genes leading to tumorigenesis. Hence, it is easily understandable that some individuals, who have inherited a germline mutation, may never develop cancer, because they never acquire the second hitwhich is necessary to start the process of tumour formation. This can explain why, when a certain germline mutation is present, cancer may appear to skip generations in a family.

In this study, a total of 103 affected patients were enrolled, including 57 Pheos, 9 PGLs, 29 HNPGLs and 8 suspected VHL cases. Among Pheo/PGL/HNPGL cohort (n=95), we observed a predominance of female to male ratio (1.5:1 in Pheos, 2:1 in PGLs and 3:1 in HNPGLs), in line with most of the previous studies (Lai et al., 2008; Audenet et al., 2013; Alesina and Walz, 2020). Similarly, in accordance with the literature, our suspected VHL cases (n=8) show a similar prevalence in males and females.

In order to determine the genetic diagnostic rate, 95 patients affected by Pheo/PGL/HNPGLand 8 individuals with suspected VHL syndrome were subjected to our custom NGS paneland to the *VHL* mutational screening, respectively. The custom NGS panel contains 15 susceptibility genes (*EGLN1, EPAS1, FH, KIF1B, MAX, MDH2, RET, SDHA, SDHAF2, SDHB, SDHC, SDHD, SLC25A11, TMEM127, VHL*).

In the entire cohort, we have identified a total of 20 variants predicted as VUSs/likely pathogenics/pathogenics, of which 11 were previously reported as disease-causing, whereas9 were novel. In line with the literature, the most commonly found variants mapped to the *SDHD* gene (35%), followed by *SDHB* and *VHL* gene defects, accounting for 20% and 15%, respectively. Finally, variants in the *EGLN1, KIF1B, SDHA, SDHAF2, SDHC* and *TMEM127*represent the 5% of the total identified variants.

Calculating the detection rate in our Pheo/PGL/HNPGL cohort, *SDHD* variants were detected most commonly, in a slightly higher average than that reported in the literature

(9.5% versus 5-7%) (Burnichon et al., 2011; Curras-Freixes et al., 2015). On the other hand, the detection rate of *SDHB* and *VHL* gene defects being 5.3% and 2%, respectively, is lowerthan expected, given that in other studies the frequency was of 8-10% and 7-10% concerning*SDHB* and *VHL* genes, respectively (Burnichon et al., 2011; Curras-Freixes et al., 2015). Similarly to the literature data, the other gene defects were found in approximately 1% of affected patients (The NGS in PPGL Study Group et al., 2017). Concerning our cohort with a clinical suspect for VHL, only 25% of patients carried a disease-causing variant in the *VHL*gene, hence received the diagnosis of VHL syndrome. This finding will be discussed in detail in paragraph 9.2.3.

Clinical characterization and genotype-phenotype correlations among Pheos, PGLs, HNPGLs and VHLs is reported below.

6.2.1. Pheos/PGLs/HNPGLs

In our cohort, the mean age of disease-onset is 57.4 years (±14.6), 50.4 years (±12.0) and 52.5years (±15.3) in Pheos, PGLs and HNPGLs, respectively. This is in accordance with the literature data reporting an incidence in sporadic cases between the fourth and the fifth decade of life (Guerrero et al., 2009).

Among the totality of patients affected by sympathetic tumours (Pheo/PGL), for whom clinical data was available (n=50), we observed that 42% (n=21/50) presented symptoms at clinical diagnosis, whereas 58% (n=29/50) of cases was detected as incidentaloma, similarlyto what has been previously reported (Haissaguerre et al., 2013). Concerning our symptomatic patients, 81% (n=17/21) showed hypertension, whilst 19% (n=4/21) manifestedsigns related to a mass effect. The increase in blood pressure is caused by the actions of tumour catecholamines on the adrenergic receptors (Canu et al., 2019). In fact,

all our patients showing hypertension were characterized by high urinary catecholamine levels. Among the totality of Pheo/PGL patients with available urinary catecholamine values (n=45), 91% (n=41/45) showed elevated concentrations indicating a secreting tumour. This figure is in line with what reported for the sympathetic tumours (Erickson et al., 2001).

Among HNPGL patients with available clinical data (n=22), we observed 36% (n=8/22) of them presenting with symptoms mainly due to a mass-related effect. However, 3/22 patientsshowed hypertension. It is worth noting that 2 of these hypertensive patients did not exhibited elevated urinary catecholamines, thus they presented a hypertension not differentfrom patients with essential hypertension, i.e. they were not affected by the characteristic symptomatic crises due to sudden release of catecholamines. Only one patient showed hypertension due to high urinary catecholamine levels, indicating a 4.5% frequency of secreting tumours among our HNPGL patients. Our data are in accordance with the literature, as, in contrast to Pheos/PGL, only about 5% of parasympathetic paragangliomashas been reported to release catecholamines (Erickson et al., 2001; Baysal and Myers, 2002).

Concerning the so called "malignant parameters" in Pheos/PGLs, i.e. the presence of multiple lesions and metastases at clinical diagnosis, 10% (n=5/50) and 12% (n=6/50) of cases were reported, respectively. According to the literature, multiple tumours in Pheo/PGL/HNPGL are more frequently identified in carriers of mutations in *SDHx*, *EPAS1*and *EGLN1* (see references in Martucci and Pacak, 2014). In our cohort, 1/5 patient with multiple lesions carried a *SDHB* variant (see below). Regarding the metastatic potential, it is well known that approximately 10% of Pheos and 35% of PGLs are metastatic at the diagnosis (Scholz et al., 2007; Baudin et al., 2014). Concerning Pheos, our findings confirm the literature data, as 13.6% (n=6/44) of patients presented with distal

metastases involvingbone, lymph nodes, liver, lung and colon. Concerning PGLs, none of the patients was foundto manifest metastases as at initial clinical presentation, and this is likely due to the low number of our PGL cases (n=5). However, we cannot exclude that these PGL patients may develop metastases in the future, given that distant spread can also occur many years later (Scholz et al., 2006, Baudin et al., 2014).

Concerning the HNPGL cohort, multiple lesions and metastases occurred in 27% (n=6/22) and 4.5% (n=1/22) of cases, respectively. Interestingly, almost all patients with multiple lesions (n=5/6) were carriers of a germline mutation in *SDHD* and *SDHAF2* genes, in line with what has been stated above (see reference in Martucci and Pacak, 2014). According to the literature, the occurrence of metastases in HNPGLs ranges from 2% to 19% based on primary tumour localization (Lee et al., 2002). In our cohort, the only patient with lung metastases was found to be wild type for germline variant in all 15 susceptibility genes. On the contrary, in the literature it is reported that metastases arise most frequently in regional lymph nodes (70%), followed by the spinal canal, lung, liver and skin (Papaspyrou et a., 2012), and they are most commonly related to *SDHB* variants, followed by those affecting the *SDHD* gene.

6.2.1.1. Genetic diagnostic yield in Pheos/PGLs/HNPGLs

Among Pheos, 14% of patients (n=8/57) were found to be carriers of pathogenic/likely pathogenic germline variants in a total of 7 genes (*EGLN1, KIF1B, SDHA, SDHB, SDHD, TMEM127* and *VHL*). Notably, one of these patients (n=1/8) carried two variants in two different genes, i.e. *SDHD* and *VHL*, consisting of a digenic mode of inheritance. Among PGLs, 33% of patients (n=3/9) were carriers of different disease-causing germline variants within the *SDHB* gene. Concerning HNPGLs, 31% of patients (n=9/29) carried germline

variants in the *SDHAF2*, *SDHC* and *SDHD* genes. 8/9 of these mutations are considered as pathogenic, leading to a diagnostic yield of 27.6% (n=8/29) in HNPGLs. In addition, one of these carriers has proved to be a digenic case, as both his *SDHC* and *VHL* genes were affected.

Therefore, by estimating the genetic diagnostic yield for the all cohort of Pheo/PGL/HNPGLwe obtain 20%, which is slightly lower than the one reported in the literature (about 30%) (The NGS in PPGL Study Group et al., 2017).

Here we report the genotype-phenotype correlations, according to the type of affected gene,ordered by their frequency.

-SDHD

Germline *SDHD* pathogenic mutations are frequently found (5-7%) in patients affected by neuroendocrine tumours (The NGS in PPGL Study Group et al., 2017). Similarly to *SDHC*, mutations in this gene were associated with a higher risk of HNPGL than Pheo/PGL (Neumann et al., 2004; Burnichon et al., 2009; Mannelli et al., 2009). In addition, a high penetrance for symptomatic tumours in *SDHD* mutation carriers has been reported (Andrews et al., 2018). Other tumours are very rarely associated with *SDHD* gene defects, almost exclusively renal cell carcinoma (Andrews et al., 2018). Malignant tumours defined by distal metastases vary between 0% to 22.7% in SDHD mutation carriers (van Hulsteijn etal., 2012). The estimated risk of HNPGL/Pheo/PGL at the age of 60 in non-index *SDHD* mutation carriers is 43.2% (Andrews et al., 2018). It is worth noting that, as for the *SDHAF2*gene, germline *SDHD* mutations show a "parent-of-origin" expression phenotype (imprinted gene), with tumour development occurring only when mutations are inherited via the paternal line (Van der Mey et al., 1989). However, in rare instances, *SDHD* mutations via the maternal line can also result in

tumorigenesis (Bayley et al., 2014).

In our cohort, we have identified a total of 9 patients carrying *SDHD* gene defects, with thehighest incidence in HNPGLs, as described in the literature. This gene resulted to be the most mutated: 6/9 HNPGLs and 3/8 Pheos. Among the 9 *SDHD* mutation carriers, only onepatient affected by pheochromocytoma also showed bone metastases at initial clinical presentation, accounting for 11% of metastatic *SDHD* mutation carriers.

Concerning the variants, a total of 7 variants have been identified, including stop gain (n=2),frameshift (n=2), missense (n=2) and one stop loss variant.

Among stop gain variants, a pathogenic founder mutation (p.Gln109X) has been detected in two female patients affected by HNPGL. One 33-yrs-old patient showed multiple HNPGLs without secreting catecholamines, whereas for the other 66-yrs-old patient no clinical data was available. Concerning the stop gain p.Tyr157X identified in a 67-yrs-old woman with Pheo, it is a novel variant predicted as pathogenic by the Varsome tool. To notethat in this patient the *SDHD* variant co-segregates with a known pathogenic *VHL* variant (p.Tyr175Cys), hence this patient represents a case of digenicity and it will be discussed in the specific paragraph.

Among frameshift variants, one (p.Asp92fs*21) was found to be novel, whereas the other one (p.Asp113Metfs*21) is known to be pathogenic. The former was identified in a 78-yrs- old man affected by a unilateral non-secreting pheochromocytoma, whereas the latter has been detected in a HNPGL woman aged 28 years. By performing variant segregation in thefamily, we can confirm its paternal origin. To note, both her 57-yrs-old father and her 24- yrs-old brother carrying the mutated allele showed the disease, suggesting a full penetrance of this variants in this family. However, it has been previously reported that relatives carrying this variant may not manifest the disease at the time of genetic diagnosis (Cascon et al., 2002; Lima et al., 2007).

Concerning the two missense variants, both were previously reported in patients with HNPGL/Pheo/PGL (Andrews et al., 2018). The founder mutation p.Pro81Leu has been almost exclusively associated with HNPGLs, in contrast with p.Thr112Ile which predisposes to both HNPGL and Pheo/PGL (Andrews et a., 2018). In our cohort, two unrelated patients (a 50-yrs-old man and a 61-yrs-old woman) carrying the p.Pro81Leu variant were found to be affected by multiple non-secreting HNPGLs. On the other hand, the p.Thr112Leu carrier was a 35-yrs-old man affected by a unilateral secreting pheocromocytoma.

Finally, a novel stop loss variant (p.Ter160Thrext*29) has been detected in a 34-yrs-old woman with multiple secreting HNPGLs. However, the variant significance is still unclear,as both Varsome and RENOVO tools have classified it as a variant of uncertain significance.Thus, we cannot state that this germline *SDHD* variant predisposes to the development of HNPGLs and, for this reason, we excluded this patient from the calculation of the genetic diagnostic yield.

-SDHB

Gerrmline *SDHB* pathogenic mutations are the most commonly found (8-10%) in patients affected by neuroendocrine tumours (The NGS in PPGL Study Group et al., 2017). In particular, mutations in this gene mainly predispose to extra-adrenal PGLs and to a lesser extent to adrenal Pheos (Neumann et al., 2004; Burnichon et al., 2009; Mannelli et al., 2009; Ricketts et al., 2010). Interestingly, male mutation carriers are at higher risk of disease than females (Jochmanova et al., 2017; Andrews et al., 2018), although the reason of this phenomenon is not clear. *SDHB* pathogenic variants also give rise to renal cell carcinoma, gastrointestinal stromal tumours and pituitary/ parathyroid adenomas (Andrews et al., 2018; Mannelli et al., 2018). Moreover, *SDHB* pathogenic variants have been associated with a high risk of metastases and poor prognosis (Amar et al., 2007; van Hulsteijn et al.,

2012). The more frequently detected mutations in this gene are missense, followed by frameshift indels, splicing and stop gain ones (Ben Aim et al., 2021). Clinical disease penetrance in non-proband *SDHB* mutation carriers is estimated to be 16%, 22% and 44% at the age of 50, 60 and 80 years, respectively (Andrews et al., 2018). Given the relatively high malignancy risk,a surveillance programme based on biochemical and radiological tests is mandatory for asymptomatic *SDHB* mutation carriers (Lenders et al., 2014).

In our cohort, we have identified a total of 5 patients carrying *SDHB* gene defects, includingthe totality of mutated PGLs (n=3/3), one case out of 8 Pheo mutated patients and one patientamong a total of 9 HNPGL mutated cases, in line with a higher risk of PGL observed in caseof *SDHB* mutations. In contrast with the literature data, none of the patients carrying *SDHB* variants presented with metastases at the clinical diagnosis. Given the high risk of disease recurrence, the possible emergence of other tumours (renal cell carcinoma and gastrointestinal stromal tumours) and the metastatic evolution, it is essential that our mutation carriers receive a strict follow-up.

Concerning the type of *SDHB* variants, a total of 4 variants have been identified, including splicing (n=2), frameshift (n=1) and missense (n=1) variants.

Among splicing mutations, we have observed the presence of a Dutch founder mutation (c.423+1G>A) in one PGL and one Pheo case, confirming the variable expressivity associatedwith this variant (Bayley et al., 2006; Ercolino et al., 2009; Erlic et al., 2009; Hes et al., 2010; Hensen et al., 2012; van Hulsteijn et al., 2014; Imamura et al., 2016, Niemeijer et al., 2017; Rijken et al., 2018). The 61-yrs-old PGL female patient was affected by secreting multiple lesions, thus also showing hypertension. She has transmitted the *SDHB* mutated allele to one of her sons, who manifested PGL by the age of 29 years, suggesting a complete penetrance of this *SDHB* splice site variant. The other mutation carrier was a 57-yrs-old man, who showed a unilateral non-secreting pheochromocytoma. By performing family segregation study, we could trace the mutation in 3 generations.

The variant was inherited from his healthy 88-yrs-old mother and then our index case transmitted it to one of his sons,who did not manifest the disease at the age of 30. This refutes the hypothesis of c.423+1G>A-related complete penetrance, further complicating its interpretation regarding its contribution to the disease-onset. However, as stated at the beginning of the discussion, allthese susceptibility genes show an incomplete and age-related penetrance, as also somatic mutations should be acquired to develop these types of tumours.

Also the other *SDHB* splicing variant (c.541-2A>G) identified in a PGL case has been previously reported in association with PGLs and Pheos (Timmers et al., 2007; Ricketts et al., 2012; Choat et al., 2014; Helman et al., 2016; Jochmanova et al., 2017; Huang et al., 2018).Additionally, an infant carrier was diagnosed with leukoencephalopaty without Pheo/PGL(Helman et al., 2016), and a 11-yrs-old boy was affected by polycythemia and abdominal PGL (Choat et al., 2014). Our patient showing an abdominal PGL has inherited the variant from her 59-yrs-old mother who did not yet manifest the disease.

Beside the splicing mutations, we identified another known pathogenic frameshift mutation(p.Ser198Alafs*22) in a 40-yrs-old woman with a secreting abdominal PGL. This variant hasbeen previously found in sporadic PGLs/Pheos with a malignant potential (Amar et al., 2005; Andrews et al., 2018).

Finally, a novel missense variant (p.Cys191Tyr) predicted as pathogenic was identified in a57-yrs-old man affected by Pheo. We have found that the variant has been transmitted to both of his sons aged 29 and 25 years. Interestingly, the older son has developed a HNPGL,suggesting a sort of genetic anticipation, in which the signs and symptoms of a certain genetic condition tend to become more severe and/or appear at an earlier age as the disorderis passed from one generation to the next. Based on what we observed in the older affectedbrother, the other 25-yrs-old healthy son carrying the mutation should undergo to a stricter follow-up.

- SDHA

Pathogenic germline *SDHA* variants account for approximately 12% of HNPGLs, 7.6% of PGLs and 2% of Pheos (Van der Tuin et al., 2018). As for all *SDH*-associated syndromes characterized by the development of PGLs, also *SDHA* gene variants confer an additional risk for developing other tumour types, i.e. clear cell renal cancer, gastrointestinal stromal tumours and, more rarely, neuroendocrine tumours and pituitary adenomas (Oudijk et al.,2013; Dwight et al., 2013; Yakirevich et al., 2015). Malignant Pheos and PGLs seem to feature across 12% of *SDHA* mutation carriers (Bausch et al., 2017). The age-related penetrance for *SDHA* mutation carriers is of 50% by the age of 70 years (Van der Tuin et al., 2018). However,the penetrance for *SDHA* mutation carriers was significantly lower in relatives compared with index patients at the age of 40 years: 13% (95% CI, 0%-33%) and 45% (95% CI, 23%-
60%), respectively. (Bausch et al., 2017).

In our cohort, a 79-yrs-old woman affected by a secreting unilateral pheochromocytoma wasfound to carry a novel *SDHA* missense variant (p.Ile319Leu), which was predicted as likelypathogenic after consulting RENOVO tool. To note, the patient did not present neither metastases nor any other tumours.

- SDHAF2

Germline *SDHAF2* mutations have been reported in <1% of patients affected by pheochromocytomas and paragangliomas (Bayley et al., 2010). Several founder mutations have been reported in this gene, including the most commonly found p.Gly78Arg in Dutchand southern European populations, which segregates with early onset of HNPGL familialcases (Bayley et al., 2010; Hensen et al., 2012). It is worth noting that mutations in this geneare inherited almost exclusively via the paternal line, since a

maternally imprinting mechanism beside the autosomal dominant inheritance has been demonstrated (Van der Mey et al., 1989).

In our cohort, we have identified the afore-mentioned founder mutation of the *SDHAF2* gene (p.Gly78Arg) in a 53-yrs-old woman with multiple HNPGLs and without metastases or other types of tumour, similarly to previously reported p.Gly78Arg carriers (Bausch e al., 2017). Unfortunately, no family members were available to confirm the paternal segregationof this variant.

- SDHC

Germline *SDHC* mutations have been reported in 1-2% of patients affected by HNPGLs, PGLs and Pheos (The NGS in PPGL Study Group et al., 2017). The phenotype associated with *SDHC* mutations has not been well defined. Early reports described benign HNPGLs (Niemann and Müller, 2000; Baysal et al., 2004; Schiavi et al., 2005), but more recent studies have also identified sympathetic PGLs and more invasive tumours (Bickmann et al., 2014; Bourdeau et al., 2016; Andrews et al., 2018). Nevertheless, we can state that mutations in *SDHC* are associated with a higher risk of HNPGLs than Pheos/PGLs (Andrews et al., 2018).Other rare tumours related to *SDHC* gene defects include pituitary adenoma and gastrointestinal stromal tumour (Andrews et al., 2018). The estimated risk of Pheo/PGL/HNPGL at the age of 60 years in non-index *SDHC* mutation carriers is 25% (Andrews et al., 2018).

In our cohort, we have identified a novel *SDHC* stop gain variant (p.Trp129X) in a HNPGLmale case, who was found to carry another stop gain variant in the *VHL* gene (p.Glu52X). This case will be discussed in the paragraph on digenic cases.

- EGNL1

Mutations in this gene have been implicated in the pathogenesis of polycythemia in humans(Ang et al., 2002; Percy et al., 2008; Prchal and Gordeuk, 2008). In 2008, Ladroue and colleagues first described a germline *EGLN1* mutation in a patient presenting with recurrentabdominal PGLs and polycythemia (Ladroue et al., 2008). In addition, a 60-year-old woman with a germline *EGLN1* variant and a diagnosis of polycythemia has developed multiple PGLs and a right adrenal Pheo (Yang et al, 2015). To note, beside polycythemia, germline *EGLN1* mutations seem to be associated with multiple or recurrent Pheos/PGLscharacterized by noradrenergic phenotypes (Yang et al., 2015).

In our cohort, a 16-yrs-old female patient affected by a secreting unilateral pheochromocytoma was found to carry a novel stop gain variant (p.Trp51X) in the *EGLN1* gene which was inherited from her healthy father. It is known that mutations in the Trp^{51} amino acid residue abrogates binding to the p23 peptide, which is a key co-chaperone for the HSP90 in order to promote HIF-A degradation due to hydroxylation (Arsenault et al., 2016). Interesting to note, this patient did not show clinical signs of polycythemia, althoughthe *EGLN1* gene is affected. A possible explanation is that *EGLN* mutation carriers generally present with borderline or mildly elevated serum erythropoietin levels and may develop polycythemia later in life (Yang et al., 2015). Thus, a late-onset polycythemia may still occurin our patient. Based on these observations, both patient and her 61-yrs-old father should undergo follow-up not only for Pheo/PGL but also for polycythemia.

- KIF1B

Mutations in this gene have been associated with the Charcot-Marie-Tooth disease, type

2A(Zhao et al., 2001), an autosomal dominant disease which is characterized by weakness and atrophy of distal muscles, depressed or absent deep tendon reflexes, and mild sensory loss(Shy et al. 1999). Beside this syndromic condition, *KIF1B* gene defects have been associated with the development of neural and non-neural tumours (Yeh et al., 2008). Germline variants in this gene have been reported in < 5% of patients affected by Pheo, while somaticvariants without germline *KIF1B* variants have been more frequently found in tumour tissues (Schlisio et al., 2008; De Filpo et al., 2020).

In out cohort, a 46-yrs old man affected by a left incidental Pheo was found to carry a novelmissense variant predicted as likely pathogenic (p.Pro1351Leu) in the *KIF1B* gene. The patient had no symptom at the first visit, although during the diagnostic workup high levels of urinary metanephrine and normetanephrine were found. To note, lymph node metastases have been identified in the patient at initial clinical presentation. By performingthe family segregation study, we observed that *KIF1B* variant was transmitted from his father, who did not manifest the disease. Both patient and his father will undergo a lifelongfollow-up in view of their genetic status, looking for Pheo recurrence/onset, metastatic disease development and the occurrence of other *KIF1B*-linked tumours.

-TMEM127

The first comprehensive series of *TMEM127* mutations revealed that these gene defects werealmost always associated with Pheos, with a frequency of 1-2% of cases (Yao et al., 2010; Curras-Freixes et al., 2015). Since than, other clinical features, such as paraganglioma and renal cell carcinoma, were detected in association with *TMEM27* variants, suggesting that TMEM127 dysfunction may lead to a wider clinical spectrum (Qin et al., 2014; Hernandez et al., 2015; Deng et al., 2017; Casey et al., 2017). According to a very recent publication, the majority of detected *TMEM127* variants leads to a truncating product, i.e. stop gain, splicingand frameshift indels (Armaiz-Pena et al., 2021).

Interestingly, patients with PGLs or renal cell carcinomas but without Pheo were more likely to have non-truncating variants than patients presenting with Pheo, suggesting that truncating *TMEM127* variants are a typical feature of Pheos (Armaiz-Pena et al., 2021). The penetrance of a driver mutation in this geneis approximately 30% at the age of 65 years (Toledo and Dahia, 2015).

In our cohort, a 53-yrs-old female patient affected by a pheochromocytoma was found to carry a known pathogenic frameshift variant (p.Ile41Argfs*39) in the *TMEM127* gene, confirming the observation that loss of function variants only result in the Pheo phenotype(Takeichi et al., 2012; Armaiz-Pena et al., 2021).

- Digenic cases carrying SDHx and VHL variants

In our cohort, two patients were revealed to be digenic cases, as carriers of two mutations in two different genes. To date, no case of digenic inheritance has been reported in pheochromocytoma/paraganglioma. Our first index case was a 33-yrs-old man affected by a unilateral non-secreting HNPGL. He was found to be a carrier both of a novel stop gain variant (p.Trp129X) in the *SDHC* gene inherited from his father and a known stop gain one(p.Glu52X) in the *VHL* gene inherited from his mother. Both parents were healthy. After identifying the *VHL* stop gain variant, the maternal family history for VHL-related phenotypes has been carefully evaluated leading to the exclusion of this variant from determining the VHL syndrome. In fact, HNPGL is not a typical VHL syndrome-related tumour, whereas germline *SDHC* mutations have been mainly associated with HNPGLs. Inaddition, although the *VHL* variant (p.Glu52X) has been identified in VHL cases, an alternative VHL gene product could arise from alternative translation initiation at a secondstart codon at the site of amino acid residue 54 within the *VHL* open reading frame (Blankenship et al., 1999). For all these reasons, the proband and his family

members carrying the *VHL* gene variant should not follow the standard early screening and close monitoring of VHL syndrome. Conversely, both proband and relatives carrying the germline *SDHC* variant, i.e. his father and his younger brother, will undergo a lifelong follow-up for recurrence/onset of paragangliomas.

The other index case was a 67-yrs-old female affected by a pheochromocytoma. She was found to carry a novel stop gain variant in the *SDHD* gene (p.Trp157X) and a known pathogenic missense one in the *VHL* gene (p.Tyr175Cys). It is well known that Pheo is a typical manifestation of VHL syndrome related to *VHL* missense variants. In addition, the identified *VHL* variant (p.Tyr175Cys) has been previously reported as disease-causing. Hence, the genotype-phenotype correlation of our index case indicates a type 2 VHL disease, instead of an isolated Pheo. Thus, thanks to the genetic screening, a strict clinical follow-up of VHL syndrome is mandatory.

6.2.2. Suspected Von Hippel-Lindau cases

The mean age of disease onset in our cohort with clinical VHL is 37 years (±28.6), which is higher than that one reported in the literature (approximately 26 years) (Maher et al., 1990).However, it has been observed that the onset age varies according to the type of affected organ(s), i.e. ≤ 30 years in case of central nervous system tumours and >30 years in case of renal cell carcinoma (RCC), pheochromocytoma (Pheo), pancreatic cystic lesion/pancreatic neuroendocrine tumours (PCL/PNET) (Zhou et al., 2019). In our cohort, clinical data was available for 6 patients. Among them, 4 were affected by abdominal tumours, such as RCC,Pheo, PCL/PNET, neuroendocrine tumour of the small intestine (SINET), whereas 2 presented with central nervous system tumours, i.e. retinal and cerebellum hemangioblastomas. Indeed, the latter patients were the youngest ones, aged 14 and 13.

Two patients manifested symptoms and multiple lesions. Symptoms differ according to theaffected organ(s), and in our study they were related to the mass effect, related to a small bowel neuroendocrine mass and to a pancreatic neoplasia. Multiple lesions affected the central nervous system (retina and cerebellum) and kidneys in a 14-yrs-old boy and in a 69-yrs-old man, respectively. Concerning metastases, 50% of cases (n=3/6) was affected: 1) a patient presenting with SINET had metastases but no information about the site(s) was available in the clinical record. According to the literature, most SINET patients already suffer from metastatic disease at the time of diagnosis, as our case (Weber and Dralle, 2019); 2) a patient affected by RCC and PCL showing bone and lymph nodes metastases. It is worthnoting that the most frequent malignant tumour in VHL disease is RCC, and a potential metastatic risk in presence of lesion diameter greater than 3 cm as in this case (10 cm) has been reported (Steinbach et al., 1994; Walther et al., 1999; Duffey et al., 2004): 3) a patient with pancreatic neoplasia showing liver metastases. PNET are known to harbour a malignant potential (Libutti et al., 1998) with two main prognostic factors, i.e. neoplasm size > 3 cm and tumour doubling time of less than 500 days (Blansfield et al., 2007; Corcos et al., 2008). Unfortunately, none of these clinical parameters was available for this patient. After a diagnosis of suspected VHL syndrome, only the detection of a germline mutation inthe *VHL* gene confirm the clinical suspicion, allowing to diagnose this syndromic condition and to carry out a close monitoring indicated in VHL cases.

6.2.2.1. Genetic diagnosis of VHL

A total of 2/8 patients were found to be carriers of germline variants in the *VHL* gene, accounting for 25% of cases with a clear diagnosis of VHL syndrome. It is interesting to notethat, according to a previous study, the detection rate of germline *VHL* mutations

ranges from 95-100% in suspected VHL cases characterized by multi-organ involvement to 24% insuspected VHL cases with single-organ involvement (Hes et al., 2007). Based on this observation, the reason we found only 25% of "true" affected cases is probably because our cohort included only half of patients with multi-organ tumours. Interestingly, both mutation carriers belonged to this group.

- VHL

The germline mutations of the *VHL* gene, as well as the disease phenotypes, are highly variable (Wong et al., 2007). According to the typical genotype–phenotype correlation, lossof function variants are responsible for the type 1 disease with a high risk of central nervoussystem hemangioblastoma and a low risk of Pheo, whereas missense mutations are responsible for the type 2 disease with a high risk of Pheo (Ong et al., 2007). However, a recent study reports that missense VHL mutations in the HIF-α binding site show a high risk both of retinal/central hemangioblastoma and pancreatic tumour, similarly to *VHL* truncating mutations (Liu et al., 2018). Thus, this novel genotype-phenotype correlation proposes to distinguish missense variants according to the involvement of the HIF-α binding site (HM mutations) and to consider both truncating and HM mutations as responsible for the type 1 VHL disease (Liu et al., 2018). Nevertheless, it is worth noting that, patients with truncating mutations are more likely to develop renal cell carcinoma than those with HM mutations (Liu et al., 2018).

In our cohort, a 14-yrs-old boy and a 28-yrs-old woman were found to be carriers of a *VHL* germline mutation. The affected boy presenting with multiple retinal and cerebellar capillary hemangioblastomas also showed facial dysmorphism, unilateral renal agenesis and hypospadias. He carried a known pathogenic stop gain variant (p.Arg113X) in the *VHL*,which has been previously associated with the early-onset of retinal lesions and multiple cerebellar and spinal hemangioblastomas (Murro et al., 2021). The affected

woman presented a unilateral pheochromyctoma and a pancreatic neuroendocrine neoplasia. She carried a known missense variant (p.Tyr175Cys), which was reported in patients affected by unilateral or bilateral Pheo (Ruiz-Llorente et al., 2004; Astapova et al., 2018). To note, thisvariant does not lie within the HIF-α binding site of the VHL protein. The genotype- phenotype correlation of our index cases confirm the previously reported classification, by which loss of function variants, i.e. the identified stop gain mutation (p.Arg113X), cause thetype 1 VHL disease, whereas missense variants in the non-HIF-α binding site of the VHL, i.e. the detected p.Tyr175Cys, are responsible for the type 2 VHL disease, characterized by the presence of Pheo.

7. Conclusions

Next-generation sequencing (NGS) methods have been rapidly adopted also in a diagnosticsetting. In fact, multi-gene panels based on NGS technology became valuable diagnostic tools for several clinical conditions, including Congenital Hpogonadotropic Hypogonadism (CHH) and rare endocrine tumours, such as Pheochromocytoma/Paraganglioma (Pheo/PGL) and von Hippel-Lindau syndrome (VHL).

Concerning CHH, the genetic diagnostic yield of our gene panel based on 34 candidate genes was 23%. By stratifying the study population according to the main CHH phenotypes, we have observed the highest diagnostic rate (42%) in classic forms of CHH (syndromic cases) and the lowest one (7%) in the milder form, i.e. Adult-Onset Hypogonadism (AOH).In this latter instance, a multifactorial origin seems to be the most plausible, given the detection of rare variants with a minor pathogenic impact which may interact with environmental factors. Thus, the targeted diagnostic CHH panel does not seem to be an appropriate tool for AOH. In our study, the most frequently mutated gene was *FGFR1*. An interesting finding is related to the *DMXL2* gene, since it is very rarely reported as the causeof CHH with a mild reproductive phenotype. Our unique case of AOH with a clear geneticdiagnosis carried a mutation in the *DMXL2* gene, contributing to clarify the role of this genein the aetiology of CHH. To note, in approximately 60% of classic CHH cases the genetic diagnosis remained unknown. We expect that ongoing large-scale exome sequencing in theframe of international networks will discover novel candidate genes which then can be included in the diagnostic panel. It is also plausible that epimutations in regulatory elementsof known candidate genes can be responsible for the

disease. However, methylation analysisin CHH genes has not been reported so far. The clinical impact of discovering novel disease-causing genes in this condition is especially relevant for diagnosing and genetic counselling,since the majority of these patients can generate their own biological child with the risk of transmission of the identified mutation(s).

Concerning Pheochromocytoma and Paraganglioma (Pheo/PGL/HNPGL), the application of our gene panel including 15 susceptibility genes, allowed us to identify the presence of agermline mutation in 20% of cases. Based on our study, PGLs show the highest hereditability among all neuroendocrine tumours, with more than 30% of patients carryinga pathogenic germline variant in one of the known susceptibility genes. Conversely, the genetic contribution for Pheos was revealed in only 14% of patients. The most frequently mutated gene is *SDHD*, followed by *SDHB*. Interestingly, mutated patients showed an earlier disease-onset and a higher presence of multiple lesions compared to the "wild type"patients. It is worth noting that, in the majority of cases, the molecular aetiology of sporadicPheo/PGL remains undefined, and exome sequencing in selected "wild type" patients witha positive family history could reveal new players involved in tumorigenic pathways. In this context, the identification of a germline mutation can lead to an early diagnosis, appropriate treatment and regular surveillance not only for the patient but also for their family members carrying the mutation.

Regarding the diagnosis of von Hippel-Lindau (VHL) syndrome in suspected cases, the genetic diagnostic rate was 25%. Interestingly, all affected patients showed a multi-organ involvement at the initial clinical presentation. It is worth noting that, after the identification of a germline pathogenic variant in the *VHL* gene, a careful active surveillance must be conducted aiming at early tumour detection and appropriate treatment. In our cohort of Pheos, one patient carrying a known pathogenic germline

mutation in the *VHL* in additionto a *SDHD* germline defect, has been subjected to the standard ophthalmologic follow-up, which revealed the presence of retinal capillary hemangioblastomas. Hence, thanks to the genetic screening, it was possible to diagnose the VHL syndrome, even in the presence of an isolated pheochromocytoma at the initial clinical presentation.

A major challenge in a clinical setting is represented by the attribution of a pathogenic roleto the detected variants, especially if they are classified as VUSs. In our study, we have identified a total of 4 variants of unknown significance and future *in vitro* studies will be carried out in order to demonstrate their potential pathogenic role.

In conclusion, this study aimed to report the genetic diagnostic yield of selected rare endocrine diseases based on our 7-years experience in targeted NGS gene panels. We confirmed that genetic testing is a powerful diagnostic tool with relevant implications in genetic counselling and clinical management. Concerning CHH, given the high probabilityof fertility induction by hormonal therapy, diagnosing the genetic defect also allows to carry out preventive measures, such as Preimplantation Genetic Test for monogenic diseases (PGT-M) in syndromic cases. Concerning rare endocrine tumours, the molecular genetic results are relevant for correct and early diagnosis leading to a better prognosis, and for appropriate treatment of the patient and the other family members through an active surveillance.

8. Bibliography

Alesina PF, Walz MK. Adrenal Tumors: Are Gender Aspects Relevant? Visc Med. 2020 Feb;36(1):15-19.

Amar L, Bertherat J, Baudin E, Ajzenberg C, Bressac-de Paillerets B, Chabre O, Chamontin B, Delemer B, Giraud S, Murat A, et al. Genetic testing in pheochromocytoma or functional paraganglioma. J Clin Oncol. 2005 Dec 1;23(34):8812-8.

Amar L, Baudin E, Burnichon N, Peyrard S, Silvera S, Bertherat J, Bertagna X, Schlumberger M, Jeunemaitre X, Gimenez-Roqueplo AP, et al. Succinate dehydrogenase B gene mutations predict survival in patients with malignant pheochromocytomas or paragangliomas. J Clin Endocrinol Metab. 2007 Oct;92(10):3822-8.

Amato LGL, Montenegro LR, Lerario AM, Jorge AAL, Guerra Junior G, Schnoll C, Renck AC, Trarbach EB, Costa EMF, Mendonca BB, et al. New genetic findings in a large cohort of congenital hypogonadotropic hypogonadism. Eur J Endocrinol. 2019 Aug 1;181(2):103-119.

Andrews KA, Ascher DB, Pires DEV, Barnes DR, Vialard L, Casey RT, Bradshaw N, Adlard J, Aylwin S, Brennan P, et al. Tumour risks and genotype-phenotype correlations associated with germline variants in succinate dehydrogenase subunit genes SDHB, SDHC and SDHD. J Med Genet. 2018 Jun;55(6):384-394.

Ang SO, Chen H, Hirota K, Gordeuk VR, Jelinek J, Guan Y, Liu E, Sergueeva AI, Miasnikova GY, Mole D, et al. Disruption of oxygen homeostasis underlies congenital Chuvash polycythemia. Nat Genet. 2002 Dec;32(4):614-21.

Aoyama K, Mizuno H, Tanaka T, Togawa T, Negishi Y, Ohashi K, Hori I, Izawa M, Hamajima T, Saitoh S. Molecular genetic and clinical delineation of 22 patients with congenital hypogonadotropic hypogonadism. J Pediatr Endocrinol Metab. 2017 Oct 26;30(10):1111-1118.

Armaiz-Pena G, Flores SK, Cheng ZM, Zhang X, Esquivel E, Poullard N, Vaidyanathan A, Liu Q, Michalek J, Santillan-Gomez AA, et al. Genotype-Phenotype Features of Germline Variants of the TMEM127 Pheochromocytoma Susceptibility Gene: A 10-Year Update. J Clin Endocrinol Metab. 2021 Jan 1;106(1):e350-e364.

Arsenault PR, Song D, Bergkamp M, Ravaschiere AM, Navalsky BE, Lieberman PM, Lee FS. Identification of Small-Molecule PHD2 Zinc Finger Inhibitors that Activate Hypoxia Inducible Factor. Chembiochem. 2016 Dec 14;17(24):2316-2323.

Asa SL, Ezzat S, Mete O. The Diagnosis and Clinical Significance of Paragangliomas in Unusual Locations. J Clin Med. 2018 Sep 13;7(9):280.

Astapova O, Biswas A, DiMauro A, Moalem J, Hammes SR. Bilateral Pheochromocytomas in a Patient with Y175C Von Hippel-Lindau Mutation. Case Rep Endocrinol. 2018 Jul 10; 2018: 8967159.

Audenet F, Méjean A, Chartier-Kastler E, Rouprêt M. Adrenal tumours are more predominant in females regardless of their histological subtype: a review. World J Urol. 2013 Oct;31(5):1037-43.

Baudin E, Habra MA, Deschamps F, Cote G, Dumont F, Cabanillas M, Arfi-Roufe J, Berdelou A, Moon B, Al Ghuzlan A, et al. Therapy of endocrine disease: treatment of malignant pheochromocytoma and paraganglioma. Eur J Endocrinol. 2014 Sep;171(3):R111-22.

Bausch B, Borozdin W, Mautner VF, Hoffmann MM, Boehm D, Robledo M, Cascon A, Harenberg T, Schiavi F, Pawlu C, et al. Germline NF1 mutational spectra and loss-of-heterozygosity analyses in patients with pheochromocytoma and neurofibromatosis type 1. J Clin Endocrinol Metab. 2007 Jul;92(7):2784-92.

Bausch B, Schiavi F, Ni Y, Welander J, Patocs A, Ngeow J, Wellner U, Malinoc A, Taschin E, BarbonG, et al. Clinical Characterization of the Pheochromocytoma and Paraganglioma Susceptibility Genes SDHA, TMEM127, MAX, and SDHAF2 for Gene-Informed Prevention. JAMA Oncol. 2017 Sep 1;3(9):1204-1212.

Bayley JP, van Minderhout I, Weiss MM, Jansen JC, Oomen PH, Menko FH, Pasini B, Ferrando B, Wong N, Alpert LC, et al. Mutation analysis of SDHB and SDHC: novel germline mutations in sporadic head and neck paraganglioma and familial paraganglioma and/or pheochromocytoma. BMC Med Genet. 2006 Jan 11;7:1.

Bayley JP, Kunst HP, Cascon A, Sampietro ML, Gaal J, Korpershoek E, Hinojar-Gutierrez A, Timmers HJ, Hoefsloot LH, Hermsen MA, et al. SDHAF2 mutations in familial and sporadic paraganglioma and phaeochromocytoma. Lancet Oncol. 2010 Apr;11(4):366-72.

Bayley JP, Oldenburg RA, Nuk J, Hoekstra AS, van der Meer CA, Korpershoek E, McGillivray B, Corssmit EP, Dinjens WN, de Krijger RR, et al. Paraganglioma and pheochromocytoma upon maternal transmission of SDHD mutations. BMC Med Genet. 2014 Oct 10;15:111.

Baysal BE, Myers EN. Etiopathogenesis and clinical presentation of carotid body tumors. Microsc Res Tech. 2002 Nov 1;59(3):256-61.

Baysal BE, Willett-Brozick JE, Filho PA, Lawrence EC, Myers EN, Ferrell RE. An Alu-mediated partial SDHC deletion causes familial and sporadic paraganglioma. J Med Genet. 2004 Sep;41(9):703-9.

Bender BU, Eng C, Olschewski M, Berger DP, Laubenberger J, Altehöfer C, Kirste G, Orszagh M, van Velthoven V, Miosczka H, Schmidt D, Neumann HP. VHL c.505 T>C mutation confers a high age related penetrance but no increased overall mortality. J Med Genet. 2001 Aug;38(8):508-14.

Ben Aim L, Maher ER, Cascon A, Barlier A, Giraud S, Ercolino T, Pigny P, Clifton-Bligh RJ, Mirebeau-Prunier D, Mohamed A, et al. International initiative for a curated SDHB variant database

improving the diagnosis of hereditary paraganglioma and pheochromocytoma. J Med Genet. 2021 Aug 27: jmedgenet-2020-107652.

Benn DE, Zhu Y, Andrews KA, Wilding M, Duncan EL, Dwight T, Tothill RW, Burgess J, Crook A,Gill AJ, et al. Bayesian approach to determining penetrance of pathogenic SDH variants. J Med Genet. 2018 Nov;55(11):729-734.

Berends AMA, Buitenwerf E, de Krijger RR, Veeger NJGM, van der Horst-Schrivers ANA, Links TP, Kerstens MN. Incidence of pheochromocytoma and sympathetic paraganglioma in the Netherlands:A nationwide study and systematic review. Eur J Intern Med. 2018 May; 51:68-73.

Bianco SD, Kaiser UB. The genetic and molecular basis of idiopathic hypogonadotropic hypogonadism. Nat Rev Endocrinol. 2009 Oct;5(10):569-76.

Bickmann JK, Sollfrank S, Schad A, Musholt TJ, Springer E, Miederer M, Bartsch O, Papaspyrou K, Koutsimpelas D, Mann WJ, et al. Phenotypic variability and risk of malignancy in SDHC-linked paragangliomas: lessons from three unrelated cases with an identical germline mutation (p.Arg133*). J Clin Endocrinol Metab. 2014 Mar;99(3):E489-96.

Blansfield JA, Choyke L, Morita SY, Choyke PL, Pingpank JF, Alexander HR, Seidel G, Shutack Y, Yuldasheva N, Eugeni M, et al. Clinical, genetic and radiographic analysis of 108 patients with von Hippel-Lindau disease (VHL) manifested by pancreatic neuroendocrine neoplasms (PNETs). Surgery. 2007 Dec;142(6):814-8.

Boedeker CC. Paragangliomas and paraganglioma syndromes. GMS Curr Top Otorhinolaryngol Head Neck Surg. 2011;10: Doc03.

Boehm U, Bouloux PM, Dattani MT, de Roux N, Dodé C, Dunkel L, Dwyer AA, Giacobini P, Hardelin JP, Juul A, et al. Expert consensus document: European Consensus Statement on congenital hypogonadotropic hypogonadism--pathogenesis, diagnosis and treatment. Nat Rev Endocrinol. 2015 Sep;11(9):547-64.

Bonomi M, Vezzoli V, Krausz C, Guizzardi F, Vezzani S, Simoni M, Bassi I, Duminuco P, Di Iorgi N, Giavoli C, et al. Characteristics of a nationwide cohort of patients presenting with isolated hypogonadotropic hypogonadism (IHH). Eur J Endocrinol. 2018 Jan;178(1):23-32.

Bourdeau I, Grunenwald S, Burnichon N, Khalifa E, Dumas N, Binet MC, Nolet S, Gimenez- Roqueplo AP. A SDHC Founder Mutation Causes Paragangliomas (PGLs) in the French Canadians:New Insights on the SDHC-Related PGL. J Clin Endocrinol Metab. 2016 Dec;101(12):4710-4718.

Brouillet S, Hoffmann P, Alfaidy N, Feige JJ. Prokinéticines - De nouveaux peptides régulateurs dela reproduction humaine [Prokineticins: new regulatory peptides in human reproduction]. Med Sci (Paris). 2014 Mar;30(3):274-9.

Burnichon N, Rohmer V, Amar L, Herman P, Leboulleux S, Darrouzet V, Niccoli P, Gaillard D, Chabrier G, Chabolle F, et al. The succinate dehydrogenase genetic testing in a large prospective series of patients with paragangliomas. J Clin Endocrinol Metab. 2009 Aug;94(8):2817-27.

Burnichon N, Vescovo L, Amar L, Libé R, de Reynies A, Venisse A, Jouanno E, Laurendeau I, ParfaitB, Bertherat J, et al. Integrative genomic analysis reveals somatic mutations in pheochromocytoma and paraganglioma. Hum Mol Genet. 2011 Oct 15;20(20):3974-85.

Butz H, Nyírő G, Kurucz PA, Likó I, Patócs A. Molecular genetic diagnostics of hypogonadotropic hypogonadism: from panel design towards result interpretation in clinical practice. Hum Genet. 2021 Jan;140(1):113-134.

Cangiano B, Duminuco P, Vezzoli V, Guizzardi F, Chiodini I, Corona G, Maggi M, Persani L, Bonomi M. Evidence for a Common Genetic Origin of Classic and Milder Adult-Onset Forms of Isolated Hypogonadotropic Hypogonadism. J Clin Med. 2019 Jan 21;8(1):126.

Cangiano B, Swee DS, Quinton R, Bonomi M. Genetics of congenital hypogonadotropic hypogonadism: peculiarities and phenotype of an oligogenic disease. Hum Genet. 2021 Jan;140(1):77-111.

Canu L, Parenti G, De Filpo G, Mannelli M. Pheochromocytomas and Paragangliomas as Causes of Endocrine Hypertension. Front Endocrinol (Lausanne). 2019 Jun 4;10:333.

Cariboni A, Davidson K, Rakic S, Maggi R, Parnavelas JG, Ruhrberg C. Defective gonadotropin-releasing hormone neuron migration in mice lacking SEMA3A signalling through NRP1 and NRP2: implications for the aetiology of hypogonadotropic hypogonadism. Hum Mol Genet. 2011 Jan 15;20(2):336-44.

Casey RT, Warren AY, Martin JE, Challis BG, Rattenberry E, Whitworth J, Andrews KA, Roberts T, Clark GR, West H, et al. Clinical and Molecular Features of Renal and Pheochromocytoma/Paraganglioma Tumor Association Syndrome (RAPTAS): Case Series and Literature Review. J Clin Endocrinol Metab. 2017 Nov 1;102(11):4013-4022.

Cascon A, Ruiz-Llorente S, Cebrian A, Telleria D, Rivero JC, Diez JJ, Lopez-Ibarra PJ, Jaunsolo MA, Benitez J, Robledo M. Identification of novel SDHD mutations in patients with phaeochromocytoma and/or paraganglioma. Eur J Hum Genet. 2002 Aug;10(8):457-61.

Cassatella D, Howard SR, Acierno JS, Xu C, Papadakis GE, Santoni FA, Dwyer AA, Santini S, Sykiotis GP, Chambion C, et al. Congenital hypogonadotropic hypogonadism and constitutional delay of growth and puberty have distinct genetic architectures. Eur J Endocrinol. 2018 Apr;178(4):377-388.

Castinetti F, Qi XP, Walz MK, Maia AL, Sansó G, Peczkowska M, Hasse-Lazar K, Links TP, Dvorakova S, Toledo RA, et al. Outcomes of adrenal-sparing surgery or total adrenalectomy in phaeochromocytoma associated with multiple endocrine neoplasia type 2: an international retrospective population-based study. Lancet Oncol. 2014 May;15(6):648-55.

Chen F, Kishida T, Yao M, Hustad T, Glavac D, Dean M, Gnarra JR, Orcutt ML, Duh FM, Glenn G, et al. Germline mutations in the von Hippel-Lindau disease tumor suppressor gene: correlations with phenotype. Hum Mutat. 1995;5(1):66-75.

Chen H, Sippel RS, O'Dorisio MS, Vinik AI, Lloyd RV, Pacak K; North American Neuroendocrine Tumor Society (NANETS). The North American Neuroendocrine Tumor Society consensus guideline for the diagnosis and management of neuroendocrine tumors: pheochromocytoma, paraganglioma, and medullary thyroid cancer. Pancreas. 2010 Aug;39(6):775-83.

Chen DY, Liu XF, Lin XJ, Zhang D, Chai YC, Yu DH, Sun CL, Wang XL, Zhu WD, Chen Y, et al. A dominant variant in DMXL2 is linked to nonsyndromic hearing loss. Genet Med. 2017 May;19(5):553-558.

Choat H, Derrevere K, Knight L, Brown W, Mack EH. SDHB-Associated Paraganglioma in a Pediatric Patient and Literature Review on Hereditary Pheochromocytoma-Paraganglioma Syndromes. Case Rep Endocrinol. 2014: 502734.

Chong JX, Buckingham KJ, Jhangiani SN, Boehm C, Sobreira N, Smith JD, Harrell TM, McMillin MJ, Wiszniewski W, Gambin T, et al. The Genetic Basis of Mendelian Phenotypes: Discoveries, Challenges, and Opportunities. Am J Hum Genet. 2015 Aug 6;97(2):199-215.

Cioppi F, Riera-Escamilla A, Manilall A, Guarducci E, Todisco T, Corona G, Colombo F, Bonomi M, Flanagan CA, Krausz C. Genetics of ncHH: from a peculiar inheritance of a novel GNRHR mutationto a comprehensive review of the literature. Andrology. 2019 Jan;7(1):88-101.

Cioppi F, Rosta V, Krausz C. Genetics of Azoospermia. Int J Mol Sci. 2021 Mar 23;22(6):3264.

Corcos O, Couvelard A, Giraud S, Vullierme MP, Dermot O'Toole, Rebours V, Stievenart JL, Penfornis A, Niccoli-Sire P, Baudin E, et al. Endocrine pancreatic tumors in von Hippel-Lindau disease: clinical, histological, and genetic features. Pancreas. 2008 Jul;37(1):85-93.

Currás-Freixes M, Inglada-Pérez L, Mancikova V, Montero-Conde C, Letón R, Comino-Méndez I, Apellániz-Ruiz M, Sánchez-Barroso L, Aguirre Sánchez-Covisa M, Alcázar V, et al. Recommendations for somatic and germline genetic testing of single pheochromocytoma and paraganglioma based on findings from a series of 329 patients. J Med Genet. 2015 Oct;52(10):647-56.

De Filpo G, Contini E, Serio V, Valeri A, Chetta M, Guasti D, Bani D, Mannelli M, Rapizzi E, LuconiM, et al. Germline Mutation in KIF1Bβ Gene Associated with Loss of Heterozygosity: Usefulness ofNext-Generation Sequencing in the Genetic Screening of Patients with Pheochromocytoma. Int J Endocrinol. 2020 May 30; 2020: 3671396.

de Roux N, Young J, Misrahi M, Genet R, Chanson P, Schaison G, Milgrom E. A family with hypogonadotropic hypogonadism and mutations in the gonadotropin-releasing hormone receptor.N Engl J Med. 1997 Nov 27;337(22):1597-602.

Delahaye A, Sznajer Y, Lyonnet S, Elmaleh-Bergès M, Delpierre I, Audollent S, Wiener-Vacher S, Mansbach AL, Amiel J, Baumann C, et al. Familial CHARGE syndrome because of CHD7 mutation: clinical intra- and interfamilial variability. Clin Genet. 2007 Aug;72(2):112-21.

Deng Y, Flores SK, Cheng Z, Qin Y, Schwartz RC, Malchoff C, Dahia PLM. Molecular and phenotypic evaluation of a novel germline TMEM127 mutation with an uncommon clinical presentation. Endocr Relat Cancer. 2017 Nov;24(11): L79-L82.

Dodé C, Levilliers J, Dupont JM, De Paepe A, Le Dû N, Soussi-Yanicostas N, Coimbra RS, Delmaghani S, Compain-Nouaille S, Baverel F, et al. Loss-of-function mutations in FGFR1 cause autosomal dominant Kallmann syndrome. Nat Genet. 2003 Apr;33(4):463-5.

Dodé C, Teixeira L, Levilliers J, Fouveaut C, Bouchard P, Kottler ML, Lespinasse J, Lienhardt- Roussie A, Mathieu M, Moerman A, et al. Kallmann syndrome: mutations in the genes encoding prokineticin-2 and prokineticin receptor-2. PLoS Genet. 2006 Oct 20;2(10):e175.

Duffey BG, Choyke PL, Glenn G, Grubb RL, Venzon D, Linehan WM, Walther MM. The relationship between renal tumor size and metastases in patients with von Hippel-Lindau disease. J Urol. 2004 Jul;172(1):63-5.

Dwight T, Benn DE, Clarkson A, Vilain R, Lipton L, Robinson BG, Clifton-Bligh RJ, Gill AJ. Loss of SDHA expression identifies SDHA mutations in succinate dehydrogenase-deficient gastrointestinal stromal tumors. Am J Surg Pathol. 2013 Feb;37(2):226-33.

Dwyer AA, Raivio T, Pitteloud N. MANAGEMENT OF ENDOCRINE DISEASE: Reversible hypogonadotropic hypogonadism. Eur J Endocrinol. 2016 Jun;174(6):R267-74.

Dwyer DC, Tu RK. Genetics of Von Hippel-Lindau Disease. AJNR Am J Neuroradiol. 2017 Mar;38(3):469-470.

Eggermann T, Elbracht M, Kurth I, Juul A, Johannsen TH, Netchine I, Mastorakos G, Johannsson G, Musholt TJ, Zenker M, et al. Genetic testing in inherited endocrine disorders: joint position paper ofthe European reference network on rare endocrine conditions (Endo-ERN). Orphanet J Rare Dis. 2020 Jun 8;15(1):144.

Eisenhofer G, Walther MM, Huynh TT, Li ST, Bornstein SR, Vortmeyer A, Mannelli M, Goldstein DS, Linehan WM, Lenders JW, et al. Pheochromocytomas in von Hippel-Lindau syndrome and multiple endocrine neoplasia type 2 display distinct biochemical and clinical phenotypes. J Clin Endocrinol Metab. 2001 May;86(5):1999-2008.

Eisenhofer G, Rivers G, Rosas AL, Quezado Z, Manger WM, Pacak K. Adverse drug reactions in patients with phaeochromocytoma: incidence, prevention and management. Drug Saf. 2007;30(11):1031-62.

Ercolino T, Taurino C, Sestini R, Bacca AV, Genuardi M, Mannelli M, Bernini GP. Malignant extra-adrenal pheochromocytoma caused by an SDHB intronic variation leading to a 54-bp deletion in exon 4. J Endocrinol Invest. 2009 Feb;32(2):111-4.

Erickson D, Kudva YC, Ebersold MJ, Thompson GB, Grant CS, van Heerden JA, Young WF Jr. Benign paragangliomas: clinical presentation and treatment outcomes in 236 patients. J Clin Endocrinol Metab. 2001 Nov;86(11):5210-6.

Erlic Z, Rybicki L, Peczkowska M, Golcher H, Kann PH, Brauckhoff M, Müssig K, Muresan M, Schäffler A, Reisch N, et al. Clinical predictors and algorithm for the genetic diagnosis of pheochromocytoma patients. Clin Cancer Res. 2009 Oct 15;15(20):6378-85.

Esden-Tempska Z, Lewczuk A, Tobias ES, Borozdin W, Kohlhase J, Sworczak K. Delayed diagnosisof adrenal hypoplasia congenita in a patient with a new mutation in the NR0B1 gene. J Pediatr Endocrinol Metab. 2012;25(1-2):147-8.

Evain-Brion D, Gendrel D, Bozzola M, Chaussain JL, Job JC. Diagnosis of Kallmann's syndrome in early infancy. Acta Paediatr Scand. 1982 Nov;71(6):937-40.

Forni PE, Taylor-Burds C, Melvin VS, Williams T, Wray S. Neural crest and ectodermal cells intermixin the nasal placode to give rise to GnRH-1 neurons, sensory neurons, and olfactory ensheathing cells. J Neurosci. 2011 May 4;31(18):6915-27.

Forni PE, Wray S. GnRH, anosmia and hypogonadotropic hypogonadism--where are we? Front Neuroendocrinol. 2015 Jan;36:165-77.

Franke G, Bausch B, Hoffmann MM, Cybulla M, Wilhelm C, Kohlhase J, Scherer G, Neumann HP. Alu-Alu recombination underlies the vast majority of large VHL germline deletions: Molecular characterization and genotype-phenotype correlations in VHL patients. Hum Mutat. 2009 May;30(5):776-86.

Gruber LM, Erickson D, Babovic-Vuksanovic D, Thompson GB, Young WF Jr, Bancos I. Pheochromocytoma and paraganglioma in patients with neurofibromatosis type 1. Clin Endocrinol (Oxf). 2017 Jan;86(1):141-149.

Grumbach MM. A window of opportunity: the diagnosis of gonadotropin deficiency in the male infant. J Clin Endocrinol Metab. 2005 May;90(5):3122-7.

Guerrero MA, Schreinemakers JM, Vriens MR, Suh I, Hwang J, Shen WT, Gosnell J, Clark OH, DuhQY. Clinical spectrum of pheochromocytoma. J Am Coll Surg. 2009 Dec;209(6):727-32.

Gutmann DH. Molecular insights into neurofibromatosis 2. Neurobiol Dis. 1997;3(4):247-61.

Haissaguerre M, Courel M, Caron P, Denost S, Dubessy C, Gosse P, Appavoupoulle V, Belleannée G, Jullié ML, Montero-Hadjadje M, et al. Normotensive incidentally discovered pheochromocytomas display specific biochemical, cellular, and molecular characteristics. J Clin Endocrinol Metab. 2013 Nov;98(11):4346-54.

Hall BJ. "Autocharge"--closing the wound of lost surgical charges. Hosp Financ Manage. 1979 Aug;33(8):58-9.

Hammel PR, Vilgrain V, Terris B, Penfornis A, Sauvanet A, Correas JM, Chauveau D, Balian A, Beigelman C, O'Toole D, et al. Pancreatic involvement in von Hippel-Lindau disease. The Groupe Francophone d'Etude de la Maladie de von Hippel-Lindau. Gastroenterology. 2000 Oct;119(4):1087-95.

Hanchate NK, Giacobini P, Lhuillier P, Parkash J, Espy C, Fouveaut C, Leroy C, Baron S, CampagneC, Vanacker C, et al. SEMA3A, a gene involved in axonal pathfinding, is mutated in patients with Kallmann syndrome. PLoS Genet. 2012 Aug;8(8):e1002896.

Hayashi, T.; Mete, O. Head and neck paragangliomas: What does the pathologist need to know? Diagn. Histopathol. 2014, 20, 316–325.

Heldwein FL, Escudier B, Smyth G, Souto CA, Vallancien G. Metastatic renal cell carcinoma management. Int Braz J Urol. 2009 May-Jun;35(3):256-70.

Helman G, Caldovic L, Whitehead MT, Simons C, Brockmann K, Edvardson S, Bai R, Moroni I, Taylor JM, Van Haren K, et al. Magnetic resonance imaging spectrum of succinate dehydrogenase- related infantile leukoencephalopathy. Ann Neurol. 2016 Mar;79(3):379-86.

Hensen EF, van Duinen N, Jansen JC, Corssmit EP, Tops CM, Romijn JA, Vriends AH, van der Mey AG, Cornelisse CJ, Devilee P, Bayley JP. High prevalence of founder mutations of the succinate dehydrogenase genes in the Netherlands. Clin Genet. 2012 Mar;81(3):284-8.

Hernandez KG, Ezzat S, Morel CF, Swallow C, Otremba M, Dickson BC, Asa SL, Mete O. Familial pheochromocytoma and renal cell carcinoma syndrome: TMEM127 as a novel candidate gene for the association. Virchows Arch. 2015 Jun;466(6):727-32.

Hero M, Laitinen EM, Varimo T, Vaaralahti K, Tommiska J, Raivio T. Childhood growth of females with Kallmann syndrome and FGFR1 mutations. Clin Endocrinol (Oxf). 2015 Jan;82(1):122-6.

Hes FJ, van der Luijt RB, Janssen AL, Zewald RA, de Jong GJ, Lenders JW, Links TP, Luyten GP, Sijmons RH, Eussen HJ, et al. Frequency of Von Hippel-Lindau germline mutations in classic and non-classic Von Hippel-Lindau disease identified by DNA sequencing, Southern blot analysis and multiplex ligation-dependent probe amplification. Clin Genet. 2007 Aug;72(2):122-9.

Hes FJ, Weiss MM, Woortman SA, de Miranda NF, van Bunderen PA, Bonsing BA, Stokkel MP, Morreau H, Romijn JA, Jansen JC, Vriends AH, Bayley JP, Corssmit EP. Low penetrance of a SDHB mutation in a large Dutch paraganglioma family. BMC Med Genet. 2010 Jun 11;11:92.

Hittner HM, Hirsch NJ, Kreh GM, Rudolph AJ. Colobomatous microphthalmia, heart disease, hearing loss, and mental retardation--a syndrome. J Pediatr Ophthalmol Strabismus. 1979 Mar- Apr;16(2):122-8.

Huang Y, Wang LA, Xie Q, Pang J, Wang L, Yi Y, Zhang J, Zhang Y, Chen R, Lan W, Zhang D, Jiang J. Germline SDHB and SDHD mutations in pheochromocytoma and paraganglioma patients. Endocr Connect. 2018 Dec 1;7(12):1217-1225.

Janssen N, Bergman JE, Swertz MA, Tranebjaerg L, Lodahl M, Schoots J, Hofstra RM, van Ravenswaaij-Arts CM, Hoefsloot LH. Mutation update on the CHD7 gene involved in CHARGE syndrome. Hum Mutat. 2012 Aug;33(8):1149-60. doi: 10.1002/humu.22086. Epub 2012 Apr 16. PMID: 22461308.

Imamura H, Muroya K, Tanaka E, Konomoto T, Moritake H, Sato T, Kimura N, Takekoshi K, Nunoi H. Sporadic paraganglioma caused by de novo SDHB mutations in a 6-year-old girl. Eur J Pediatr. 2016 Jan;175(1):137-41.

Jochmanova I, Wolf KI, King KS, Nambuba J, Wesley R, Martucci V, Raygada M, Adams KT, Prodanov T, Fojo AT, et al. SDHB-related pheochromocytoma and paraganglioma penetrance and genotype-phenotype correlations. J Cancer Res Clin Oncol. 2017 Aug;143(8):1421-1435.

Kaelin WG Jr. Molecular basis of the VHL hereditary cancer syndrome. Nat Rev Cancer. 2002 Sep;2(9):673-82.

Kaplan JD, Bernstein JA, Kwan A, Hudgins L. Clues to an early diagnosis of Kallmann syndrome. Am J Med Genet A. 2010 Nov;152A(11):2796-801.

Kim SH. Congenital Hypogonadotropic Hypogonadism and Kallmann Syndrome: Past, Present, and Future. Endocrinol Metab (Seoul). 2015 Dec;30(4):456-66. doi: 10.3803/EnM.2015.30.4.456. PMID: 26790381; PMCID: PMC4722398.

Kim JH, Seo GH, Kim GH, Huh J, Hwang IT, Jang JH, Yoo HW, Choi JH. Targeted Gene Panel Sequencing for Molecular Diagnosis of Kallmann Syndrome and Normosmic Idiopathic Hypogonadotropic Hypogonadism. Exp Clin Endocrinol Diabetes. 2019 Sep;127(8):538-544.

Koenigkam-Santos M, Santos A, C, Versiani B, R, Diniz P, R, B, Junior J, E, de Castro M. Quantitative Magnetic Resonance Imaging Evaluation of the Olfactory System in Kallmann Syndrome: Correlation with a Clinical Smell Test. Neuroendocrinology 2011; 94:209-217.

Lack, E.E. Tumors of the Adrenal Gland and Extraadrenal Paraganglia. Armed Forces Institute of Pathology. In Atlas of Tumor Pathology; Series 4, Faccicle 8; American Registry of Pathology Press: Washington, DC, USA, 2007.

Ladroue C, Carcenac R, Leporrier M, Gad S, Le Hello C, Galateau-Salle F, Feunteun J, Pouysségur J, Richard S, Gardie B. PHD2 mutation and congenital erythrocytosis with paraganglioma. N Engl J Med. 2008 Dec 18;359(25):2685-92.

Lai EW, Perera SM, Havekes B, Timmers HJ, Brouwers FM, McElroy B, Adams KT, Ohta S, WesleyRA, Eisenhofer G, Pacak K. Gender-related differences in the clinical presentation of malignant andbenign pheochromocytoma. Endocrine. 2008 Aug-Dec;34(1-3):96-100.

Lalani SR, Safiullah AM, Fernbach SD, Harutyunyan KG, Thaller C, Peterson LE, McPherson JD, Gibbs RA, White LD, Hefner M, et al. Spectrum of CHD7 mutations in 110 individuals with CHARGE syndrome and genotype-phenotype correlation. Am J Hum Genet. 2006 Feb;78(2):303-14.

Lawaetz JG, Hagen CP, Mieritz MG, Blomberg Jensen M, Petersen JH, Juul A. Evaluation of 451 Danish boys with delayed puberty: diagnostic use of a new puberty nomogram and effects of oral testosterone therapy. J Clin Endocrinol Metab. 2015 Apr;100(4):1376-85.

Leaños-Miranda A, Janovick JA, Conn PM. Receptor-misrouting: an unexpectedly prevalent and rescuable etiology in gonadotropin-releasing hormone receptor-mediated hypogonadotropic hypogonadism. J Clin Endocrinol Metab. 2002 Oct;87(10):4825-8.

Lee JH, Barich F, Karnell LH, Robinson RA, Zhen WK, Gantz BJ, Hoffman HT; American College of Surgeons Commission on Cancer; American Cancer Society. National Cancer Data Base report on malignant paragangliomas of the head and neck. Cancer. 2002 Feb 1;94(3):730-7.

Lenders JW, Eisenhofer G, Mannelli M, Pacak K. Phaeochromocytoma. Lancet. 2005 Aug 20-26;366(9486):665-75.

Lenders JW, Duh QY, Eisenhofer G, Gimenez-Roqueplo AP, Grebe SK, Murad MH, Naruse M, PacakK, Young WF Jr; Endocrine Society. Pheochromocytoma and paraganglioma: an endocrine society clinical practice guideline. J Clin Endocrinol Metab. 2014 Jun;99(6):1915-42.

Libutti SK, Choyke PL, Bartlett DL, Vargas H, Walther M, Lubensky I, Glenn G, Linehan WM, Alexander HR. Pancreatic neuroendocrine tumors associated with von Hippel Lindau disease: diagnostic and management recommendations. Surgery. 1998 Dec;124(6):1153-9.

Lima J, Feijão T, Ferreira da Silva A, Pereira-Castro I, Fernandez-Ballester G, Máximo V, Herrero A, Serrano L, Sobrinho-Simões M, Garcia-Rostan G. High frequency of germline succinate dehydrogenase mutations in sporadic cervical paragangliomas in northern Spain: mitochondrial succinate dehydrogenase structure-function relationships and clinical-pathological correlations. J Clin Endocrinol Metab. 2007 Dec;92(12):4853-64.

Liu SJ, Wang JY, Peng SH, Li T, Ning XH, Hong BA, Liu JY, Wu PJ, Zhou BW, Zhou JC, et al. Genotype and phenotype correlation in von Hippel-Lindau disease based on alteration of the HIF- α binding site in VHL protein. Genet Med. 2018 Oct;20(10):1266-1273.

- Lo CY, Lam KY, Wat MS, Lam KS. Adrenal pheochromocytoma remains a frequently overlooked diagnosis. Am J Surg. 2000 Mar;179(3):212-5. doi: 10.1016/s0002-9610(00)00296-8. PMID: 10827323.

- Main KM, Schmidt IM, Skakkebaek NE. A possible role for reproductive hormones in newborn boys: progressive hypogonadism without the postnatal testosterone peak. J Clin Endocrinol Metab. 2000 Dec;85(12):4905-7. doi: 10.1210/jcem.85.12.7058. PMID: 11134160.

Manger WM. The protean manifestations of pheochromocytoma. Horm Metab Res. 2009 Sep;41(9):658-63.

Maher ER, Yates JR, Ferguson-Smith MA. Statistical analysis of the two stage mutation model in von Hippel-Lindau disease, and in sporadic cerebellar haemangioblastoma and renal cell carcinoma. J Med Genet. 1990 May;27(5):311-4.

Maher ER, Kaelin WG Jr. von Hippel-Lindau disease. Medicine (Baltimore). 1997 Nov;76(6):381-91.

Mannelli M, Castellano M, Schiavi F, Filetti S, Giacchè M, Mori L, Pignataro V, Bernini G, Giachè V, Bacca A, et al. Italian Pheochromocytoma/Paraganglioma Network. Clinically guided genetic screening in a large cohort of italian patients with pheochromocytomas and/or functional or nonfunctional paragangliomas. J Clin Endocrinol Metab. 2009 May;94(5):1541-7.

Mannelli M, Canu L, Ercolino T, Rapizzi E, Martinelli S, Parenti G, De Filpo G, Nesi G. DIAGNOSIS of ENDOCRINE DISEASE: SDHx mutations: beyond pheochromocytomas and paragangliomas. Eur J Endocrinol. 2018 Jan;178(1): R11-R17.

Margolin DH, Kousi M, Chan YM, Lim ET, Schmahmann JD, Hadjivassiliou M, Hall JE, Adam I, Dwyer A, Plummer L, et al. Ataxia, dementia, and hypogonadotropism caused by disordered ubiquitination. N Engl J Med. 2013 May 23;368(21):1992-2003.

Martucci VL, Pacak K. Pheochromocytoma and paraganglioma: diagnosis, genetics, management, and treatment. Curr Probl Cancer. 2014 Jan-Feb;38(1):7-41.

McNeil AR, Blok BH, Koelmeyer TD, Burke MP, Hilton JM. Phaeochromocytomas discovered during coronial autopsies in Sydney, Melbourne and Auckland. Aust N Z J Med. 2000 Dec;30(6):648-52.

McNicol AM. Update on tumours of the adrenal cortex, phaeochromocytoma and extra-adrenal paraganglioma. Histopathology. 2011 Jan;58(2):155-68.

Men M, Wu J, Zhao Y, Xing X, Jiang F, Zheng R, Li JD. Genotypic and phenotypic spectra of FGFR1, FGF8, and FGF17 mutations in a Chinese cohort with idiopathic hypogonadotropic hypogonadism. Fertil Steril. 2020 Jan;113(1):158-166.

Mikhail MI, Singh AK. Von Hippel Lindau Syndrome. [Updated 2021 Feb 4]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 Jan. Available from: https://www.ncbi.nlm.nih.gov/books/NBK459242/

Mitchell AL, Dwyer A, Pitteloud N, Quinton R. Genetic basis and variable phenotypic expression of Kallmann syndrome: towards a unifying theory. Trends Endocrinol Metab. 2011 Jul;22(7):249-58.

Murro V, Lippera M, Mucciolo DP, Canu L, Ercolino T, De Filpo G, Giorgio D, Traficante G, Sodi A, Virgili G, et al. Outcome and genetic analysis of patients affected by retinal capillary hemangioblastoma in von Hippel Lindau syndrome. Mol Vis. 2021 Sep 2;27: 542-554.

Neumann HP, Wiestler OD. Clustering of features of von Hippel-Lindau syndrome: evidence for a complex genetic locus. Lancet. 1991 May 4;337(8749):1052-4.

Neumann HP, Pawlu C, Peczkowska M, Bausch B, McWhinney SR, Muresan M, Buchta M, Franke G, Klisch J, Bley TA, et al. Distinct clinical features of paraganglioma syndromes associated with SDHB and SDHD gene mutations. JAMA. 2004 Aug 25;292(8):943-51.

Neumann HP, Vortmeyer A, Schmidt D, Werner M, Erlic Z, Cascon A, Bausch B, Januszewicz A, Eng C. Evidence of MEN-2 in the original description of classic pheochromocytoma. N Engl J Med. 2007 Sep 27;357(13):1311-5.

Neumann HPH, Young WF Jr, Eng C. Pheochromocytoma and Paraganglioma. N Engl J Med. 2019 Aug 8;381(6):552-565.

NGS in PPGL (NGSnPPGL) Study Group, Toledo RA, Burnichon N, Cascon A, Benn DE, Bayley JP, Welander J, Tops CM, Firth H, Dwight T, et al. Consensus Statement on next-generation- sequencing-based diagnostic testing of hereditary phaeochromocytomas and paragangliomas. Nat Rev Endocrinol. 2017 Apr;13(4):233-247.

Niemann S, Müller U. Mutations in SDHC cause autosomal dominant paraganglioma, type 3. Nat Genet. 2000 Nov;26(3):268-70.

Niemeijer ND, Rijken JA, Eijkelenkamp K, van der Horst-Schrivers ANA, Kerstens MN, Tops CMJ,van Berkel A, Timmers HJLM, Kunst HPM, Leemans CR, et al. The phenotype of SDHB germline mutation carriers: a nationwide study. Eur J Endocrinol. 2017 Aug;177(2):115-125.

Ong KR, Woodward ER, Killick P, Lim C, Macdonald F, Maher ER. Genotype-phenotype correlations in von Hippel-Lindau disease. Hum Mutat. 2007 Feb;28(2):143-9.

Oudijk L, Gaal J, Korpershoek E, van Nederveen FH, Kelly L, Schiavon G, Verweij J, Mathijssen RH,den Bakker MA, Oldenburg RA, et al. SDHA mutations in adult and pediatric wild-type gastrointestinal stromal tumors. Mod Pathol. 2013 Mar;26(3):456-63.

Oudijk, L.; de Krijger, R.R.; Pacak, K.; Tischler, A.S. Adrenal medulla and extra-adrenal paraganglia.In Endocrine Pathology; Mete, O., Asa, S.L., Eds.; Cambridge University Press: Cambridge, UK, 2016; pp. 628–676.

Pacak K, Linehan WM, Eisenhofer G, Walther MM, Goldstein DS. Recent advances in genetics, diagnosis, localization, and treatment of pheochromocytoma. Ann Intern Med. 2001 Feb 20;134(4):315-29.

Pacak K. Preoperative management of the pheochromocytoma patient. J Clin Endocrinol Metab. 2007 Nov;92(11):4069-79.

Palmert MR, Dunkel L. Clinical practice. Delayed puberty. N Engl J Med. 2012 Feb 2;366(5):443-53.

Papaspyrou K, Mewes T, Rossmann H, Fottner C, Schneider-Raetzke B, Bartsch O, SchreckenbergerM, Lackner KJ, Amedee RG, Mann WJ. Head and neck paragangliomas: Report of 175 patients (1989-2010). Head Neck. 2012 May;34(5):632-7.

Percy MJ, Beer PA, Campbell G, Dekker AW, Green AR, Oscier D, Rainey MG, van Wijk R, Wood M, Lappin TR, et al. Novel exon 12 mutations in the HIF2A gene associated with erythrocytosis. Blood. 2008 Jun 1;111(11):5400-2.

Pierce A, Bliesner B, Xu M, Nielsen-Preiss S, Lemke G, Tobet S, Wierman ME. Axl and Tyro3 modulate female reproduction by influencing gonadotropin-releasing hormone neuron survival andmigration. Mol Endocrinol. 2008 Nov;22(11):2481-95

Pitteloud N, Hayes FJ, Boepple PA, DeCruz S, Seminara SB, MacLaughlin DT, Crowley WF Jr. The role of prior pubertal development, biochemical markers of testicular maturation, and genetics in elucidating the phenotypic heterogeneity of idiopathic hypogonadotropic hypogonadism. J Clin Endocrinol Metab. 2002 Jan;87(1):152-60.

Pitteloud N, Meysing A, Quinton R, Acierno JS Jr, Dwyer AA, Plummer L, Fliers E, Boepple P, HayesF, Seminara S, et al. Mutations in fibroblast growth factor receptor 1 cause Kallmann syndrome witha wide spectrum of reproductive phenotypes. Mol Cell Endocrinol. 2006 Jul 25;254-255:60-9.

Pitteloud N, Quinton R, Pearce S, Raivio T, Acierno J, Dwyer A, Plummer L, Hughes V, Seminara S, Cheng YZ, et al. Digenic mutations account for variable phenotypes in idiopathic hypogonadotropic hypogonadism. J Clin Invest. 2007 Feb;117(2):457-63.

Prchal JT, Gordeuk VR. The HIF2A gene in familial erythrocytosis. N Engl J Med. 2008 May 1;358(18):1966; author reply 1966-7.

Qin Y, Deng Y, Ricketts CJ, Srikantan S, Wang E, Maher ER, Dahia PL. The tumor susceptibility gene TMEM127 is mutated in renal cell carcinomas and modulates endolysosomal function. Hum Mol Genet. 2014 May 1;23(9):2428-39.

Quaynor SD, Bosley ME, Duckworth CG, Porter KR, Kim SH, Kim HG, Chorich LP, Sullivan ME, Choi JH, Cameron RS, et al. Targeted next generation sequencing approach identifies eighteen new candidate genes in normosmic hypogonadotropic hypogonadism and Kallmann syndrome. Mol Cell Endocrinol. 2016 Dec 5;437: 86-96.

Reisch N, Peczkowska M, Januszewicz A, Neumann HP. Pheochromocytoma: presentation, diagnosis and treatment. J Hypertens. 2006 Dec;24(12):2331-9.

Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015 May;17(5):405-24.

Ricketts CJ, Forman JR, Rattenberry E, Bradshaw N, Lalloo F, Izatt L, Cole TR, Armstrong R, KumarVK, Morrison PJ, et al. Tumor risks and genotype-phenotype-proteotype analysis in 358 patients with germline mutations in SDHB and SDHD. Hum Mutat. 2010 Jan;31(1):41-51.

Ricketts CJ, Shuch B, Vocke CD, Metwalli AR, Bratslavsky G, Middelton L, Yang Y, Wei MH, PautlerSE, Peterson J, et al. Succinate dehydrogenase kidney cancer: an aggressive example of the Warburgeffect in cancer. J Urol. 2012 Dec;188(6):2063-71.

Rijken JA, Niemeijer ND, Jonker MA, Eijkelenkamp K, Jansen JC, van Berkel A, Timmers HJLM, Kunst HPM, Bisschop PHLT, Kerstens MN, et al. The penetrance of paraganglioma and pheochromocytoma in SDHB germline mutation carriers. Clin Genet. 2018 Jan;93(1):60-66.

Ruiz-Llorente S, Bravo J, Cebrián A, Cascón A, Pollan M, Tellería D, Letón R, Urioste M, Rodríguez-López R, de Campos JM, et al. Genetic characterization and structural analysis of VHL Spanish families to define genotype-phenotype correlations. Hum Mutat. 2004 Feb;23(2):160-9.

Salian-Mehta S, Xu M, Knox AJ, Plummer L, Slavov D, Taylor M, Bevers S, Hodges RS, Crowley WF Jr, Wierman ME. Functional consequences of AXL sequence variants in hypogonadotropic hypogonadism. J Clin Endocrinol Metab. 2014 Apr;99(4):1452-60.

Sarfati J, Fouveaut C, Leroy C, Jeanpierre M, Hardelin JP, Dodé C. Greater prevalence of PROKR2 mutations in Kallmann syndrome patients from the Maghreb than in European patients. Eur J Endocrinol. 2013 Oct 21;169(6):805-9.

Schiavi F, Boedeker CC, Bausch B, Peçzkowska M, Gomez CF, Strassburg T, Pawlu C, Buchta M, Salzmann M, Hoffmann MM, et al. Predictors and prevalence of paraganglioma syndrome associated with mutations of the SDHC gene. JAMA. 2005 Oct 26;294(16):2057-63.

Schlisio S, Kenchappa RS, Vredeveld LC, George RE, Stewart R, Greulich H, Shahriari K, Nguyen NV, Pigny P, Dahia PL, et al. The kinesin KIF1Bbeta acts downstream from EglN3 to induce apoptosis and is a potential 1p36 tumor suppressor. Genes Dev. 2008 Apr 1;22(7):884-93.

Scholz T, Schulz C, Klose S, Lehnert H. Diagnostic management of benign and malignant pheochromocytoma. Exp Clin Endocrinol Diabetes. 2007 Mar;115(3):155-9.

Schwanzel-Fukuda M, Bick D, Pfaff DW. Luteinizing hormone-releasing hormone (LHRH)-expressing cells do not migrate normally in an inherited hypogonadal (Kallmann) syndrome. BrainRes Mol Brain Res. 1989 Dec;6(4):311-26.

Schwarting GA, Kostek C, Ahmad N, Dibble C, Pays L, Püschel AW. Semaphorin 3A is required for guidance of olfactory axons in mice. J Neurosci. 2000 Oct 15;20(20):7691-7.

Shaw ND, Seminara SB, Welt CK, Au MG, Plummer L, Hughes VA, Dwyer AA, Martin KA, QuintonR, Mericq V, et al. Expanding the phenotype and genotype of female GnRH deficiency. J Clin Endocrinol Metab. 2011 Mar;96(3): E566-76.

Shendure J, Balasubramanian S, Church GM, Gilbert W, Rogers J, Schloss JA, Waterston RH. DNA sequencing at 40: past, present and future. Nature. 2017 Oct 19;550(7676):345-353.

- **Shi** CH, Schisler JC, Rubel CE, Tan S, Song B, McDonough H, Xu L, Portbury AL, Mao CY, True C,et al. Ataxia and hypogonadism caused by the loss of ubiquitin ligase activity of the U box protein CHIP. Hum Mol Genet. 2014 Feb 15;23(4):1013-24.

Seminara SB, Hayes FJ, Crowley WF Jr. Gonadotropin-releasing hormone deficiency in the human (idiopathic hypogonadotropic hypogonadism and Kallmann's syndrome): pathophysiological and genetic considerations. Endocr Rev. 1998 Oct;19(5):521-39.

Seminara SB, Acierno JS Jr, Abdulwahid NA, Crowley WF Jr, Margolin DH. Hypogonadotropic hypogonadism and cerebellar ataxia: detailed phenotypic characterization of a large, extended kindred. J Clin Endocrinol Metab. 2002 Apr;87(4):1607-12.

Settas N, Faucz FR, Stratakis CA. Succinate dehydrogenase (SDH) deficiency, Carney triad and the epigenome. Mol Cell Endocrinol. 2018 Jul 5; 469:107-111.

Shy ME, Kamholz J, Lovelace RE. Charcot-Marie-Tooth Disorders Ann. New York Acad. Sci. 1999, pp. 34-56.

Simonis N, Migeotte I, Lambert N, Perazzolo C, de Silva DC, Dimitrov B, Heinrichs C, Janssens S, Kerr B, Mortier G, et al. FGFR1 mutations cause Hartsfield syndrome, the unique association of holoprosencephaly and ectrodactyly. J Med Genet. 2013 Sep;50(9):585-92.

Soltani A, Pourian M, Davani BM. Does this patient have Pheochromocytoma? a systematic reviewof clinical signs and symptoms. J Diabetes Metab Disord. 2016 Mar 17;15:6.

Steinbach F, Novick AC, Shoskes D. Renal transplantation in patients with renal cell carcinoma andvon Hippel-Lindau disease. Urology. 1994 Nov;44(5):760-3.

Stamou MI, Varnavas P, Plummer L, Koika V, Georgopoulos NA. Next-generation sequencing refines the genetic architecture of Greek GnRH-deficient patients. Endocr Connect. 2019 May 1;8(5):468-480.

Taïeb D, Kaliski A, Boedeker CC, Martucci V, Fojo T, Adler JR Jr, Pacak K. Current approaches and recent developments in the management of head and neck paragangliomas. Endocr Rev. 2014 Oct;35(5):795-819.

Takeichi N, Midorikawa S, Watanabe A, Naing BT, Tamura H, Wakakuri-Kano T, Ishizaki A, Sugihara H, Nissato S, Saito Y, et al. Identical germline mutations in the TMEM127 gene in two unrelated Japanese patients with bilateral pheochromocytoma. Clin Endocrinol (Oxf). 2012 Nov;77(5):707-14.

Tata B, Huijbregts L, Jacquier S, Csaba Z, Genin E, Meyer V, Leka S, Dupont J, Charles P, ChevenneD, et al. Haploinsufficiency of Dmxl2, encoding a synaptic protein, causes infertility associated witha loss of GnRH neurons in mouse. PLoS Biol. 2014 Sep 23;12(9): e1001952.

Teixeira L, Guimiot F, Dodé C, Fallet-Bianco C, Millar RP, Delezoide AL, Hardelin JP. Defective migration of neuroendocrine GnRH cells in human arrhinencephalic conditions. J Clin Invest. 2010 Oct;120(10):3668-72.

Timmers HJ, Kozupa A, Eisenhofer G, Raygada M, Adams KT, Solis D, Lenders JW, Pacak K. Clinical presentations, biochemical phenotypes, and genotype-phenotype correlations in patients with succinate dehydrogenase subunit B-associated pheochromocytomas and paragangliomas. J Clin Endocrinol Metab. 2007 Mar;92(3):779-86.

Tischler, A.S. The adrenal medulla and extra-adrenal paraganglia. In Functional Endocrine Pathology; Kovacs, K., Asa, S.L., Eds.; Blackwell Science: Hoboken, NJ, USA, 1998; pp. 550–595.

Toledo RA, Dahia PL. Next-generation sequencing for the diagnosis of hereditarypheochromocytoma and paraganglioma syndromes. Curr Opin Endocrinol Diabetes Obes. 2015 Jun;22(3):169-79.

van Buuren S, Ooms JC. Stage line diagram: an age-conditional reference diagram for tracking development. Stat Med. 2009 May 15;28(11):1569-79.

van der Mey AG, Maaswinkel-Mooy PD, Cornelisse CJ, Schmidt PH, van de Kamp JJ. Genomic imprinting in hereditary glomus tumours: evidence for new genetic theory. Lancet. 1989 Dec 2;2(8675):1291-4.

van der Tuin K, Mensenkamp AR, Tops CMJ, Corssmit EPM, Dinjens WN, van de Horst-Schrivers ANA, Jansen JC, de Jong MM, Kunst HPM, Kusters B, et al. Clinical Aspects of SDHA-Related Pheochromocytoma and Paraganglioma: A Nationwide Study. J Clin Endocrinol Metab. 2018 Feb 1;103(2):438-445.

van Hulsteijn LT, Dekkers OM, Hes FJ, Smit JW, Corssmit EP. Risk of malignant paraganglioma in SDHB-mutation and SDHD-mutation carriers: a systematic review and meta-analysis. J Med Genet. 2012 Dec;49(12):768-76.

van Hulsteijn LT, Niemeijer ND, Hes FJ, Bayley JP, Tops CM, Jansen JC, Corssmit EP. Phenotype of SDHB mutation carriers in the Netherlands. Fam Cancer. 2014 Dec;13(4):651-7.

Walther MM, Choyke PL, Glenn G, Lyne JC, Rayford W, Venzon D, Linehan WM. Renal cancer in families with hereditary renal cancer: prospective analysis of a tumor size threshold for renal parenchymal sparing surgery. J Urol. 1999 May;161(5):1475-9.

Wang Y, Gong C, Qin M, Liu Y, Tian Y. Clinical and genetic features of 64 young male paediatric patients with congenital hypogonadotropic hypogonadism. Clin Endocrinol (Oxf). 2017 Dec;87(6):757-766.

Weber F, Dralle H. Value of ileus-prophylactic surgery for metastatic neuroendocrine midgut tumours. Best Pract Res Clin Endocrinol Metab. 2019 Oct;33(5):101342.

Welander J, Söderkvist P, Gimm O. Genetics and clinical characteristics of hereditary pheochromocytomas and paragangliomas. Endocr Relat Cancer. 2011 Dec 1;18(6): R253-76.

Whalen RK, Althausen AF, Daniels GH. Extra-adrenal pheochromocytoma. J Urol. 1992 Jan;147(1):1-10.

Williams MD. Paragangliomas of the Head and Neck: An Overview from Diagnosis to Genetics. Head Neck Pathol. 2017 Sep;11(3):278-287.

Wonkam-Tingang E, Schrauwen I, Esoh KK, Bharadwaj T, Nouel-Saied LM, Acharya A, Nasir A, Leal SM, Wonkam A. A novel variant in DMXL2 gene is associated with autosomal dominant non-syndromic hearing impairment (DFNA71) in a Cameroonian family. Exp Biol Med (Maywood). 2021 Jul;246(13):1524-1532.

Xekouki P, Brennand A, Whitelaw B, Pacak K, Stratakis CA. The 3PAs: An Update on the Association of Pheochromocytomas, Paragangliomas, and Pituitary Tumors. Horm Metab Res. 2019Jul;51(7):419-436.

Xu N, Qin Y, Reindollar RH, Tho SP, McDonough PG, Layman LC. A mutation in the fibroblast growth factor receptor 1 gene causes fully penetrant normosmic isolated hypogonadotropic hypogonadism. J Clin Endocrinol Metab. 2007 Mar;92(3):1155-8.

Xu C, Cassatella D, van der Sloot AM, Quinton R, Hauschild M, De Geyter C, Flück C, Feller K, Bartholdi D, Nemeth A, et al. Evaluating CHARGE syndrome in congenital hypogonadotropic hypogonadism patients harboring CHD7 variants. Genet Med. 2018 Aug;20(8):872-881.

Yakirevich E, Ali SM, Mega A, McMahon C, Brodsky AS, Ross JS, Allen J, Elvin JA, Safran H, Resnick MB. A Novel SDHA-deficient Renal Cell Carcinoma Revealed by Comprehensive Genomic Profiling. Am J Surg Pathol. 2015 Jun;39(6):858-63.

Yang C, Zhuang Z, Fliedner SM, Shankavaram U, Sun MG, Bullova P, Zhu R, Elkahloun AG, KourlasPJ, Merino M, et al. Germ-line PHD1 and PHD2 mutations detected in patients with pheochromocytoma/paraganglioma-polycythemia. J Mol Med (Berl). 2015 Jan;93(1):93-104.

Yao L, Schiavi F, Cascon A, Qin Y, Inglada-Pérez L, King EE, Toledo RA, Ercolino T, Rapizzi E, Ricketts CJ, et al. Spectrum and prevalence of FP/TMEM127 gene mutations in pheochromocytomasand paragangliomas. JAMA. 2010 Dec 15;304(23):2611-9.

Yeh IT, Lenci RE, Qin Y, Buddavarapu K, Ligon AH, Leteurtre E, Do Cao C, Cardot-Bauters C, PignyP, Dahia PL. A germline mutation of the KIF1B beta gene on 1p36 in a family with neural and nonneural tumors. Hum Genet. 2008 Oct;124(3):279-85.

Young J. Approach to the male patient with congenital hypogonadotropic hypogonadism. J Clin Endocrinol Metab. 2012 Mar;97(3):707-18.

Young WFJ. Endocrine hypertension. In: Melmed S, Polonsky KS, Larsen PR, Kronenbreg HM. Williams textbook of endocrinology. 13th ed. Philadelphia: Elsevier, 2016:556-88.

Young J, Xu C, Papadakis GE, Acierno JS, Maione L, Hietamäki J, Raivio T, Pitteloud N. Clinical Management of Congenital Hypogonadotropic Hypogonadism. Endocr Rev. 2019 Apr 1;40(2):669- 710.

Zanni G, Barresi S, Travaglini L, Bernardini L, Rizza T, Digilio MC, Mercuri E, Cianfarani S, Valeriani M, Ferraris A, et al. FGF17, a gene involved in cerebellar development, is downregulated in a patient with Dandy-Walker malformation carrying a de novo 8p deletion. Neurogenetics. 2011 Aug;12(3):241-5.

Zhao C, Takita J, Tanaka Y, Setou M, Nakagawa T, Takeda S, Yang HW, Terada S, Nakata T, Takei Y, et al. Charcot-Marie-Tooth disease type 2A caused by mutation in a microtubule motor KIF1Bbeta.Cell. 2001 Jun 1;105(5):587-97.

Zhou C, Niu Y, Xu H, Li Z, Wang T, Yang W, Wang S, Wang DW, Liu J. Mutation profiles and clinical characteristics of Chinese males with isolated hypogonadotropic hypogonadism. Fertil Steril. 2018 Aug;110(3):486-495.e5.

Zhou B, Wang J, Liu S, Peng X, Hong B, Zhou J, Ma K, Zhang J, Cai L, Gong K. Hemangioblastoma Instead of Renal Cell Carcinoma Plays a Major Role in the Unfavorable Overall Survival of Von Hippel-Lindau Disease Patients. Front Oncol. 2019 Oct 9; 9:1037.