Genetics in Medicine (2023) 25, 100881

ELSEVIER

Genetics in Medicine
An Official Journal of the ACMG

www.journals.elsevier.com/genetics-in-medicine

ARTICLE

Identification of genetic variants and phenotypic characterization of a large cohort of patients with congenital hypopituitarism and related disorders

Louise C. Gregory[1], Cecilia Cionna[2], Manuela Cerbone[1,3], GOSgene, Mehul T. Dattani[1,3,*]

[1]*Genetics and Genomic Medicine Research and Teaching Department, UCL Great Ormond Street Institute of Child Health, London, United Kingdom;* [2]*Pediatric Unit, Department of Mother and Child Health, G. Salesi Children's Hospital, Ancona, Italy;* [3]*Department of Endocrinology, Great Ormond Street Hospital for Children, Great Ormond Street, United Kingdom*

ARTICLE INFO

Article history:
Received 4 August 2022
Received in revised form
28 April 2023
Accepted 30 April 2023
Available online 8 May 2023

Keywords:
Congenital hypopituitarism
Endocrinology
Genetic analysis
Hypothalamo-pituitary development

ABSTRACT

Purpose: Congenital hypopituitarism (CH) disorders are phenotypically variable. Variants in multiple genes are associated with these disorders, with variable penetrance and inheritance.
Methods: We screened a large cohort ($N = 1765$) of patients with or at risk of CH using Sanger sequencing, selected according to phenotype, and conducted next-generation sequencing (NGS) in 51 families within our cohort. We report the clinical, hormonal, and neuroradiological phenotypes of patients with variants in known genes associated with CH.
Results: We identified variants in 178 patients: *GH1/GHRHR* (51 patients of 414 screened), *PROP1* (17 of 253), *POU1F1* (15 of 139), *SOX2* (13 of 59), *GLI2* (7 of 106), *LHX3/LHX4* (8 of 110), *HESX1* (8 of 724), *SOX3* (9 of 354), *OTX2* (5 of 59), *SHH* (2 of 64), and *TCF7L1*, *KAL1*, *FGFR1*, and *FGF8* (2 of 585, respectively). NGS identified 26 novel variants in 35 patients (from 24 families). Magnetic resonance imaging showed prevalent hypothalamo-pituitary abnormalities, present in all patients with *PROP1*, *GLI2*, *SOX3*, *HESX1*, *OTX2*, *LHX3*, and *LHX4* variants. Normal hypothalamo-pituitary anatomy was reported in 24 of 121, predominantly those with *GH1*, *GHRHR*, *POU1F1*, and *SOX2* variants.
Conclusion: We identified variants in 10% (178 of 1765) of our CH cohort. NGS has revolutionized variant identification, and careful phenotypic patient characterization has improved our understanding of CH. We have constructed a flow chart to guide genetic analysis in these patients, which will evolve upon novel gene discoveries.

The Article Publishing Charge (APC) for this article was paid by University College London (UCL).

Louise C. Gregory, Cecilia Cionna, and Manuela Cerbone contributed equally to this work.

*Correspondence and requests for materials should be addressed to Mehul T. Dattani, UCL Great Ormond Street Institute of Child Health, 30 Guilford Street, London, WC1N 1EH United Kingdom. *Email address:* m.dattani@ucl.ac.uk

doi: https://doi.org/10.1016/j.gim.2023.100881

Introduction

Congenital hypopituitarism (CH) is a complex and highly heterogeneous disorder that is associated with highly variable clinical phenotypes that range in severity and include deficiencies in ≥1 of the 6 anterior pituitary hormones. The most common deficiency present in patients with CH is growth hormone deficiency (GHD), which may occur in isolation (IGHD) or as part of combined pituitary hormone deficiency (CPHD), including variable combinations of thyroid-stimulating hormone (TSH), luteinizing hormone and follicle-stimulating hormone, adrenocorticotropic hormone (ACTH), and prolactin (PRL) deficiencies. Central diabetes insipidus (CDI) may also be associated in some patients, usually in the presence of midline brain defects.

Pathogenic variants in a number of genes have been identified over the past 30 years or so in patients with a range of phenotypes, which include isolated hormone deficiencies, CPHD, and complex disorders, including septo-optic dysplasia (SOD) and holoprosencephaly (HPE). These genes either encode developmental proteins implicated in hypothalamo-pituitary (HP) development or hormones. However, the penetrance and inheritance may be variable, and overlapping phenotypes may be associated with pathogenic variants in a specific gene. For example, *HESX1* variants may be associated with SOD, IGHD, or CPHD. Furthermore, a particular phenotype may be associated with variants in >1 gene. For example, IGHD, which occurs in 1:4000 to 10,000 live births with up to approximately 30% of cases being familial, may be caused by autosomal dominant or recessive *GH1* deletions/pathogenic variants (22.7% of familial and 2.7% sporadic cases), as well as recessive *GHRHR* and *RNPC3* variants. The latter have also been associated more recently with complex hypopituitarism.[1]

Similarly, isolated TSH deficiency (ITSHD; prevalence 1:20,000-80,000)[1,2] may be associated with recessive variants in *TSHB* or *TRHR*.[3-5] More recently, X-linked forms of TSHD have been described, in association with variants in *TBL1X*,[6] or *IGSF1* when it is frequently (88% of cases) associated with adult macroorchidism.[7,8]

CPHD may occur in isolation with/without structural abnormalities of the hypothalamus and pituitary, or in association with craniofacial, midline structural brain, and eye abnormalities such as optic nerve hypoplasia, anophthalmia or microphthalmia, and coloboma, giving rise to specific complex disorders, such as HPE and SOD, when it is associated with significant morbidity and mortality. Although some patients have a completely normal appearance of the pituitary gland, anterior pituitary hypoplasia may be identified on magnetic resonance imaging (MRI) with or without an ectopic posterior pituitary (EPP). Other neuroradiological abnormalities may also be identified, such as agenesis of the corpus callosum or an absent septum pellucidum, as well as generalized white matter loss, cortical dysplasia, and cerebellar abnormalities.[1,9] The clinical and neuroimaging phenotypes associated with CH are extremely heterogeneous, and the evolution of endocrine deficits over time is unpredictable, particularly in patients with SOD or those with pituitary stalk interruption syndrome.[10]

Based on the type of CH disorder diagnosed and the presence/absence of brain abnormalities predisposing to the risk of CH (eg, isolated vs combined pituitary deficits; presence/absence of associated midline brain/eye abnormalities), genetic screening may be performed for variants in genes associated with particular CH phenotypes, based on previously published reports in the literature. Table 1 summarizes the range of CH clinical phenotypes and a list of the most frequently altered genes associated with each phenotype.

In this study, we report the results of screening our cohort of 1765 index cases for variants in genes associated with CH and related disorders. We propose a protocol that can be used to direct molecular analysis in this rare condition. We also present preliminary data showing the potential impact of next-generation sequencing (NGS) on future molecular diagnosis.

Materials and Methods

Cohorts

DNA was extracted from blood samples taken from a total of 1765 patients with or at risk of CH from 1563 unrelated families (consisting of 1511 families with 1 affected child, and 52 families with >1 affected child), recruited from national and international centers between 1998 and 2020. Patients were considered "at risk of CH" if they had one or more of the following: HP structural abnormalities, midline brain abnormalities, optic nerve hypoplasia, and/or severe eye defects or HPE. The numbers of patients in each CH subcohort are noted in Table 1.

Polymerase chain reaction and direct sequencing analysis

Patients in each CH subcohort were screened for variants in genes associated with CH that were selected based on their clinical features (Table 1) by polymerase chain reaction (PCR) and Sanger sequencing. Of note, not every patient in a given subcohort was screened for every phenotypically relevant gene listed in Table 1. The exact numbers of patients screened to date, respective of each gene, are presented in Table 2. The coding regions of the genes were amplified by PCR using exon flanking primers with BIOTAQ DNA Polymerase reagents (Bioline, BIO-21060) on an Eppendorf Thermocycler. PCR products were cleaned using Microclean (Web Scientific, 2MCL-10) following manufacturer's instructions, and precipitates were resuspended and sequenced with the forward or reverse primer using BigDye Terminator v1.1 Cycle Sequencing Kit (Life Technologies Ltd., 4337450) on an Eppendorf

Table 1 Our congenital hypopituitarism patient cohort and the CH genes associated

Congenital Hypopituitarism Phenotype	Incidence	Number of Patient Samples From Our Total Patients With CH ($N = 1765$)	Description	Genes Associated With CH
CPHD without midline defects	1/4000	621	Deficiencies in ≥1 of the 6 anterior pituitary hormones: GH, TSH, LH, FSH, PRL, ACTH	*LHX3*, *LHX4*, *PROP1*, *POU1F1*, *HESX1*, *SOX3*, *OTX2*, *GLI2*, *KAL1*
IGHD	1/4000-1/10,000	414	The most common isolated deficiency: short stature, delayed growth velocity, and delayed skeletal maturation	*GH1*, *GHRHR*, *RNPC3*, *HESX1*, *OTX2*, *SOX3*, *POU1F1*
SOD	1/10,000	585	Optic nerve hypoplasia, midline neuroradiological abnormalities. Structural HP abnormalities with endocrine deficits	*SOX2*, *OTX2*, *HESX1*, *FGF8*, *FGFR1*, *KAL1*, *TCF7L1*
SED	1/10,000-1/20,000	59[a]	Anophthalmia/microphthalmia	*SOX2*, *OTX2*
HPE	1/10,000-1/20,000	64	Incomplete cleavage of the prosencephalon, affecting both the forebrain and the face: Alobar (no forebrain division) Semilobar (some separation) Lobar (complete separation) Microcephaly, hypotelorism, a single central maxillary incisor, cleft lip and/or palate	*SHH*, *GLI2*, *ZIC2*, *SIX3*, *TGIF1*, *PCTH1*, *FGF8* Submicroscopic deletions at a number of loci
ITSHD	1/20,000-1/80,000	0	Low T4/FT4 and low/normal TSH. Macroorchidism in males with *IGSF1* pathogenic variants	*TSHβ*, *TRHR*, *TBL1X*, *IGSF1*
IACTHD	Unknown	0	Neonatal hypoglycemia	*TBX19 (TPIT)*, *POMC*, *PC1*

A breakdown of our total CH cohort into subcohorts with a standard description of their main clinical features and incidence in the general population. A list of the most common genes associated with CH that are associated with each subcohort according to the literature.

ACTH, adrenocorticotropic hormone; *CH*, congenital hypopituitarism; *CPHD*, combined pituitary hormone deficiency; *ES*, exome sequencing; *FSH*, follicle-stimulating hormone; *GH*, growth hormone; *HP*, hypothalamo-pituitary; *HPE*, holoprosencephaly; *IACTHD*, isolated adrenocorticotropic hormone deficiency; *IGHD*, isolated growth hormone deficiency; *ITSHD*, isolated thyroid stimulating hormone deficiency; *LH*, luteinizing hormone; *PRL*, prolactin; *SED*, severe eye defect; *SOD*, septo-optic dysplasia.

[a]Fifty-nine patients with SEDs are part of the SOD ($n = 585$) cohort. The 81 patients defined as having a "unique" phenotype in our paper, are not included in this table because they do not fit clearly into any of these categories.

Thermocycler. Precipitates were washed and re-suspended in 1M TE buffer before analysis on a 3730XL DNA Analyzer (Applied Biosystems 625-0020). Primer sequences, annealing temperatures, and conditions for PCR and direct sequencing analysis are available upon request.

Next-generation sequencing

Exome sequencing (ES) and genome sequencing (GS) in a few cases were performed on 81 patients from 51 unrelated pedigrees. These patients/families were selected for NGS either because they had a completely unique CH phenotype ($n = 11$ families) that did not fit clearly within one of the subcohorts described, for example, 5 families with a duplicated pituitary, or they fitted loosely within one of the specified CH subcohorts (14 IGHD, 21 CPHD, or 5 SOD) but had additional unique features that are not usually seen in combination with CH (note that these 81 patients have not been included under any subcohort in Table 1). In some patients within the latter subcohorts, genes previously associated with the relevant CH phenotype had been screened but no variants had been identified, for example, if they had IGHD, they would have had *GH1* and *GHRHR* screened before NGS. The families with unique CH phenotypes did not have an obvious known candidate gene associated with the relevant phenotype. Thus, selected families were submitted for NGS using either ES or GS. Although we have many families within our cohort in whom no genetic etiology has been defined as yet, funding issues necessitated a selection of 51 of 1563 unrelated families. Variants were filtered and analyzed by GOSgene, a UCL Great Ormond Street Institute of Child Health in-house genetic service. In the majority of these cases, trios (the patient and both parents) were sequenced simultaneously. However, parental DNA samples were unavailable in some instances. Other affected family members and/or unaffected siblings were also sequenced simultaneously with the patient/parents where possible.

Table 2 The number of variants in genes associated with congenital hypopituitarism and related disorders in each subcohort

Gene	Diagnosis	Number of Variants Identified Out of Total Number of Patients Sanger Sequenced to Date (%)
GH1	IGHD	29/414 (7)
GHRHR	IGHD	22/414 (5)
PROP1	CPHD	17/253 (7)
POU1F1/PIT1	CPHD	15/139 (11)
SOX2	SED	13/59 (22)
GLI2	IGHD (5) CPHD (2)	7/106[a] (5)
HESX1	CPHD (5) IGHD (1) SOD (2)	8/724 (1)
SOX3	CPHD (6) IGHD (3)	9/354 (2)
OTX2	SED	5/59 (8)
LHX4	CPHD (2) IGHD (1) SOD (1)	4/110 (4)
LHX3	CPHD	4/110 (4)
SHH	HPE	2/64 (3)
TCF7L1	SOD	2/526 (<1)
KAL1 (ANOS1)	SOD	2/526 (<1)
FGFR1	SOD	2/701 (<1)
FGF8	SOD (1/526) HPE (1/64)	2/590 (<1)

The number of variants presented in this table were identified through Sanger sequencing which commenced between 1998 and 2020. The total number of patients screened to date for each respective gene, and percentages of those with a variant identified so far, is shown in the third column.

CPHD, combined pituitary hormone deficiency; *ES*, exome sequencing; *HPE*, holoprosencephaly; *IGHD*, isolated growth hormone deficiency; *SED*, severe eye defect; *SOD*, septo-optic dysplasia.

[a]Four of the 8 variants in the *GLI2* gene were identified through ES (3 IGHD, 1 CPHD). These individuals were submitted directly for ES and were not Sanger sequenced for *GLI2* before these findings. In addition to the 106 patients screened, the 64 patients with HPE were also screened for variants in *GLI2*; however, none were identified.

Exons and splice sites (ES) and introns (GS) were captured using the Agilent, SureSelect version 4 kit and sequenced on the high-throughput Illumina HiSeq2000. Raw sequencing data were mapped against the GRCh37/hg19 reference genome using Burrows-Wheeler Aligner algorithm. During variant calling, the structured programming framework, Genome Analysis Toolkit (GATK),[11] was used for base quality score recalibration, INDEL realignment, and duplicate removal. Standard hard-filtering parameters and variant quality scores were used for genotyping and SNP and INDEL identification, respectively. Data were annotated and analyzed using the Ingenuity Variant Analysis software (https://www.qiagenbioinformatics.com/products) from Qiagen, Inc. All possible inheritance models for each respective submitted pedigree were used to analyze variants that may have resulted in the CH phenotype in the patient. These included autosomal recessive (homozygous, hemizygous, or compound heterozygous), autosomal dominant, and de novo analysis. Incomplete or variable penetrance of variants in phenotypically associated genes and pathways was also considered by analyzing novel heterozygous variants in the patient that were inherited from an unaffected parent. A biological panel list of genes related to patient phenotype was uploaded into the Ingenuity Variant Analysis software using human phenotype ontology terms. This allowed the biologically filtered genes to be analyzed first, before proceeding to the analysis of all filtered genes in the genome using an unbiased approach to ensure that no potential pathogenic variants were overlooked.

For any variants identified either through Sanger sequencing or NGS, control databases such as 1000 Genomes (www.1000genomes.org), dbSNP (www.ncbi.nlm.nih.gov/SNP/), Exome Variant Server (evs.gs.washington.edu/EVS/), and the gnomAD Browser (https://gnomad.broadinstitute.org/) were consulted for their presence or frequency. Those with a frequency of 1% or higher on these databases were excluded, with the exception of compound heterozygous variants, in which it is possible that a variant may occur at a higher frequency in the general population but only be pathogenic in the presence of another variant in the same gene.

Variants were confirmed in patients via Sanger sequencing using specifically designed exon-spanning primers that amplify the DNA region containing the variant. In some cases, Sanger sequencing was also performed on other family members to screen for the presence of the variant in question.

Data collection

The following retrospective clinical, biochemical, and neuroradiological data were collected for all patients who had a gene variant identified through Sanger sequencing in this study: prevalence of pituitary hormonal deficiencies, GH peak after dynamic stimulation or at the time of hypoglycemia, prevalence of pituitary and extrapituitary brain abnormalities on MRI, and presence of associated phenotypic features (distinctive facial features, skeletal malformations, neurological, ear-nose-throat, genital, cardiac and gastrointestinal abnormalities, and eye defects). The hormonal evaluation was mostly made at the referral center in accordance with local diagnostic guidelines. We considered a diagnosis of gonadotropin deficiency (GnD) only in patients who had reached the pubertal age. If the patients had undergone >1 GH stimulation test, the highest GH peak value was used for the statistical analysis. Patients evaluated at our institution were tested by glucagon or insulin tolerance tests, whereas a range of different stimulation tests were used in other national and international referring centers. In the absence of universally accepted reference standards for the size of the HP structures in the pediatric age

group, the neuroimaging data collected were qualitative and descriptive. The following HP abnormalities (HPAs) were described: anterior pituitary (absent/hypoplastic/enlarged), posterior pituitary (absent/ectopic), and pituitary stalk (absent/thin/thick). Corpus callosal (absent/hypoplastic) or septum pellucidum (cavum/absent/hypoplastic) abnormalities were reported as midline forebrain defects. Other associated extrapituitary brain anomalies were also noted. When reporting "distinctive facial features," we did not include the facial dysmorphisms commonly observed in patients with GH deficiency (frontal bossing, saddle nose, and maxillary hypoplasia), but we included only other features that could possibly be related to the potential underlying pathogenic variant.

Statistical analysis

Categorial data are expressed as numbers and percentages. Continuous data are expressed as mean ± standard deviation. The differences of GH peak between subgroups of patients with variants were analyzed using a nonparametric test (Kruskal Wallis). Statistical significance was defined as $P < .05$. All analyses were performed using the Statistical Package for the Social Sciences Version 20.0.

We compared clinical, endocrine, and imaging phenotypes in the patients with variants in the following 3 groups of genes: (1) early developmental genes (*SOX2*, *SOX3*, *GLI2*, *LHX3*, *LHX4*, *HESX1*, *OTX2*, *TCF7L1*, *FGF8*, and *FGFR1*), (2) genes involved in GH secretion (*GH1* or *GHRHR)*, and (3) late developmental genes involved in regulating pituitary cell differentiation (*PROP1* or *POU1F1).*

We used the WGlab online tool (https://wintervar.wglab.org/) to calculate the American College of Medical Genetics and Genomics classifications and scores for each variant identified through Sanger sequencing (Supplemental Table 1). Despite some unshaded variants being depicted as having an "unknown significance" as an American College of Medical Genetics and Genomics classification, these variants are those that we have shown a functional compromise in and have been classified as pathogenic/likely pathogenic in our overall conclusions in this manuscript.

Results

Overview: Phenotypes in patients with an identified variant in a gene known to be associated with CH, and the prevalence of these variants in a given gene

The total number of patients with variants identified in genes associated with CH in our overall cohort ($n = 1765$) to date is 143. The phenotypic category of patients with identified variants and the relevant gene are shown in Table 2, and a list of specific variants and the number of patients harboring these variants is presented in Supplemental Table 1. The vast majority, including the gene deletions, were identified through Sanger sequencing that prevailed from 1998 to 2020, with the exception of 4 *GLI2* variants that were identified through ES. The percentage occurrence rate of variants discussed in this paper (shown in Table 2) has been calculated from the number of patients who were screened for each respective gene to date, rather than from the total number of patients in that subcohort.

Pituitary deficiencies in patients with an identified variant

Only a small number of patients with variants identified (6 of 143, 4.2%) had preserved pituitary function at diagnosis (4 patients with SOD: 1 *KAL1*, 1 *FGFR1*, 1 *OTX2*, and 1 *TCF7L1* variant), and 2 patients with HPE (2 *SHH* variants). In the remaining 137 of 143 (95.8%), the most frequently identified deficiency was GHD (124 of 143, 86.7%) followed by GnD (36 of 86, 41.9%), TSHD (51 of 143, 35.7%), and ACTHD (28 of 143, 19.6%). Among 79 patients in whom PRL measurements were available, 21 (26.6%) had PRL deficiency. In patients with identified variants, only 1 patient had CDI. Various combinations of pituitary hormone deficiencies were present in patients with different altered genes (Table 3). GHD was present in the majority of patients except in those with HPE due to SHH variants, who had preserved pituitary function, and in the majority (12 of 13) of patients with *SOX2* variants, who had normal GH secretion. Isolated GHD was the most common occurrence in our patients with *GH1*, *GHRHR*, or *OTX2* variants. Patients with a *SOX2* variant invariably presented with isolated GnD. The prevalence of TSHD and ACTHD was variable in patients with different variants: the highest was associated with *LHX3* variants (100% and 100%, respectively), and the lowest prevalence for these deficiencies was in patients with *GLI2* variants (28.6% and 14.3%, respectively). In addition to patients with *SOX2* variants, GnD was also present in most of the patients with *LHX3* and *LHX4* variants and in the majority (≥80%) of patients with *PROP1* variants. PRL deficiency was present in all patients with *POU1F1* and *LHX3* variants, whereas it was less frequent in patients with *LHX4* (67%) and *GLI2* (33.3%) variants. CDI was present in only 1 patient, with an *FGF8* variant. The GH peaks after stimulation or at the time of hypoglycemia in patients with GHD were mostly <4 μg/L or ng/mL. The mean GH peaks were significantly higher in patients with variants in early developmental genes (*SOX2*, *SOX3*, *GLI2*, *LHX3*, *LHX4*, *HESX1*, *OTX2*, *TCF7L1*, *FGF8*, and *FGFR1*; 2.00 ± 1.79 μg/L or ng/mL) than in patients with variants in genes involved in GH secretion (*GH1*, *GHRHR*; 0.95 ± 0.97 μg/L or ng/mL) or in late developmental genes involved in regulating pituitary cell differentiation (*PROP1*, *POU1F1*; 0.51 ± 0.58 μg/L or ng/mL) ($P = .002$).

Imaging in patients with an identified variant

HPAs on MRI were highly prevalent in this cohort (at least 50% of patients in all subgroups) (Table 4). In particular,

Table 3 The prevalence of each hormonal pituitary deficiency in patients with an identified variant in genes associated with CH

Patient phenotype	*GH1* ($n = 29$)	*GHRHR* ($n = 22$)	*PROP1* ($n = 17$)	*POU1F1* ($n = 15$)	*SOX2* ($n = 13$)	*GLI2* ($n = 7$)	*SOX3* ($n = 9$)	*HESX1* ($n = 8$)	*LHX3* ($n = 4$)	*LHX4* ($n = 4$)	*OTX2* ($n = 5$)	*SHH* ($n = 2$)	*TCF7L1* ($n = 2$)	*FGFR1* ($n = 2$)	*FGF8* ($n = 2$)	*KAL1* ($n = 2$)
GHD *n* (%)	29/29 (100)	22/22 (100)	17/17 (100)	15/15 (100)	1/13 (7.6)	7/7 (100.0)	9/9 (100)	8/8 (100)	4/4 (100)	4/4 (100)	4/5 (80)	0/2 (0)	1/2 (50)	1/2 (50)	1/2 (50)	1/2 (50)
TSHD *n* (%)	0/29 (0)	0/22 (0)	15/17 (88.2)	12/15 (80.0)	0/13 (0)	2/7 (28.6)	6/9 (66.7)	7/8 (87.5)	4/4 (100.0)	3/4 (75)	0/5 (0)	0/2 (0)	0/2 (0)	1/2 (50)	1/2 (50)	0/2 (0)
ACTHD *n* (%)	0/29 (0)	0/22 (0)	7/17 (41.2)	0/15 (0)	0/13 (0)	1/7 (14.3)	4/9 (44.4)	6/8 (75.0)	4/4 (100.0)	3/4 (75)	0/5 (0)	0/ 2 (0)	1/2 (50)	1/2 (50)	1/2 (50)	0/2 (0)
GnD[a] *n* (%)	0/11 (0)	0/15 (0)	10/12 (83.3)	0/11 (0)	13/13 (100)	1/5 (20.0)	5/7 (71.4)	4/5 (80)	2/2 (100)	1/1 (100)	0/0 (0)	0/2 (0)	0/1 (0)	0/0 (0)	0/0 (0)	0/0 (0)
PRLD *n* (%)	0/7 (0)	0/6 (0)	0/12 (0)	14/14 (100)	0/10 (0)	1/3 (33.3)	0/4 (0)	0/6 (0)	2/2 (100)	2/3 (66.7)	0/5 (0)	0/2 (0)	0/2 (0)	1/2 (50)	1/1 (100)	0/0 (0)
DI *n* (%)	0/29 (0)	0/22 (0)	0/17 (0)	0/15 (0)	0/13 (0)	0/7 (0)	0/6 (0)	0/8 (0)	0/4 (0)	0/4 (0)	0/5 (0)	0/2 (0)	0/2 (0)	0/2 (0)	1/2 (50.0)	0/2 (0)
Peak GH mcg/L/ ng/mL mean ± SD (*n*)	1.13 ± 1.11 (20)	0.74 ± 0.76 (17)	0.80 ± 0.79 (8)	0.30 ± 0.21 (11)	4.2 (1)	2.73 ± 2.07 (7)	1.49 ± 1.2 (5)	1.01 ± 1.25 (2)	0.71 ± 0.53 (4)	1.64 ± 2.4 (3)	4.75 ± 0.35 (2)	NA	3.4 (1)	0.9 (1)	5.1 (1)	NA

The numbers and percentages are based on patients with an identified variant in a known causative gene only. The total number of patients with a variant in each gene is noted in parentheses in the column headings. The mean GH peak following a stimulation test or at the time of hypoglycemia is shown for patients with confirmed GHD and variants in each gene.

ACTHD, adrenocorticotrophic hormone deficiency; *DI*, diabetes insipidus; *GHD*, growth hormone deficiency; *GnD*, gonadotrophin deficiency; *n*, number of patients; *NA*, not applicable; *PRLD*, prolactin deficiency; *TSHD*, thyroid-stimulating hormone deficiency.

[a]For the prevalence of GnD, we only considered the patients who have reached pubertal age.

they were present in 100% of patients with *PROP1*, *GLI2*, *SOX3*, *HESX1*, *LHX4*, *OTX2*, and *LHX3* variants. A normal HP axis anatomy was reported in only 24 of 121 (19.8%) patients, mainly in a subset of those with *GH1*, *GHRHR*, *POU1F1*, and *SOX2* variants. A small or aplastic anterior pituitary was observed in 88 of 121 (72.7%), whereas a minority of patients (8 of 121, 6.6%) had anterior pituitary enlargement (4 patients with *PROP1*, 2 patients with *SOX2*, 1 with *FGF8*, and 1 with a *SOX3* variant). An ectopic or absent posterior pituitary gland was observed in 25 of 121 (20.7%), and only in patients with variants in early developmental genes, including *GLI2*, *SOX3*, *HESX1*, *LHX4*, *OTX2*, *FGFR1*, and *TCF7L1* variants. Pituitary stalk abnormalities were reported in 9 of 121 (7.4%) patients, mainly in those with *HESX1*, *GLI2*, *FGFR1*, *SOX3*, and *POU1F1* variants.

Among the 6 patients with preserved pituitary function, HP structural abnormalities were present in only 1 patient with HPE and an *SHH* variant (small anterior pituitary) and in 1 patient with SOD, including a small anterior pituitary with an EPP and an *OTX2* variant. Midline forebrain defects were described in 22 of 121 (15.7%) patients and only in those with variants in early developmental genes (*HESX1*, *OTX2*, *SOX2*, *SHH*, *TCF7L1*, *FGF8*, *FGFR1*, and *KAL1*), whereas other brain malformations were found in 16 of 121 (13.2%) patients (Table 4).

Extrapituitary phenotypes in patients with an identified variant

In this cohort, we reported the following extrapituitary phenotypic features that may or may not be associated with the underlying genotype (Supplemental Table 2). Neurological abnormalities, such as developmental delay, autistic spectrum disorder, and epilepsy, were described in some patients with variants in all of the reported genes discussed in this manuscript, with the exception of *KAL1*, but were more common in patients with variants in early developmental genes (*LHX3*, *SOX2*, *OTX2*, *SHH*, *TCF7L1*, and *FGF8*). Distinctive facial features were described in patients with *FGFR1*, *SHH*, *TCF7L1*, and *FGF8* variants. Skeletal malformations were found in all patients with variants in *LHX3*, but they were also seen in patients with *PROP1*, *POU1F1*, *SOX3*, *GLI2*, *HESX1*, *FGFR1*, and *TCF7L1* variants. Ear-nose-throat abnormalities were present in all patients with *LHX3* variants, but they were also described in patients with variants in *SOX2* and *OTX2*. Genital abnormalities were commonly reported in patients with variants associated with GnD, but they were also described in patients with variants in genes linked to IGHD, such as *GH1* and *GHRHR*, or CPHD without GnD, such as *POU1F1*, and in 1 patient with a variant in *SHH* and preserved pituitary function. Gastrointestinal abnormalities were found in only 1 patient with a *SOX2* variant, whereas cardiac abnormalities were reported in 1 patient with a *GH1*, 1 with a *GHRHR*, 1 with an *FGFR1*, and 1 with a *SOX2* variant. Six patients had other associated genetic conditions: 4 patients with *GH1* variants from the same pedigree had ichthyosis, 1 patient with a *HESX1* variant had a carnitine deficit, and 1 patient with a *SOX3* deletion had hemophilia B.

Novel variants

In total, we have identified 74 novel variants in our cohort, of which 42 are in genes associated with CH and have been published by us previously (Supplemental Table 3). An additional 6 variants were identified through NGS in genes that have not previously been associated with CH. These variants have been functionally tested, and recently published by us (see details below). The remaining 26 of 74 novel variants will be functionally tested before they can be termed as pathogenic. These 26 include variants in *LHX4* ($n = 2$), *GLI2* ($n = 2$), and *GH1* ($n = 2$) identified through Sanger sequencing and 20 other (including 4 in *GLI2*) novel variants identified through NGS studies.

Therefore, 26 of 74 novel variants were identified through NGS, 22 of which were in genes that are not previously associated with CH. These 26 variants were identified in 35 patients from 24 unrelated families out of a total of 81 patients from 51 unrelated pedigrees that were submitted for NGS to GOSgene. Following the variant analysis filtering of ES or GS data, these 26 variants were shortlisted as those considered most likely to be causative of the disease in the respective patients and that segregated with the disease in the pedigree. As mentioned above, only 6 of these 26 novel NGS findings have been published to date, and these include the genes *ARNT2*,[12] *EIF2S3*,[13] *MAGEL2*, *L1CAM*,[14] *RNPC3*,[15] and *PRDM13*.[16] The remaining 20 of 26 novel, potentially pathogenic, variants identified are unpublished and are currently under further investigation through expression and functional analysis. The remaining 27 of 51 pedigrees that were submitted are either yet to have their NGS data analyzed or they did not have a variant in a gene that passed the variant calling or that segregated with the disease.

Discussion

To our knowledge, our overall patient cohort with CH and related disorders is the largest that has been screened for variants in a large number of known causative genes, including both genes implicated in early HP development as well as those implicated in cellular differentiation and proliferation. To date, we have identified variants (Supplemental Table 1) in these known causative genes associated with CH in 143 of 1765 patients with CH and related disorders, who were referred to our center for genetic analysis from national and international centers over a 20-year period. This overall variant detection rate using Sanger sequencing amounts to 8%. This is consistent with other smaller screening studies,[17-20] such as that of Jullien

Table 4 Structural HPAs, midline brain defects, and other associated brain abnormalities seen on magnetic resonance imaging in patients with an identified variant in genes associated with CH

	GH1 ($n = 29$)	*GHRHR* ($n = 22$)	*PROP1* ($n = 17$)	*POU1F1* ($n = 15$)	*SOX2* ($n = 13$)	*GLI2* ($n = 7$)	*SOX3* ($n = 9$)
HPA present	11/19 (57.9)	16/19 (84.2)	15/15 (100)	8/12 (66.7)	8/12 (66.6)	7/7 (100.0)	8/8 (100)
AP normal	8/19 (42.1)	3 /19 (15.8)	0/15 (0)	4/12 (33.3)	4/12 (33.3)	0/7 (0)	0/8 (0)
AP absent	0/19 (0)	0 /19 (0)	0/15 (0)	1/12 (8.3)	0/12 (0)	1/7 (14.3)	0/8 (0)
Small AP (SAP)	11/19 (57.9)	16/19 (84.2)	11/15 (73.3)	7/12 (58.3)	6/12 (50.0)	6/7 (85.7)	7/8 (87.5)
AP enlargement	0/19 (0)	0 /19 (0)	4/15 (26.7)	0/12 (0)	2/12 (16.7)	0/7 (0)	1/8 (12.5)
PP normal	19/19 (100)	19/19 (100)	15/15 (100)	12/12 (100)	12/12 (100)	4/8 (50.0)	2/8 (25.0)
PP absence (PPA)	0/19 (0)	0/19 (0)	0/15 (0)	0/12 (0)	0/12 (0)	1/8 (12.5)	0/8 (0)
Ectopic PP (EPP)	0/19 (0)	0/19 (0)	0/15 (0)	0/12 (0)	0/12 (0)	3/8 (37.5)	6/8 (75.0)
PS normal	19/19 (100)	19/19 (100)	15/15 (100)	11/12 (91.7)	12/12 (100)	6/8 (75)	5/8 (62.5)
PS absence (PSA)	0/19 (0)	0/19 (0)	0/15 (0)	0/12 (0)	0/12 (0)	1/8 (12.5)	1/8 (12.5)
Thin PS	0/19 (0)	0/19 (0)	0/15 (0)	0/12 (0)	0/12 (0)	1/8 (12.5)	1/8 (12.5)
Thick PS	0/19 (0)	0/19 (0)	0/15 (0)	1/12 (8.3)	0/12 (0)	0/8 (0)	0/8 (0)
Midline brain defects	0/19 (0)	0/19 (0)	0/15 (0)	0/12 (0)	8/12 (66.7)	0/8 (0)	0/8 (0)
Other brain abnormalities	2/19 (6.9)	0/19 (0)	0/15 (0)	0/12(0)	8/12 (66.7)	0/8 (0)	1/8 (12.5)
	HESX1 ($n = 8$)	*LHX4* ($n = 4$)	*OTX2* ($n = 5$)	*LHX3* ($n = 4$)	*SHH* ($n = 2$)	*TCF7L1* ($n = 2$)	*FGFR1* ($n = 2$)	*FGF8* ($n = 2$)	*KAL1* ($n = 2$)
HPA present	8/8 (100)	4/4 (100)	5/5 (100)	2/2 (100)	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)
AP normal	0/8 (0)	0/4 (0)	1/5 (20.0)	0/2 (0)	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)
AP absent	1/8 (12.5)	0/4 (0)	0/5 (0)	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)
Small AP (SAP)	7/8 (87.5)	4/4 (100)	4/5 (80.0)	2/2 (100)	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	0/2 (0)	1/2 (50.0)
AP enlargement	0/8 (0)	0/4 (0)	0/5 (0)	0/2 (0)	0/2 (0)	0/2 (0)	0	1/2 (50.0)	0/2 (0)
PP normal	3/8 (37.5)	1/4 (25.0)	0/5 (0)	2/2 (100)	2/2 (100)	1/2 (50.0)	1/2 (50.0)	2/2 (100)	2/2 (100)
PP absence (PPA)	0/8 (0)	0/4 (0)	0/5 (0)	0/2 (0)	0/2 (0)	0/2 (0)	1/2 (50.0)	0/2 (0)	0/2 (0)
Ectopic PP (EPP)	5/8 (62.5)	3/4 (75.0)	5/5 (100)	0/2 (0)	0/2 (0)	1/2 (50.0)	0/2 (0)	0/2 (0)	0/2 (0)
PS normal	5/8 (62.5)	4/4 (100)	5/5 (100)	2/2 (100)	2/2 (100)	2/2 (100)	1/2 (50.0)	2/2 (100)	2/2 (100)
PS absence (PSA)	1/8 (12.5)	0/4 (0)	0/5 (0)	0/2 (0)	0/2 (0)	0/2 (0)	1/2 (50.0)	0/2 (0)	0/2 (0)
Thin PS	2/8 (25.0)	0/4 (0)	0/5 (0)	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)
Thick PS	0/8 (0)	0/4 (0)	0/5 (0)	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)
Midline brain defects	2/8 (25.0)	0/4 (0)	1/5 (20)	0/2 (0)	1/2 (50)	2/2 (100)	1/2 (50.0)	2/2 (100)	2/2 (100)
Other brain abnormalities[a]	0/8 (0)	1/4 (25.0)	3/5 (60.0)	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	1/2 (50.0)	0/2 (0)

Values are presented as *n/N* (%).

AP, anterior pituitary; *EPP*, ectopic PP; *HPA*, hypothalamo-pituitary abnormality; *PP*, posterior pituitary; *PPA*, PP absence; *PS*, pituitary stalk; *PSA*, PS absence.

[a]Other brain abnormalities are as follows: *GH1* (globally reduced white matter and pachygyria/Chiari type 1 anomaly), *SOX2* (hippocampal abnormalities, hypothalamic hamartoma, generalized white matter reduction), *SOX3* (persistent craniopharyngeal canal), *OTX2* (abnormal rotation of the cerebellar vermis/hamartoma in the floor of the third ventricle), and *FGF8* (semilobar holoprosencephaly).

et al[20] who screened 8 known associated CH genes in 1143 patients and identified genetic variants in 7.3% of cases, and Blum et al[18] who screened 9 known genes in 917 patients and identified variants in 10% of cases, confirming that the majority of patients with CH do not currently have an identified genetic etiology. Comparison of variant prevalence in individual patients with various phenotypes between our cohort and previous studies is difficult, given the differing inclusion criteria and screening strategies, with most of the previous studies being multicenter in nature. One of the largest published studies from the Genesis program[18] included patients with documented GHD only, whereas, of those with an identified variant in our cohort, 14.5% of the patients had preserved GH function. Another recent study[20] recruited, through the GENHYPOPIT network, both pediatric and adult patients presenting with at least 1 anterior pituitary hormone deficiency, whereas we only included pediatric cases. Additionally, we reported on some complex and unusual CH-associated phenotypes. Some of these now have an identified molecular cause and have been published, for example, CH with primary ovarian insufficiency[15] and X-linked CH with an unusual form of glucose dysregulation that fluctuates between hyperinsulinemic hypoglycemia and postprandial hyperglycemia.[13]

The optimal Sanger sequencing approach involves screening only genes that are known to be associated with CH. For this reason, it is important to collect as much information as possible about the phenotypes of these patients. There are a number of challenges to this approach, for example, patients with *PROP1* variants may only manifest GHD initially, with other hormone deficiencies evolving with time. Similarly, *POU1F1* variants may present with isolated GHD, and TSHD may evolve, thus making it unclear as to which gene may need to be screened in some patients, and demonstrating how some genes can give rise to variable phenotypes. Furthermore, autosomal dominant *GH1* variants may be associated with CPHD[21]; therefore, these patients may not be routinely screened for *GH1* variants. Phenotypic variability and incomplete or variable penetrance can further complicate the picture,[22,23] which we discuss in more detail later in this section.

With respect to the hormonal phenotypes of these patients evolving over time,[10] intact function of some pituitary hormones can sometimes be interpreted as excluding the presence of variants in certain genes. Of note, 6 patients in our cohort had preserved pituitary function at the time of data collection. However, some of these patients might still develop pituitary hormone deficiencies over time.[10,24] This has important clinical implications. Careful monitoring for additional evolving pituitary hormone deficiencies is essential in these patients. In accordance with previous reports,[18] GHD (86.7%) was the most common endocrinopathy seen in our patients who harbored variants in genes associated with CH, followed by GnD (41.9%), TSHD (35.7%), and ACTHD (19.6%). A similar prevalence of GHD (85.8%) was reported in a large population study of 1213 patients recruited through the GENHYPOPIT network,[20] whereas the prevalence of remaining anterior pituitary deficits was higher than in our study (58.6% for TSHD, 50.5% for GnD, and 49.3% for ACTHD). This is expected because the study from Jullien et al included adult cases, and the risk of developing additional pituitary deficits increases with the patients' age and the years of follow-up.[20,24] Although GH deficiency is more often diagnosed in childhood, confirmation of GnD can only be made in adolescence or adult life in some cases. PRL deficiency seemed to be more common in our patient cohort than in the cohort reported by Blum et al[18] (24.4 % vs 13%). The higher prevalence could be explained by the single-center vs multicenter nature of the 2 studies, with PRL concentrations not being routinely monitored at all centers.

The patients had GH responses that were on average <4 μg/L or ng/mL following dynamic stimulation, or at the time of hypoglycemia. In our cohort, patients with *GHRHR*, *POU1F1*, *PROP1*, *LHX3*, and *SOX3* variants seemed to homogeneously have the lowest GH peak concentrations, followed by those with *GH1* and *HESX1* variants, whereas patients with *GLI2* and *LHX4* variants had a wider range. Conversely, the study by Blum et al[18] reported on a wide range of GH peaks (between 3 and 6 μg/L or ng/mL) in patients with *GH1*, *PROP1*, and *SOX3* variants. However, the study by Blum et al was multicenter in nature and included patients from >30 countries. Hence, the variability between different GH stimulation tests and different assays used for the GH deficiency diagnosis might have contributed to the higher range of GH peaks seen. Additionally, in the study by Blum et al, when >1 stimulation test was conducted to diagnose GH deficiency, the highest GH peak value was collected. We had a similar approach, but our patients did not necessarily receive >1 stimulation test, when other pituitary deficiencies were already present, growth factors were low, and there was clear evidence of linear growth failure.

With respect to the MRI findings, the prevalence of HPA (80.5%) in our cohort was consistent with that (79.7%) of a large cohort of patients[20] with CH, in which 7.3% had variants in CH-associated genes. We have previously reported[10] on the MRI findings of our overall cohort of patients with hypopituitarism +/− midline/optic nerve defects (mainly including patients in whom variants in CH-associated genes were not identified). Similarly, we found a high prevalence of structural HPAs (83.3% of patients with SOD and 98% of patients with CPHD had anterior pituitary abnormalities). The study from Blum et al[18] reported on a much lower prevalence of morphological abnormalities of the pituitary gland (39% of patients without a variant and 24% of those with a variant), but the study included patients with isolated GHD, in whom normal MRI findings and reversal of GHD are more frequent than in patients with multiple hormonal deficiencies.[25]

In our cohort, a small/absent anterior pituitary gland was variably noted in patients with variants in 15 of 16 genes screened (Table 4). An ectopic or absent posterior pituitary

gland was described (with differing prevalence) in patients with variants in *OTX2*, *LHX4*, *SOX3*, *HESX1*, *GLI2*, *FGFR1*, and *TCF7L1*. *SOX3* variants are usually associated with an EPP and no other brain abnormalities; however, we previously described a patient with a eutopic posterior pituitary and a persistent craniopharyngeal canal.[26]

Pituitary stalk abnormalities were only identified in a few patients with *GLI2*, *HESX1*, *POU1F1*, *FGFR1*, *SOX3*, and *OTX2* variants, although mild stalk anomalies are not always reported by neuroradiologists, possibly resulting in an underestimation of their prevalence. We found that pituitary enlargement was found in 4 of 15 (23.5%) of our patients with *PROP1* variants, as previously described,[27] but also in 2 patients with *SOX2*, in 1 patient with an *FGF8*, and in 1 patient with a *SOX3* variant. This finding is very important because in previous work,[18] it has been described as a very strong indicator of *PROP1* variants only. Conversely, pituitary enlargement was not detected in our patients with *LHX3* variants.[28]

Generally, patients with variants in genes involved in pituitary development (*HESX1*, *GLI2*, *SOX2*, *SOX3*, *SHH*, *TCF7L1*, and *FGF8*) were more likely to have structural abnormalities of the HP region than variants in genes regulating GH synthesis and secretion (*GH1* and *GHRHR*). However, patients with variants in genes involved in early HP development can also present with a normal pituitary anatomy. It could be speculated that in these cases, the variants are possibly causing maldevelopment/malfunction of the hypothalamus rather than the pituitary gland, or a functional disconnection between the 2 structures that is not clearly visible on MRI. However, it is important to note that the magnetic resonance images in this cohort were acquired at a specific point in time given the cross-sectional nature of the study. Some of these patients will have received their scans early in life when the images are more difficult to interpret, and some HPAs might be easier to visualize later in life.

Based on the phenotypes described in our patients and those summarized in more recent reviews[1,20,28,29] on this topic, we have proposed an algorithm that the clinician and the geneticist can use to investigate the molecular basis of CH and related disorders. This will obviously be in conjunction with the Sanger sequencing approach, in which there are strong phenotypic features that raise the suspicion of specific genetic defects (eg, it is well established that severe eye defects may be associated with *OTX2* and *SOX2* variants and skeletal malformations and deafness with *LHX3* variants). Some phenotypes may be associated with a number of genes associated with CH, in which case, the cost of screening all of them via Sanger sequencing may not be deemed to be cost-effective in comparison with ES/GS, and this needs to be taken into consideration when considering each individual case. Unfortunately, screening all patients with CH for all phenotypically relevant (Tables 1 and 2) or all HP-related genes, in an unbiased approach using Sanger sequencing, will have significant manpower, cost, and time implications.

Aside from guiding the Sanger sequencing approach, a detailed phenotypic (clinical, hormonal, and MRI) characterization of these patients could also aid the use of a targeted genetic panel approach, a compromise between Sanger and ES/GS approaches. For example, differing panels could be developed depending on the presence of IGHD or CPHD and the different combinations of HP, midline brain, and optic nerve MRI anomalies detected (Figure 1), similar to the HP-associated genes used in the panel-approach 100,000 Genomes project (Genomics England). Upon identification of any potential pathogenic variant, family members can then be Sanger sequenced for that particular change, taking into consideration incomplete or variable penetrance in some cases. The list of these genes is rapidly increasing as our knowledge of HP development in humans and animal models advances. It is becoming clear that CH is a highly heterogeneous disorder.

Incomplete penetrance poses further challenges. Some examples of cases in which incomplete penetrance has been apparent in our cohort are in *HESX1* and *TCF7L1*. First, the functionally significant novel heterozygous missense *HESX1* p.Glu149Lys variant was present in a patient with IGHD and digital abnormalities; however, it was also present in his asymptomatic son, mother, and brother.[30] Second, 2 unrelated patients with forebrain/pituitary defects had novel functionally significant *TCF7L1* variants, p.Arg92Pro and p.Arg400Gln, which were present in their asymptomatic father and paternal uncle (p.Arg92Pro) and asymptomatic mother and 2 siblings (p.Arg400Gln), respectively (Supplemental Table 3).[31]

Furthermore, oligogenicity is an emerging phenomenon in many of these patients. Therefore, it is likely that variants in >1 gene may contribute to different aspects of the phenotype and the severity of the disease.[32-34]

Therefore, this algorithm will need to be constantly updated with the evolving knowledge of these disorders, the discovery of new genes, and the description of new related phenotypes. If adopted by other national and international pediatric endocrine and genetic centers, this would be an interesting tool to audit whether its use can improve the detection rate of variants in known causative genes for these disorders.

Clearly, many pathogenic variants would remain undetected if we continued to perform Sanger sequencing alone using a set clinically based algorithm. Furthermore, it would impede new discoveries or the development of restructured and updated algorithms because, although rarely the case, we know that some genes are occasionally altered in patients with a phenotype that is at variance with a previously published phenotype related to that gene. For example, variants in the Kallmann genes *KAL1*, *FGFR1*, and *FGF8*, that are known to cause Kallmann syndrome/hypogonadotropic hypogonadism, have now been reported in association with SOD or HPE.[35-37] Before these studies, patients with SOD would not have been routinely screened for variants in these genes, which resulted in such potential pathogenic variants remaining undetected. Similarly, *POU1F1* variants

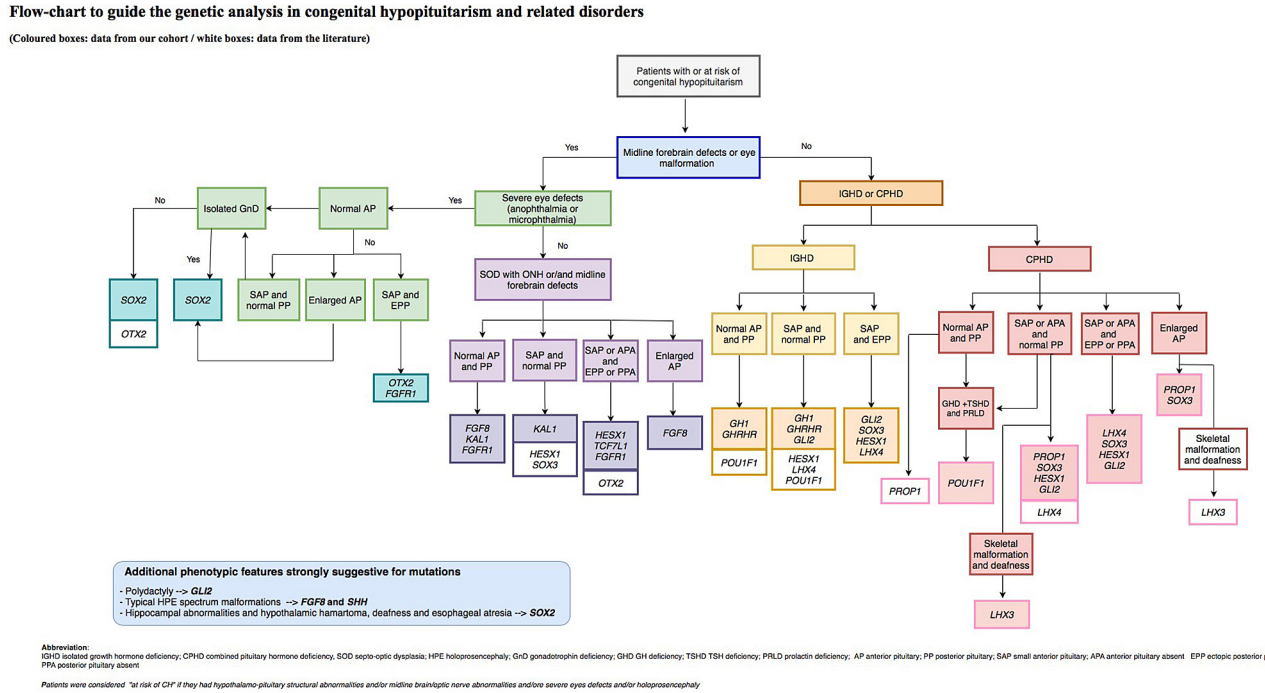

Figure 1 A flowchart to guide clinicians. Based on our sequencing results, this flowchart provides a guide to what known hypothalamo-pituitary associated genes could be Sanger sequenced in patients with certain phenotypes.

are most commonly associated with CPHD but have also been reported in patients with IGHD, and *HESX1* variants, as mentioned, have been identified in patients from different CH subcohorts. The Sanger sequencing approach will therefore only identify variants in genes known to be associated with CH related to a particular phenotype.

In recent years, the science of genomics has been revolutionized by NGS and the constant technological improvements and speed with which we are able to screen an individual's genome. These cost-effective techniques have rapidly replaced the more laborious Sanger sequencing approach in identifying pathogenic variants, allowing more time for functional analysis of novel variants and pathways. Although these techniques allow more rapid validation of patient genotypes and limit the possibility of overlooking pathogenic variants, the data generated are also challenging. The identification of a single causative variant is equivalent to looking for the proverbial needle in a haystack[38] because the average person carries approximately 4 million genetic variations in total.[39]

Nevertheless, NGS has enabled researchers to analyze all genes associated with the HP axis simultaneously, and essentially the entire genome of the patient, revealing novel genes and pathways associated with CH and leading to new discoveries that older screening methods did not allow. As mentioned, we have identified 7 *GLI2* variants, 4 of which were through NGS. These latter 4 variants would not have been identified if the patients had been screened using the standard Sanger sequencing approach because *GLI2* was initially identified in patients with HPE and thus would not have necessarily been routinely screened in patients with IGHD or CPHD. The remaining 3 of 7 *GLI2* variants were identified through Sanger sequencing of *GLI2* in patients with IGHD (1 of 3) and CPHD (2 of 3)[22] (Supplemental Table 3), demonstrating once again how genes associated with CH may be altered in overlapping phenotypic cohorts. Our HPE cohort ($n = 64$) was screened for *GLI2* variants but did not reveal any variants.

Currently, it is not feasible to screen every patient in our CH cohort ($n = 1765$) via NGS because trios would need to be screened, which would have significant resource implications. Ideally trios including both parents and/or other affected or unaffected siblings would be sequenced in parallel to the patient to eliminate benign single-nucleotide variation present in both genes associated with CH and those of uncertain significance during analysis and to identify likely causative variants that segregate with the disease. It is also important to note that there are areas of the genome that have low coverage and that are unable to be comprehensively screened.

The remaining families submitted for NGS in our study that did not have a potential pathogenic variant identified may have a mosaic variant, by which the disease in the child is caused by a pathogenic variant derived from the germ cells of an unaffected parent. Alternatively, the condition could be caused by environmental or epigenetic factors. These avenues of research will no doubt be further explored in the future in an attempt to further unravel the complexity of these disorders.

To summarize, we have identified variants in 51 of 621 (8.2%) patients with CPHD, 61 of 414 (14.7%) patients with IGHD, 10 of 526 (1.9%) patients with SOD, 18 of 59

(30.5%) patients with SED, and 3 of 64 (4.7%) patients with HPE using Sanger sequencing, out of the total number of 1765 patients with CH in our overall cohort. From the patients submitted for NGS, so far we have identified novel variants ($n = 26$) in a further 35 of 81 (46%) patients from 24 of 51 families submitted. The majority of these variants are currently being investigated for functional significance related to the patient phenotype ($n = 20$ variants).

We have identified 108 different variants in a total of 10% (178 of 1765) of patients in our overall CH cohort. Of this 10%, 78% of these cases (138 of 178 patients) have variants that elicit a significant functional consequence and can be considered as pathogenic, leaving the remaining 22% (40 of 178 patients; with 37 different variants) either yet to have functional investigation performed as described above or they have had functional assays performed that have not as yet shown specific functional consequence compared with wild type and cannot technically be termed as "pathogenic" yet, despite in silico prediction models suggesting pathogenicity. The variants with unknown significance are shaded in Supplemental Tables 1 and 3. Therefore, to conclude, we have identified what we can term as pathogenic variants (with functional consequence) in 8% (138 of 1765) of our cohort to date. This is consistent with the literature, where approximately 90% of cases remain unsolved without a functionally significant identified genetic cause underlying their disease. Many other studies report the presence of possible pathogenic variants without functionally testing them, which skews the literature. The need for functional analysis remains vital in determining the pathogenicity of all novel variants. Our study excludes any benign variants or those with a high frequency on control databases. Our study accentuates the need for a high-throughput and cost-effective approach to screening, in which we move more towards targeted panels, microarrays, and ES/GS. For any novel genes, transgenesis in relevant model organisms, such as the mouse or zebrafish, as well as functional analysis using in vivo/in vitro cell- and tissue-based assays, and additionally demonstrating human and murine gene expression in relevant tissues, will further enhance our understanding of CH and related disorders.

Careful phenotyping remains vital not only for the timely identification of evolving pituitary deficiencies requiring life-saving replacement treatments and any eventual associated extrapituitary abnormalities that need specialized supportive strategies, but also for guiding the best genetic analysis approach, which will ultimately take into account differing resources available in different centers/countries.

In those families in which no cause was identified for the CH by Sanger sequencing, the disorder may be caused by variants in as-yet unidentified genes. Furthermore, many cases may be oligogenic, in which there may be multiple, often subtle, changes in different genes that when present in isolation would not elicit a phenotype; however, when present together, they may be causing the disease in the patient. Additionally, in these families as well as in those in which there were no likely genetic candidates identified through NGS, epigenetic, environmental, or other, as-yet unidentified factors may be playing a role and need to be considered. These factors need to be considered in remaining cases, which, admittedly, increases the complexity of uncovering the pathogenesis of the disorder in these patients.

Data Availability

All reported variants in this manuscript have been submitted to ClinVar. The majority of these reported variants are published in journals with a full account of patient clinical features (see Supplemental Table 3 for publication details of the novel variants identified by our laboratory group).

Acknowledgments

The authors thank GOSgene for performing the NGS in the 81 phenotypically unique families, and all previous members of Professor Dattani's laboratory for their contribution toward this research; all national and international collaborating clinicians for sending them DNA samples and clinical phenotypes; and Great Ormond Street Hospital (GOSH) Children's Charity and the Medical Research Foundation, United Kingdom, for funding this work (grant# 535963) and the National Institute for Health and Care Research (NIHR) GOSH Biomedical Research Centre for supporting this work.

Funding

This research was funded by GOSH Children's Charity and the Medical Research Foundation, United Kingdom (grant# 535963). This work is supported by the NIHR GOSH Biomedical Research Centre. The views expressed are those of the author(s) and not necessarily those of the National Health Service, the NIHR, or the Department of Health.

Author Information

Conceptualization: M.T.D.; Data Curation: L.C.G., C.C., M.C.; Formal Analysis: L.C.G., C.C., M.C.; Funding Acquisition: M.T.D.; Investigation: L.C.G., C.C., M.C.; Methodology: L.C.G., C.C., M.C., M.T.D.; Project Administration: L.C.G., C.C., M.C.; Resources: L.C.G., C.C., M.C., M.T.D.; Software: L.C.G., C.C., M.C.; Supervision: M.T.D.; Validation: L.C.G., C.C., M.C., M.T.D.; Visualization: M.T.D.; Writing-original draft: L.C.G.; Writing-review and editing: L.C.G., C.C., M.C., M.T.D.

Ethics Declaration

Ethical committee approval was obtained from UCL Great Ormond Street Institute of Child Health/Great Ormond Street Hospital for Children Joint Research Ethics Committee, and informed written consent was obtained from patients and/or parents, who gave full consent to all clinical and genetic studies carried out on their blood/DNA and any subsequent publication of clinical and genetic information.

Conflict of Interest

The authors declare no conflicts of interest.

Additional Information

The online version of this article (https://doi.org/10.1016/j.gim.2023.100881) contains supplemental material, which is available to authorized users.

References

1. Gregory LC, Dattani MT. The molecular basis of congenital hypopituitarism and related disorders. *J Clin Endocrinol Metab*. 2020;105(6):dgz184. http://doi.org/10.1210/clinem/dgz184
2. García M, Fernández A, Moreno JC. Central hypothyroidism in children. *Endocr Dev*. 2014;26:79-107. http://doi.org/10.1159/000363157
3. Koulouri O, Nicholas AK, Schoenmakers E, et al. A novel thyrotropin-releasing hormone receptor missense mutation (P81R) in central congenital hypothyroidism. *J Clin Endocrinol Metab*. 2016;101(3):847-851. http://doi.org/10.1210/jc.2015-3916
4. Karges B, LeHeup B, Schoenle E, et al. Compound heterozygous and homozygous mutations of the TSHbeta gene as a cause of congenital central hypothyroidism in Europe. *Horm Res*. 2004;62(3):149-155. http://doi.org/10.1159/000080071
5. McDermott MT, Haugen BR, Black JN, Wood WM, Gordon DF, Ridgway EC. Congenital isolated central hypothyroidism caused by a "hot spot" mutation in the thyrotropin-beta gene. *Thyroid*. 2002;12(12):1141-1146. http://doi.org/10.1089/105072502321085252
6. Heinen CA, Losekoot M, Sun Y, et al. Mutations in TBL1X are associated with central hypothyroidism. *J Clin Endocrinol Metab*. 2016;101(12):4564-4573. http://doi.org/10.1210/jc.2016-2531
7. Sun Y, Bak B, Schoenmakers N, et al. Loss-of-function mutations in IGSF1 cause an X-linked syndrome of central hypothyroidism and testicular enlargement. *Nat Genet*. 2012;44(12):1375-1381. http://doi.org/10.1038/ng.2453
8. Joustra SD, Heinen CA, Schoenmakers N, et al. IGSF1 deficiency: lessons from an extensive case series and recommendations for clinical management. *J Clin Endocrinol Metab*. 2016;101(4):1627-1636. http://doi.org/10.1210/jc.2015-3880
9. Mehta A, Hindmarsh PC, Mehta H, et al. Congenital hypopituitarism: clinical, molecular and neuroradiological correlates. *Clin Endocrinol (Oxf)*. 2009;71(3):376-382. http://doi.org/10.1111/j.1365-2265.2009.03572.x
10. Cerbone M, Güemes M, Wade A, Improda N, Dattani M. Endocrine morbidity in midline brain defects: differences between septo-optic dysplasia and related disorders. *EClinicalmedicine*. 2020;19:100224. http://doi.org/10.1016/j.eclinm.2019.11.017
11. McKenna A, Hanna M, Banks E, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res*. 2010;20(9):1297-1303. http://doi.org/10.1101/gr.107524.110
12. Webb EA, AlMutair A, Kelberman D, et al. ARNT2 mutation causes hypopituitarism, post-natal microcephaly, visual and renal anomalies. *Brain*. 2013;136(10):3096-3105. http://doi.org/10.1093/brain/awt218
13. Gregory LC, Ferreira CB, Young-Baird SK, et al. Impaired EIF2S3 function associated with a novel phenotype of X-linked hypopituitarism with glucose dysregulation. *EBiomedicine*. 2019;42:470-480. http://doi.org/10.1016/j.ebiom.2019.03.013
14. Gregory LC, Shah P, Sanner JRF, et al. Mutations in MAGEL2 and L1CAM are associated with congenital hypopituitarism and arthrogryposis. *J Clin Endocrinol Metab*. 2019;104(12):5737-5750. http://doi.org/10.1210/jc.2019-00631
15. Akin L, Rizzoti K, Gregory LC, et al. Pathogenic variants in RNPC3 are associated with hypopituitarism and primary ovarian insufficiency. *Genet Med*. 2022;24(2):384-397. http://doi.org/10.1016/j.gim.2021.09.019
16. Whittaker DE, Oleari R, Gregory LC, et al. A recessive PRDM13 mutation results in congenital hypogonadotropic hypogonadism and cerebellar hypoplasia. *J Clin Invest*. 2021;131(24):e141587. http://doi.org/10.1172/JCI141587
17. De Rienzo F, Mellone S, Bellone S, et al. Frequency of genetic defects in combined pituitary hormone deficiency: a systematic review and analysis of a multicentre Italian cohort. *Clin Endocrinol (Oxf)*. 2015;83(6):849-860. http://doi.org/10.1111/cen.12849
18. Blum WF, Klammt J, Amselem S, et al. Screening a large pediatric cohort with GH deficiency for mutations in genes regulating pituitary development and GH secretion: frequencies, phenotypes and growth outcomes. *EBiomedicine*. 2018;36:390-400. http://doi.org/10.1016/j.ebiom.2018.09.026
19. Reynaud R, Gueydan M, Saveanu A, et al. Genetic screening of combined pituitary hormone deficiency: experience in 195 patients. *J Clin Endocrinol Metab*. 2006;91(9):3329-3336. http://doi.org/10.1210/jc.2005-2173
20. Jullien N, Saveanu A, Vergier J, et al. Clinical lessons learned in constitutional hypopituitarism from two decades of experience in a large international cohort. *Clin Endocrinol (Oxf)*. 2021;94(2):277-289. http://doi.org/10.1111/cen.14355
21. Alatzoglou KS, Dattani MT. Genetic causes and treatment of isolated growth hormone deficiency-an update. *Nat Rev Endocrinol*. 2010;6(10):562-576. http://doi.org/10.1038/nrendo.2010.147
22. Gregory LC, Gaston-Massuet C, Andoniadou CL, et al. The role of the sonic hedgehog signalling pathway in patients with midline defects and congenital hypopituitarism. *Clin Endocrinol (Oxf)*. 2015;82(5):728-738. http://doi.org/10.1111/cen.12637
23. McCabe MJ, Gaston-Massuet C, Tziaferi V, et al. Novel FGF8 mutations associated with recessive holoprosencephaly, craniofacial defects, and hypothalamo-pituitary dysfunction. *J Clin Endocrinol Metab*. 2011;96(10):E1709-E1718. http://doi.org/10.1210/jc.2011-0454
24. Cerbone M, Dattani MT. Progression from isolated growth hormone deficiency to combined pituitary hormone deficiency. *Growth Horm IGF Res*. 2017;37:19-25. http://doi.org/10.1016/j.ghir.2017.10.005
25. Cerbone M, Ederies A, Losa L, Moreno C, Spoudeas HA. Neuroradiological features in a cohort of 53 children with thickened pituitary stalk (TPS) and/or idiopathic central diabetes insipidus (ICDI). Free Communication BSPED. Endocrine Abstracts. 2015. https://www.endocrine-abstracts.org/ea/0039/ea0039oc6.10
26. Alatzoglou KS, Azriyanti A, Rogers N, et al. SOX3 deletion in mouse and human is associated with persistence of the craniopharyngeal canal. *J Clin Endocrinol Metab*. 2014;99(12):E2702-E2708. http://doi.org/10.1210/jc.2014-1160
27. Riepe FG, Partsch CJ, Blankenstein O, Mönig H, Pfäffle RW, Sippell WG. Longitudinal imaging reveals pituitary enlargement preceding hypoplasia in two brothers with combined pituitary hormone deficiency attributable to PROP1 mutation. *J Clin Endocrinol Metab*. 2001;86(9):4353-4357. http://doi.org/10.1210/jcem.86.9.7828

28. Xatzipsalti M, Voutetakis A, Stamoyannou L, Chrousos GP, Kanaka-Gantenbein C. Congenital hypopituitarism: various genes, various phenotypes. *Horm Metab Res*. 2019;51(2):81-90. http://doi.org/10.1055/a-0822-3637
29. Castinetti F, Reynaud R, Saveanu A, et al. Mechanisms in endocrinology: an update in the genetic aetiologies of combined pituitary hormone deficiency. *Eur J Endocrinol*. 2016;174(6):R239-R247. http://doi.org/10.1530/EJE-15-1095
30. McNay DE, Turton JP, Kelberman D, et al. HESX1 mutations are an uncommon cause of septooptic dysplasia and hypopituitarism. *J Clin Endocrinol Metab*. 2007;92(2):691-697. http://doi.org/10.1210/jc.2006-1609
31. Gaston-Massuet C, McCabe MJ, Scagliotti V, et al. Transcription factor 7-like 1 is involved in hypothalamo-pituitary axis development in mice and humans. *Proc Natl Acad Sci U S A*. 2016;113(5):E548-E557. http://doi.org/10.1073/pnas.1503346113
32. Weiss B, Eberle B, Roeth R, et al. Evidence that non-syndromic familial tall stature has an oligogenic origin including ciliary genes. *Front Endocrinol (Lausanne)*. 2021;12:660731. http://doi.org/10.3389/fendo.2021.660731
33. Argente J, Tatton-Brown K, Lehwalder D, Pfäffle R. Genetics of growth disorders-which patients require genetic testing? *Front Endocrinol (Lausanne)*. 2019;10:602. http://doi.org/10.3389/fendo.2019.00602
34. Fang Q, George AS, Brinkmeier ML, et al. Genetics of combined pituitary hormone deficiency: roadmap into the genome era. *Endocr Rev*. 2016;37(6):636-675. http://doi.org/10.1210/er.2016-1101
35. Raivio T, Avbelj M, McCabe MJ, et al. Genetic overlap in Kallmann syndrome, combined pituitary hormone deficiency, and septo-optic dysplasia. *J Clin Endocrinol Metab*. 2012;97(4):E694-E699. http://doi.org/10.1210/jc.2011-2938
36. McCabe MJ, Hu Y, Gregory LC, et al. Novel application of luciferase assay for the in vitro functional assessment of KAL1 variants in three females with septo-optic dysplasia (SOD). *Mol Cell Endocrinol*. 2015;417:63-72. http://doi.org/10.1016/j.mce.2015.09.010
37. Fukami M, Iso M, Sato N, et al. Submicroscopic deletion involving the fibroblast growth factor receptor 1 gene in a patient with combined pituitary hormone deficiency. *Endocr J*. 2013;60(8):1013-1020. http://doi.org/10.1507/endocrj.ej13-0023
38. Eichler EE. Genetic variation, comparative genomics, and the diagnosis of disease. *N Engl J Med*. 2019;381(1):64-74. http://doi.org/10.1056/NEJMra1809315
39. 1000 Genomes Project Consortium, Auton A, Brooks LD, et al. A global reference for human genetic variation. *Nature*. 2015;526(7571):68-74. http://doi.org/10.1038/nature15393