New Genetic Findings in a Large Cohort of Congenital Hypogonadotropic Hypogonadism: Insights into Reproductive Endocrinology and Molecular Pathogenesis

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Abstract

Context: Congenital hypogonadotropic hypogonadism (CHH) is a rare condition caused by GnRH deficiency. Several genes have been associated with the pathogenesis of CHH, but most cases still remain without a molecular diagnosis. The advent of next-generation sequencing (NGS) has allowed the simultaneous genotyping of several regions, faster, making possible the extension of the genetic knowledge of CHH.

Objective: Genetic characterization of a large cohort of Brazilian CHH patients.

Design and patients: A cohort of 130 unrelated patients (91 males, 39 females) with CHH (75 normosmic CHH, 55 Kallmann syndrome) was studied using a panel containing 36 CHH associated genes.

Results: Potential pathogenic or probably pathogenic variants were identified in 43 (33%) CHH patients. The genes *ANOS1*, *FGFR1* and *GNRHR* were the most frequently affected. A novel homozygous splice-site mutation was identified in the *GNRH1* gene and a deletion of the entire coding sequence was identified in *SOX10*. Deleterious variants in the *IGSF10* gene were identified in two patients with reversible normosmic CHH. Notably, 6.9% of the patients had rare variants in more than one gene. Rare variants were also identified in *SPRY4*, *IL17RD*, *FGF17*, *IGSF1* and *FLRT3* genes.

Conclusions: This is a large study of the molecular genetics of CHH providing new genetic findings for this complex and heterogeneous genetic condition. NGS has been shown to be a fast, reliable and effective tool in the molecular diagnosis of congenital CHH and being able to targeting clinical genetic testing in the future.

Introduction

Normal pubertal development is dependent on the secretion and proper action of the gonadotropin-releasing hormone (GnRH), produced by a small number of neurons located in the ventromedial hypothalamus (1, 2). GnRH secreting neurons have their origin outside the central nervous system (CNS), in the olfactory placode, and migrate in association with olfactory neurons during the embryonic life to their final hypothalamic location (1-3). Congenital hypogonadotropic hypogonadism (CHH) is a rare condition caused by GnRH deficiency, clinically manifested as sexual infantilism, low serum steroid concentrations associated to low or normal levels of gonadotropins, and otherwise normal pituitary functions. Because of the common embryonic origin of olfactory and GnRH neurons, CHH is often associated with olfactory defects, characterizing Kallmann syndrome (KS), which corresponds to approximately 50 to 60% of CHH cases (3).

CHH is a clinical and genetically heterogeneous condition. In the last decades, a growing list of genes has been implicated in the molecular pathogenesis of CHH. These genes are involved in different stages of the regulation of GnRH production, secretion or action, as well as in the process of embryonic neuronal migration (3-5). Some of these genes have been classically known to cause CHH, such as *ANOS1* (previously termed *KAL1),* which is associated with the X-linked form of KS, with a severe reproductive phenotype, whereas *GNRHR* mutations are a common cause of autosomal recessive normosmic CHH (nCHH) (3, 6-10). On the other hand, mutations in genes such as *FGFR1*, *FGF8*, *PROK2* and *PROKR2* have been associated with substantial intra and interfamilial phenotypic variability, with affected members presenting with KS, nCHH, isolated anosmia, isolated lip-palatine cleft, constitutional delay of growth and puberty (CDGP), or even normal phenotype (5, 8, 11, 12). In addition, the advancement of genetic screening techniques allowed the identification of variants in more than one gene in the same patient, changing the previous concept of CHH as a strictly monogenic

disease (13-15). Currently, more than 30 genes have been associated with syndromic and non-syndromic forms of CHH (3-5). However, despite the vast history of the genetic causes of CHH, until recently only about 30% of these patients had a recognized molecular diagnosis [6,13].

In the last two decades, we have been studying the genetic causes of GnRH deficiency by screening CHH associated genes, including *ANOS1, GNRHR, FGFR1, FGF8, PROKR2, PROK2, TACR3, TAC3, KISS1R* and *KISS1* using classical genetic methods, such as Sanger sequencing and Multiplex Ligation-dependent Probe Amplification(9, 10, 16-23). Nevertheless, the majority of the patients remain undiagnosed from the molecular point of view. With the growing number of genes and the genetic complexity of CHH, it has become almost impossible to keep the screening of all candidate genes updated using the traditional techniques. The recent advent of next-generation sequencing (NGS) has allowed the screening of a large numbers of genes quickly and efficiently.

Materials and Methods

Patients

Patients were selected from the outpatient clinic of the Developmental Endocrinology Unit, Hospital das Clínicas da Faculdade de Medicina, Universidade de São Paulo (HCFMUSP) and from the Hospital das Clínicas da Universidade de Campinas (Unicamp). The project was approved by the ethics committee of Sao Paulo University. All patients or their caregivers signed the informed consent form for participation in the research.

A total of 130 unrelated Brazilian patients with CHH (75 nCHH and 55 KS, including 91 men and 39 women) was studied by targeted NGS. The clinical criteria for inclusion of the patients were lack of appearance or incomplete development of secondary sexual

characteristics after 16 years of age in girls and 18 years of age in boys; subnormal concentrations of sexual steroids (testosterone or estradiol), subnormal or normal luteinizing hormone (LH) and follicle stimulating hormone (FSH) concentrations, absence of other associated pituitary deficiencies, and normal imaging of CNS. The University of Pennsylvania Smell Identification Test was used to diagnose olfactory abnormalities (Smell Identification Test, Philadelphia, PA) (24). Additional phenotypic abnormalities were observed in some patients as follows: congenital hearing impairment (4.6%), cognitive deficit (3.8%), epilepsy (2.3%), heart defects (1.5%), cleft palate (1.5%), dental anomalies (1.5%), congenital renal anomalies (1.5%), skeletal anomalies (0.8%), bimanual synkinesis (0.8%). Uni or bilateral cryptorchidism was present in 29.7% of the men and all patients whose penile size could be assessed pre-treatment had micropenis. Family history of CHH or pubertal delay was present in 20 patients (15.4%).

Most patients (107 cases) were selected from a large cohort previously studied by Sanger sequencing for the classical CHH associated genes (9, 10, 17-23) (*ANOS1, GNRHR, GNRH1, FGFR1, FGF8, PROKR2, PROK2, TACR3, TAC3, KISS1R* and *KISS1*) (Supplemental table 1).

Genetic Analysis

Genomic DNA was extracted from peripheral-blood leukocytes of all patients and their relatives when available. A custom SureSelectXT DNA target enrichment panel (Agilent Technologies Inc) was designed to capture 36 known and candidate CHH genes. All exons, the 25 base pairs of intronic flanking region and 5' and 3' untranslated region of each gene were sequenced. The panel covered genes classically associated with CHH, genes rarely or anecdotally reported in CHH patients and seven candidate genes, namely *EBF2*, *GHSR, MSX1*, *MKRN3*, *OTX2*, *IGSF1* and *IGFALS*, based on data from biochemical/biological pathways, or present in correlated conditions affecting the gonadotropic axis (Table 1). Sequence capture was performed according to the SureSelectXT Target Enrichment protocol using Agilent Bravo

Automated Liquid Handling Platform; sequencing was performed on a NextSeq 500 (Illumina, Inc). All preparations and sequencing were performed at the University of Sao Paulo (Large Scale Sequencing Laboratory – SELA).

Variant Analysis

Data analysis was performed using in-house bioinformatics pipeline. Briefly, paired-end reads were aligned to the b37+decoy version of the human genome using *bwa*, followed by downstream processing of the aligned reads (sorting, merging, and indexing) using the *bamsort* tool from biobambam2 (25, 26). We used *fastqc* (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and *qualimap* to assess sequencing quality and target regions coverage metrics, respectively (27). Variant calling was performed using *freebayes.* Finally, variants were normalized with *vt* and annotated using *annovar* (28, 29).

The panel sequencing data were screened for rare (minor allele frequency less than 1% in public and in-house databases), nonsynonymous, located in exonic regions and consensus splice site variants. Subsequently, variant filtration prioritized genes on the basis of their potential to be pathogenic: loss-of-function variants and variants predicted to be pathogenic by at least two *in silico* programs (SIFT, PolyPhen2, Mutation Taster, Mutation Assessor, Functional Analysis through Hidden Markov Models - FATHMM, Protein Variation Effect Analyzer - PROVEAN, Combined Annotation Dependent Depletion - CADD). For variants affecting splicing sites, were used *in silico* prediction sites: Human Splicing Finder Version 2.4.1 (http://www.umd.be/HSF/) and NetGene2 (http: /www.cbs.dtu.dk/services/ NetGene2/). *In silico* analysis included the exclusive selection of variants with GERP (genomic evolutionary rate profiling) > 2.5.

Population data criteria were evaluated with the help two public genomic databases: Genome Aggregation Database (GnomAD: http://gnomad.broadinstitute.org/), Online Archive of Brazilian Mutations (ABraOM: http://abraom.ib.usp.br/) (30), composed of 609 exomas of healthy Brazilians elderly, and an in-house database comprising data from exomic sequencing covering 774 alleles (Large Scale Sequencing Laboratory – SELA). All rare variants considered potentially pathogenic were visually confirmed in the Integrative Genomics Viewer (IGV) program, from the BAM file. All potential disease-causing variants were confirmed by Sanger sequencing. Familial segregation was performed whenever genetic material of relatives was available.

The variants were further classified for pathogenicity according to the criteria of the American College of Medical Genetics (ACMG) and the Association for Molecular Pathology (AMP) guidelines (31). Furthermore, all these variants were interpreted based in the phenotype to which the variant was associated, pattern of genetic inheritance and segregation data.

Copy-number variation (CNV) analysis

Copy number variations (CNVs) were screened using the CONTRA (REF: PMID 22474122). To infer segmental copy-number status for each patient, each individual BAM file was compared to a pooled reference constituted of all individuals included in the analysis.

RNA isolation and RT-PCR for the GnRH1

Lymphocytes were isolated from peripheral lymphocytes from the index case, his mother and a healthy adult control with normal pubertal development using the Ficoll-Hypaque method. Total RNA was extracted using Trizol® (Invitrogen, Carlsbad, Ca) and reverse transcribed using the QuantiTect Reverse Transcription Kit (*Qiagen, Germany, 2009*) according to the manufacturer's instructions. *GNRH1* cDNA was amplified with a specific primer pair located at

exons 1 and 2, comprising the splicing region of interest between exons 1 and 2. PCR product was visualized in 2% agarose gel, purified and automatically sequenced in an ABI Prism Genetic Analyzer 3100 (Perkin-Elmer).

Multiplex ligation-dependent probe amplification (MLPA)

SOX10 gene deletion was confirmed using the SALSA MLPA P186 PAX3-MITF-SOX10 probemix (MRC Holland, Amsterdam, Netherlands) that includes probes for all four exons of *SOX10* gene. The peak area for each probe was determined with GeneScan analysis software V.3.7 and normalized by combined value of the control probes in corresponding lane. This relative value was compared to those obtained in two normal controls (DNA references) using Cofallyser. Final probe ratios of 0.5 were considered positive for heterozygous deletion.

Results

Genetic findings

The lowest coverage was 98% of the targeted regions with coverage greater than 20 times, and the vast majority of patients had coverage greater than 50 times in 99% of the target regions, indicating that the quality of the sequencing was very satisfactory.

Potentially pathogenic variants were identified in 77 (59.2%) of the 130 CHH patients studied with targeted NGS approach. A total of 89 different variants were detected in 29 genes (Table 2 and Figure 1). However, after applying the ACMG-AMP guidelines, the number of individuals considered to carry pathogenic or probably pathogenic variants dropped to 43 (33%) (Figure 2). Clinical and molecular characterization of all patients is described in Supplemental table 2. Variants in genes with known autosomal recessive inheritance were not considered causative of the phenotype when identified in the heterozygous state. Variants which did not

segregate in the family or which were present in healthy individuals databases were classified as benign. Detailed data on variants considered pathogenic and probably pathogenic are exposed in Table 3. No rare variants were identified in *KISS1*, *KISS1R*, *HS6ST1*; genes previously associated to CHH, and in the candidate genes *DUSP6*, *GHSR*, *MSX1* and *MKRN3*.

Pathogenic and probably pathogenic variants

Variants in classical CHH genes

The majority of pathogenic or probably pathogenic variants were identified in classical CHH genes. The *FGFR1* was the most prevalent gene with eleven variants, five in KS and six in nCHH patients (8.5%). Additional nonreproductive abnormalities were observed in three patients with *FGFR1* mutations: Patient 16 (p.Arg250Trp) with dental agenesis, epilepsy, mild cognitive deficit, unilateral hearing loss and synkinesia, Patient 19 (p.Ala343Val) with bilateral hearing loss and Patient 23 (p.Cys767Tyr) with ogival palate and unilateral deafness. *ANOS1* mutations were identified in six men with KS, all with complete hypogonadism phenotype (4.6%). Patient 31 (p.Ala30fs) had bilateral cryptorchidism, ogival palate and mild cognitive deficit. Biallelic mutations in the *GNRHR* gene were identified in three patients with CHH (2.3%) (Table 2). One of these patients presented with reversal hypogonadism with subsequent relapse (Patient 28, carrying the variants p.Val134Gly/p.Arg262Gln). The well-known partially inactivating variant p.Gln106Arg was identified in four patients, but not considered enough to explain the phenotype because it was in the heterozygous state in all instances (7, 10) .

A novel mutation in GNRH1

A homozygous splicing-site mutation (c.142-2A>C) in the *GNRH1* gene was identified in a male patient (Patient 52), with no other molecular variant identified. The patient presented at 18 years of age complaining of lack of pubertal development, history of bilateral cryptorchidism,

surgically corrected in childhood, and no other associated condition. He was born to consanguineous parents, and had no other affected member in the family. At physical examination, he had pubic hair Tanner stage III, micropenis, testicular size 1.5 x 1.0 cm, left and 2.0 x 1.4 right, body mass index (BMI) 27 kg/m^2, with eunuchoid proportions. He reported a normal sense of smell, confirmed by smell test. Hormonal evaluation revealed total testosterone 11 ng/dL (prepubertal < 12 ng/dL, adults 271 - 965 ng/dL), LH < 0.1 IU/L (1.5 - 8.4IU/L), FSH < 1 IU/L (1.7 - 12.4 IU/L). Other tests of anterior pituitary function were normal, as well as a magnetic resonance imaging (MRI) scan of the hypothalamo-pituitary region, establishing the diagnosis of CHH. The c.142-2A>C variant was identified in the heterozygous state in the patient's unaffected sister and his parents (Figure 3a). Human Splicing Finder Version 2.4.1 and NetGene2 *in silico* tools predicted the c.142-2A>C variant to damage the natural acceptor splicing site in intron 2-3 while simultaneously activating a cryptic acceptor site. In fact, an aberrant *GNRH1* transcript harboring a deletion of 4 bp (c.142-145delATAG) was detected in patient's lymphocytes. This deletion leads to a frameshift and a subsequent premature stop codon at aminoacid position 74. mRNA levels of the wild type and mutated *GNRH1* were detected in lymphocytes from the patient and his mother (Figure 3b).

CHD7 variants

A total of 12 distinct rare *CHD7* variants were identified in 14 patients with KS and nCHH (Table 2). However, after applying the ACMG criteria, 12 variants were lately classified as uncertain clinical significance (VUS) and two, p.G2655fs and p.2690_2692del, identified in Patients 9 and 10, were classified as pathogenic and probably pathogenic, respectively. Patient 9 had KS associated to hearing loss and body asymmetry and Patient 10, also heterozygous for the p.Gln106Arg *GNRHR* mutation, had KS, dental agenesis, high arched palate and kidney anomalies (Supplemental table 2). Five of these patients had associated pathogenic variants in known CHH genes, which would be alone sufficient to cause the phenotype (*FGFR1, ANOS1,*

TAC3, PROK2) (Table 2). Patient 3, whose only variant was a *CHD7* p.Ser699Gly, of uncertain significance, had KS associated with unilateral renal agenesis and unilateral deafness.

A large deletion of the SOX10 gene

A large deletion involving the entire coding region of the *SOX10* gene was identified in the heterozygous state in a woman with KS and bilateral sensorineural deafness (Patient 60). She presented at 19 years of age, with lack of pubertal development, primary amenorrhea and absent sense of smell. She had a severe bilateral sensorineural hearing loss. At physical examination, she had no pubic hair and breast development Tanner stage I, BMI 19 kg/m^2, and bilateral clinodactyly. Skin, hair or iris pigmentation abnormalities were absence. Olfactory test confirmed anosmia. Hormonal evaluation revealed pre-pubertal levels of serum estradiol, LH and FSH. MRI scan of CNS showed olfactory bulb aplasia and pelvic ultrasound showed hypoplasic uterus and ovaries. She was born to non-consanguineous parents, and had no family background of delayed puberty or hearing loss. The *SOX10* heterozygous deletion was confirmed by MLPA assay. An additional, *SOX10* variant (p.Val340Met) was identified in a woman with nCHH and no other associated conditions (Patient 75), who also harbored a *OTX2* variant (p.Ala55Ser), both classified as VUS.

Other rare variants

Variants predicted to be pathogenic or probably pathogenic were identified in *IGSF10, SPRY4, IL17RD, and FGF17*, some of them in oligogenicity with variants in other genes.

Rare *IGSF10* variants were initially identified in eight patients, but after applying variant selection criteria, only Patient 49 (p.Asp2277Gly and p.Thr1538Ile), and Patient 47 (p.Gln433fs), both with nCHH, were found to carry probably pathogenic and pathogenic variants, respectively.

A probably pathogenic variant was identified in *FGF17* (p.Val66Met) in a man with nCHH and no additional phenotype (Patient 72). Two different pathogenic *IL17RD* variants, one homozygous (p.Pro566Leu) and one heterozygous (p.Glu536fs) were identified in a male patient with nCHH (Patient 15), who carried heterozygous rare variants in *FGFR1* (p.Pro28Leu) and *DMXL2* (p.Ser1724Leu) as well.

Rare variants were identified in *FLRT3* (p.Tyr274Cys) in a woman with nCHH and sensorineural hearing loss (Patient 59), and in *IGSF1,* a candidate gene that has not been previously described in CHH patients. The *IGSF1* p.Pro237Ala was identified in Patient 74, a woman with nCHH and no other endocrinopathies, with a family history of pubertal delay. Both *FLRT3* and *IGSF1* variants, however, are still considered of uncertain clinical significance, since the causative role of these genes in CHH is not yet sufficiently established in the literature.

CHH reversal

CHH reversal was defined as the presence of normal adult testosterone levels after hormone replacement was discontinued for an appropriate washout period (3–6 months for testosterone injections), and no symptoms of hypogonadism after cessation of treatment [32]. Hypogonadism reversal was observed in 6/92 male patients participating in this study (6.5%). Patients 47 and 49 had nCHH and probably pathogenic variants in the *IGSF10* gene (p.Gln433fs and p.Asp2277Gly/Thr1538Ile, respectively). Patient 28, also with nCHH, had a compound heterozygous mutation in the *GNRHR* gene (p.Val134Gly/Arg262Gln). Patients 27, 57 and 90 had KS, without established molecular diagnosis.

Oligogenic findings

Nine patients (6.9%) presented with rare variants classified as pathogenic, probably pathogenic or VUS in more than one gene (Table 2). The gene most commonly identified in

association with others was *CHD7* followed by *FGFR1*. In the majority of these patients, however, one of the variants alone would be sufficient to explain the phenotype. The role of these variants together in the pathogenesis of CHH is unknown, and one cannot discard the possibility of a synergistic role for these variants in the patients' phenotype.

Discussion

We investigated the presence of rare genetic variants in patients with KS and nCHH, using a 36 genes panel, which included 29 genes previously associated with CHH and seven candidate genes. With this approach it was possible to identify pathogenic or probably pathogenic variants in 42/130 patients (32.3%). Miraoui, H *et al.* (2013) analyzed 17 genes in 350 CHH individuals and identified mutations in 35% of the cases (32). Most recently, Cassatella, D. *et al*. (2018) screened 24 genes on 116 CHH patients and identified rare variants in 51% of the cases, though with less rigorous selection criteria than ours (33). Although the selection of genes was somewhat different form ours, the most frequently affected genes were quite the same in all works (32, 33). In the literature, the percentage of patients with identified mutations varies from 30 to 50% (12, 32-36). This variation may be probably attributed to factors as choice of genes to be studied, technique used, variants selection criteria and interpretation of data. It is remarkable to observe that though targeted NGS was able to increase the percentage of patients with a molecular diagnosis, genes classically associated to CHH remains responsible for most of the cases in all studies.

In this work, *FGFR1* and *ANOS1* were the most frequently affected genes, followed by *GNRHR*. It is important to remind that 107/130 patients studied here had already been screened by Sanger sequencing for the main CHH genes, as part of a previously studied cohort of 260 patients, 22.3% of them with established molecular diagnosis. Notably, many patients with previously identified deleterious mutation in genes as *ANOS1* and *GNRHR*, among others, were

not included in the present study. Considering the new genetic findings identified in this panel in addition to the mutations previously identified by Sanger sequencing, we have a prevalence 35% (99/283) of all patients with molecular diagnosis, confirming *FGFR1* (11.2%), *ANOS1* (10.2%), *and GNRHR* (9.4%) as the most frequently affected genes, considering only nCHH patients for *GNRHR* and only KS patients for *ANOS1*. These findings strengthen the importance of these genes in the pathogenesis of CHH. The prevalence of mutations in the classical CHH genes in our cohort is in agreement to the described in the literature, although the frequency of mutations in each specific gene is somehow variable in different publications, possibly due to baseline differences in the selection of populations studied, ethnicity, frequency of familial cases and consanguineous families (5, 10, 12, 32, 34, 35, 37-41).

GNRH1 has been considered for several years an obvious candidate for mutations in patients with nCHH, but so far only three different homozygous mutations have been described (Figure 3c) (42-44). In our study, we identified a novel *GNRH1* homozygous mutation located at the consensus splicing site region upstream the start of exon 2 (c.142-2A>C). Interestingly, this mutation does not affect the transcription of GnRH decapeptide, completely encoded by exon 1. However, it predicts a loss of the GnRH-associated peptide (GAP), which corresponds to the same region that is lost in the natural animal model of deficiency of GnRH, the *hpg* mouse (45). One plausible hypothesis is that the variant, resulting in a premature stop/termination codon (PTC), lead to *GNRH1* mRNA nonsense-mediated decay (NMD), in which case no GnRH is produced, thus explaining the phenotype. Though, the recognition of the PTC depends of several factors including the distance between the nonsense codon and the downstream exon-exon junction. In the aberrant transcript described here this distance is 14 base pair, less than 50-55 nucleotides required to effective RNAm degradation triggered by Nonsense-Mediated mRNA Decay. This unprecedented finding of a putative loss-of-function variant in this position

gives us the opportunity to elucidate the functional importance of the GAP region, which has not yet a defined function (45).

The *CHD7* gene encodes a chromodomain helicase DNA binding protein 7, which plays a relevant role in the neuronal development of the olfactory bulb and GnRH neurons (46). *CHD7* loss-of-function mutations of the gene have been classically associated with CHARGE syndrome (OMIM 214800), a multisystem disorder characterized by the presence of coloboma, cardiac abnormalities, choanal atresia, developmental delay, genital and ear anomalies (46). The seminal study conducted by Kim H *et al*. (2008) first demonstrated heterozygous mutations of *CHD7* in patients with KS and no other CHARGE features (46). To date, the overall prevalence of *CHD7* mutations is approximately 7% in patients with KS, however, when associated with the deafness and KS, it can achieve nearly 40% of patients (4, 5). Notably, *CHD7* sequencing analysis had not been previously performed in this Brazilian cohort. Although *CHD7* mutations have also been reported in a high frequency in other CHH cohorts (5), this selection bias actually could partially explain the higher prevalence of *CHD7* findings in our cohort. It is important to take into consideration that the *CHD7* is a large gene with a lot of rare missense variants reported in GnomAD. It is therefore not uncommon to find a rare variant unrelated to the phenotype in such a gene simply by chance. Consequently, most of the variants identified in this gene should be interpreted with caution, and in this study most of them were classified as VUS. Three patients with *CHD7* variants had additional phenotypic features (deafness and kidney defects) corroborating the concept that these characteristics are more frequently in patients with mutations in this gene (5, 46). Segregation analysis was possible in three families and in two of them (Patients 7 and 12), the same variants were identified in healthy relatives (p.Ala2259Thr and p.Leu2806Val), weakening the importance of *CHD7* in the pathogenesis of CHH in these patients.

Recently, the knowledge about *IGSF10* functions related to the gonadotrophic axis and

GnRH neurons has been deepened. In 2016, The *IGSF10* gene has been associated with self-limited delayed puberty and functional forms of GnRH deficiency (47). In the present study, we identified probably pathogenic or pathogenic *IGSF10* variants in two male patients with nCHH. Interestingly, both patients presented with reversion of the hypogonadism. Reversibility has been described in approximately 10% of CHH cases after sex steroids replacement therapy, especially in patients with partial CHH (48). In the present study, four other patients had reversible CHH, one with a compound heterozygous mutation in *GNRHR*, a gene previously associated to reversibility of hypogonadism (10), and three patients with no identified pathogenic mutations so far. Howard, S *et al*. (2016) hypothesized that defects in *IGSF10* leads to delayed puberty through dysregulation of GnRH neuronal migration during embryonic development (47). It is possible that *IGSF10* plays a temporal role in GnRH neurons migration and plasticity modulation, allowing the immature neurons to complete their development when submitted to sex hormones stimulation. We hypothesize that *IGSF10* defects may be represent a common basis to different presentations of transient GnRH deficiency, including functional hypogonadotropic hypogonadism, self-limited delayed puberty and reversible CHH. Functional studies to assess the consequences of these specific variants could clarify these issues, but the current results already enrich our knowledge about the likely role of *IGSF10* in CHH.

The *FGFR1* signalling complex plays a crucial role in the migration of olfactory and GnRH neurons, acting in synergy with anosmin-1, and its involvement in the pathogenesis of CHH is well-known (38, 49, 50). Genes involved in the *FGFR1* signalling pathway were associated with CHH, including *IL17RD*, *HS6ST1*, *FGF17*, *FLRT3* and *SPRY4* (32, 51).

Mutations in the *IL17RD* gene were initially described in KS associated with deafness and subsequently in some cases of puberty delay (32, 52). Previous studies have suggested that only one allelic defect in the *IL17RD* gene would probably not be sufficient to cause the CHH phenotype and additional affected alleles in the same or other genes would be required for

the KS phenotype (32, 52). Therefore, it was suggested that variants in heterozygosis in *IL17RD* could result in reproductive endocrine phenotypes of varying severity (32). In Patient 15, with nCHH without deafness, two variants were identified in *IL17RD*, a homozygous missense in associated with a heterozygous frameshift. This patient also presented heterozygous variants in *FGFR1* and *DMXL2* genes. The variants of *IL17RD* and *FGFR1* were classified as pathogenic and the *DMXL2* variant as probably benign; the degree of contribution of each variants to the CHH phenotype is unknown. Until now, mutations in *IL17RD* gene had been described only in patients with KS and puberty delay, often with the associated deafness phenotype.

The *FLRT3*, *FGF17* and *SPRY4* include the set of genes expressed and regulated during development in a similar manner to the *FGF8.* Miraoui, H *et al.* (2013) described variants in *FLRT3* in KS patients and in *FGF17* and *SPRY4* in both KS and nCHH patients (32). We identified two variants in *SPRY4* in patients with nCHH: p.Ser259Phe (Patient 48), classified as probably pathogenic, and p.Ser241Tyr (Patient 17), classified as benign for its presence in the heterozygous and homozygous state in ABraOM and GnomAD databases. Indeed, Patient 17 also carried a *FGFR1* mutation (p.Pro286fs), enough to justify the phenotype. Heterozygous variants in *FGF17* (p.Val66Met) and *FLRT3* (p.Tyr274Cys) were identified in patients with nCHH and classified as probably pathogenic and VUS, respectively. Patient 59, with the *FLRT3* p.Tyr274Cys variant, had normal olfaction and unilateral hearing loss, different from the initial description of the association of this gene with KS without deafness (32). Considering the relation of *FLRT3* with the *FGF8*/*FGFR1* pathway, which is associated a variable phenotypic spectrum, we raise the possibility that the patient described here might represent a new variation of the phenotype associated with *FLRT3* defects.

SOX10 gene plays a major role in neural crest development and is known as the causative gene of Waardenburg syndrome (WS), characterized by skin/hair/iris hypopigmentation, deafness, and other variable neuronal defects. *SOX10* has recently been

associated to KS and hearing loss (53). We describe here a deletion of the whole coding sequence of *SOX10* in a patient with KS and deafness. This finding provides further evidence for the significance of *SOX10* mutations as genetic cause of KS, especially when associated with deafness, and suggests a lack of genotype-phenotype correlation since a large deletion, as described here, is not associated with the complete Waardenburg syndrome phenotype.

OTX2 mutations have been described in association with a clinical syndrome involving multiple pituitary gland deficiency or with retinal dystrophy without pituitary deficiencies (54). We identified an *OTX2* variant in association with a *SOX10* variant, both classified as VUS, in a patient with nCHH with no other phenotypic abnormalities. Both genes have been previously associated with complex phenotypes, so it is unlikely that the variants described here are responsible to this patient's phenotype.

The importance of oligogenic mutations in CHH has long been recognized (13). It has been suggested that oligogenic inheritance could at least partially explain the phenotypic variability of CHH within and across families. It is hypothesised that different genetic defects might have a synergistic effect contributing to the phenotype, but in most cases it is extremely difficult to distinguish whether both variants are sufficient and/or contributing to the phenotype. In the case of CHH, studying the interaction of these genes indeed increases the comprehension of the wide variability existing in the time of onset of normal puberty and the various forms of presentation of hypogonadotropic hypogonadism, from congenital forms, some reversible to cases of functional hypogonadism. In addition, the concept of oligogenicity may help to elucidate the molecular diagnosis of patients with isolated mutations do not justify the phenotype, such as heterozygous mutations in genes with a typical autosomal recessive inheritance. Oligogenicity was described in 2.5% to 7% of the cases in previous studies(14, 15, 32). With the advent of NGS, the frequency of detection of oligogenicity has recently increased to approximately 15% (5, 55). Here, we identified variants in more than one gene in nine

patients, corresponding to 6.9% of the total cohort. The large number of genes associated with CHH and the possible influences of the environment on some phenotypes are factors that still make it difficult to prove that multiple genotypes would lead to more severe phenotypes.

The use of targeted NGS in this large cohort provided novel molecular insights in genetic of CHH. Rare and potentially pathogenic variants were identified in more than 30% of CHH patients and the vast majority was in genes previously associated with CHH, confirming that the panel is useful in detecting known genes associated with a disease, and for identify new genes exomic sequencing certainly is a more appropriate approach. This study was able to amplify the genetic characterization of CHH and to approximate the proportion of patients with molecular diagnosis of the descriptions in the literature, in addition increasing the identification of the oligogenic cases. However, since the growth of NGS in the approach to many diseases, we must interpret very carefully the results of the large number of variants that will be identified, since even using a strict selection criterion to identify the most relevant findings, after analyzing each variant individually, 51 (66.2%) of the 77 variants initially identified were considered benign or probably benign.

The complexity of the CHH genetic model strengthens the importance of the use of new genetics technologies, in the approach of rare diseases and with multiple genetic causes such as CHH. Targeted NGS has been shown to be a powerful tool in molecular diagnosis of this rare condition.

Declaration of interest: nothing to disclose

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Figure Legends

- Figure 1. Rare variant identified by NGS in 130 patients with CHH.

- Figure 2. Flowchart for the selection of candidate variants.

- Figure 3. a. Family heredogram of patient with a *GNRH1* mutation (squares indicate male family members, circles female family members, black symbols clinically affected patient - index, white circles phenotypically normal).

Figure 3. b. Amplification and automatic sequencing of cDNA fragment (556 bp) of *GNRH1.*

Figure 3. c. Schematic structure of *GNRH1* with the localization of 4 homozygous mutations (3 previously described).

Table 1. Genes selected for the NGS panel.

Table 2. Rare variants identified in the CHH cohort.

Table 3. Pathogenic and probably pathogenic variants identified in 43 CHH patients.

Supplemental table 1: Prevalence of genetic defects in CHH patients previously studied by Sanger sequencing in our cohort.

Supplemental table 2. Clinical and genetic features of 130 patients evaluated by gene panel.

Table 1. Genes selected for the NGS panel

Gene	GeneCards ID	Reference
ANOS1	GC0XM008528	Legouis *et al.*1991 (56)
FGFR1	GC08M038411	Dodé *et al*. 2003 (49)
FGF8	GC10M101770	Falardeau *et al*. 2008 (50)
GNRH1	GC08M025419	Bouligand *et al*. 2009 (43)
GNRHR	GC04M067737	de Roux *et al*. 1997 (7)
TAC3	GC12M057009	Topaloglu *et al*. 2009 (57)
TACR3	GC04M103586	Topaloglu *et al*. 2009 (57)
KISS1	GC01M204190	Topaloglu *et al*. 2012 (58)

KISS1R	GC19P000917	de Roux *et al.* 2003/ Seminara *et al.* 2003 (59, 60)
PROK2	GC03M071820	Dodé *et al*. 2006 (61)
PROKR2	GC20M005301	Dodé *et al*. 2006 (61)
CHD7	GC08P060678	Kim *et al.* 2008 (46)
FGF17	GC08P022042	Miraoui *et al*. 2013 (32)
SEMA3A	GC07M083955	Young *et al*. 2012 (62)
SEMA7A	GC15M074409	Känsäkoski *et al*. 2014 (63)
IL17RD	GC03M057124	Miraoui *et al*. 2013 (32)
HS6ST1	GC02M128236	Tornberg *et al*. 2011 (51)
RNF216	GC07M005661	Margolin *et al*. 2013 (64)
DUSP6	GC12M089347	Miraoui *et al*. 2013 (32)
WDR11	GC10P120851	Kim *et al*. 2010 (65)
POLR3A	GC10M077969	Saitsu *et al*. 2011 (66)
POLR3B	GC12P106357	Saitsu *et al*. 2011 (66)
FLRT3	GC20M014322	Miraoui *et al.* 2013 (32)
SPRY4	GC05M142272	Miraoui *et al*. 2013 (32)
SOX10	GC22M039963	Pingault *et al*. 2013 (53)
NSMF	GC09M137447	Miura *et al*. 2004 (67)
MKRN3	GC15P024015	Abreu *et al.* 2013 (68)
MSX1	GC04P004861	Xie *et al*. 2013 (69)
OTX2	GC14M056799	Diaczok *et al.* 2008 (54)
EBF2	GC08M025841	Trarbach *et al*. 2005 (70)
GHSR	GC03M172443	Pugliese-Pires *et al*. 2011 (71)
IGSF1	GC0XM131273	Sun *et a*l. 2012 (72)
DMXL2	GC15M051447	Tata *et al*. 2014 (73)
IGSF10	GC03M151425	Howard *et al*. 2016 (47)
IGFALS	GC16M001790	Domene *et al*. 2004 (74)
PNPLA6	GC19P007534	Topaloglu *et al*. 2014 (75)

NGS, next-generation sequencing

Table 2. Rare variants identified in the CHH cohort.

ID	Sex	Diagnosis	Gene	cDNA genotype	Aminoacid genotype	Reported inheritance	GenomAD MAF (%)	ABraOM MAF (%)	In-house database MAF (%)**	Segregation data	Variant classification	Previous descriptions
1	F	nCHH	*CHD7*	c.611G>T/WT	p.Gly204Val/WT	AD	absent	absent	absent	NA	VUS	No
			FGFR1[y]	c.1825C>T/WT	p.Arg609X/WT	AD	absent	absent	absent		P	Riley *et* al. Proc Natl Acad Sci, 2007 (76)
2	M	nCHH	*CHD7*	c.1958C>T/WT	p.Pro653Leu/WT	AD	0.0297[a]	absent	absent	NA	VUS	No
3	M	nCHH	*CHD7*	c.2095A>G/WT	p.Ser699Gly/WT	AD	absent	absent	absent	NA	VUS	Jongmans *et al.* J Med Genet, 2006 (77)
4	M	nCHH	*CHD7*	c.2201_2203de/ WT	p.734_735del/ WT	AD	absent	absent	0.13[a]	NA	VUS	No
5	M	KS	*CHD7*	c.3161G>A/WT	p.Arg1054Gln/WT	AD	0.004[a]	absent	absent	NA	VUS	No
			ANOS1[y]	c.1385G>A	p.Trp462X	X-linked	absent	absent	absent		P	Montenegro *et al.* Fertil Steril, 2013 (9)
6	M	KS	*CHD7*	c.3973T>C/WT	p.Tyr1325His/WT	AD	0.0057[a]	absent	absent	NA	VUS	No
			PROK2[y]	c.297dupT/c.297dupT	p.Gly100fs/p.Gly100fs	AR	0.0104[a]	absent	absent		P	Abreu *et al.* J Clin Endocrinol Metab, 2008 (18)
7	F	nCHH	*CHD7*	c.6775G>A/WT	p.Ala2259Thr/WT	AD	0.0163[a]	absent	absent	Negative	PB	Bilan *et al.* J Mol Diagn, 2012 (78)
			IGSF10	c.2126G>T/WT	p.Gly709Val/WT	AD	0.0324[a]	0.24[a]	absent	Positive	VUS	No
8	F	nCHH	*CHD7*	c.7253G>A/WT	p.Arg2418Gln/WT	AD	0.0072[a]	absent	absent	NA	VUS	No
			TAC3	c.209-1G>C/c.209-1G>C		AR	0.0108[a]	absent	absent		P	Young *et al.* J Clin Endocrinol Metab, 2010 (79)
9	M	KS	*CHD7*	c.7963_7970del/WT	p.G2655fs/WT	AD	absent	absent	absent	NA	P	No
10	M	KS	*CHD7*	c.8068_8076del/WT	p.2690_2692del	AD	absent	absent	absent	NA	PP	No
			GNRHR	c.317A>G/WT	p.Gln106Arg/WT	AR	0.2712[a]	0.16[a]	0.26[a]		P	de Roux *et al.* N Engl J Med, 1997 (7)
11	M	KS	*CHD7*	c.8213C>T/WT	p.Thr2738Met/WT	AD	0.00016[a]	absent	absent	NA	VUS	No
			FGFR1[y]	c.289G>A/WT	p.Gly97Ser/WT	AD	absent	absent	absent		P	Dodé *et al.* Eur J Hum Genet, 2009 (80)
			WDR11	c.2305A>G/WT	p.Met769Val/WT	AD	0.0004[a]	0.16[a]	0.26[a]		VUS	No
			EBF2	c.650C>T/WT	p.Thr217Met/WT	?	0.0008[a]	absent	absent	NA	VUS	No
12	M	nCHH	*CHD7*	c.8416C>G/WT	p.Leu2806Val/WT	AD	0.1189[a]	0.41[a]	0.65[a]	Negative	B	No
13	M	nCHH	*CHD7*	c.8416C>G/WT	p.Leu2806Val/WT	AD	0.1189[a]	0.41[a]	0.65[a]	NA	B	No
14	F	nCHH	*CHD7*	c.8416C>G/WT	p.Leu2806Val/WT	AD	0.1189[a]	0.41[a]	0.65[a]	NA	B	No

15	M	nCHH	*FGFR 1* *IL17RD* *IL17RD* *DMXL2*	c.83C>T/WT c.1697C>T/c.1697C>T c.1608_1611del/WT c.5171C>T/WT	p.Pro28Leu/WT p.Pro566Leu/p.Pro566Leu p.Glu536fs/WT p.Ser1724Leu/WT	AD AD AD AR	absent 0.2024[a] absent absent	absent 0.8[a] absent absent	absent 0.39[a] absent absent	Positive Positive Positive Positive	P PP P PB	No No No No
16	M	KS	*FGFR 1*[r]	c.748C>T/WT	p.Arg250Trp/WT	AD	absent	absent	absent	NA	P	Trarbach *et al.* J Clin Endocrinol Metab 91, 2006 (17)
17	F	nCHH	*FGFR 1* *SPRY 4*	c.857dupC/WT c.722C>A/WT	p.P286fs/WT p.Ser241Tyr/WT	AD AD	absent 0.4549[b]	absent 0.6568[a]	absent absent	NA	P B	No Miraoui *et al.* Am J Hum Genet, 2013 (32)
18	M	nCHH	*FGFR 1* *IGSF10* *POLR 3B*	c.962_963del/WT c.5405A>T/WT c.926C>G/WT	p.Lys321fs/WT p.Asp1802Val/WT p.Ala309Gly/WT	AD AD AR	0.0004[a] 0.2723[a] absent	absent 0.32[a] absent	absent absent absent	Positive Negative Negative	P B B	No No No
19	M	KS	*FGFR 1*[r]	c.1028C>T/WT	p.Ala343Val/WT	AD	absent	absent	absent	NA	P	Trarbach *et al.* J Clin Endocrinol Metab 91, 2006 (17)
20	F	KS	*FGFR 1*	c.2008G>A/WT	p.Glu670Lys/WT	AD	absent	absent	absent	NA	P	No
21	M	KS	*FGFR 1*	c.2070+1G>A		AD	0.0406[a]	absent	absent	NA	P	No
22	F	nCHH	*FGFR 1*	c.2275G>T/WT	p.Glu759X/WT	AD	absent	absent	absent	NA	P	No
23	M	nCHH	*FGFR 1*	c.2300G>A/WT	p.Cys767Tyr/WT	AD	absent	absent	absent	NA	P	No
24	M	nCHH	*GNRHR*	c.30T>A; c.31C>A/c.847T>C	p.Asn10Lys; p.Gln11Lys/p.Tyr283His	AR	0.0135[a] /0.0008[a]	absent	absent	NA	P/P	Costa *et al.* J Clin Endocrinol Metab, 2001 (16)
25	M	nCHH	*GNRHR*	c.317A>G/WT	p.Gln106Arg/WT	AR	0.2712[a]	0.16[a]	0.26[a]	NA	P	de Roux *et al.* N Engl J Med, 1997 (7)
26	M	KS	*GNRHR*	c.317A>G/WT	p.Gln106Arg/WT	AR	0.2712[a]	0.16[a]	0.26[a]	NA	P	de Roux *et al.* N Engl J Med, 1997 (7)
27	M	KS	*GNRHR*	c.317A>G/WT	p.Gln106Arg/WT	AR	0.2712[a]	0.16[a]	0.26[a]	NA	P	de Roux *et al.* N Engl J Med, 1997 (7)
28	M	nCHH	*GNRHR*[r]	c.401T>G/c.785G>A	p.Val134Gly/p.Arg262Gln	AR	0.0028[a] /0.1804[a]	absent	absent	NA	P/P	de Roux *et al.* N Engl J Med, 1997 (7)
29	M	nCHH	*GNRHR*	c.416G>A/c.416G>A	p.Arg139His/p.Arg139His	AR	0.017[a]	absent	absent	NA	P	Costa *et al.* J Clin Endocrinol Metab, 2001 (16)
30	M	KS	*ANOS1*	c.153A>G	p.Met1Val	X-linked	absent	absent	absent	NA	P	No

31	M	KS	*ANOS 1*[r]	c.90_100del	p.Ala30fs	X-linked	absent	absent	absent	NA	P	Montenegro *et al*. Fertil Steril, 2013 (9)
32	M	KS	*ANOS 1*[r]	c.1062+1G>T		X-linked	absent	absent	absent	NA	P	Montenegro *et al*. Fertil Steril, 2013 (9)
33	M	KS	*ANOS 1*	c.566_567del	p.E189fs	X-linked	absent	absent	absent	NA	P	No
34	M	KS	*ANOS 1*	c.1632_1635del	p.Leu544fs	X-linked	absent	absent	absent	NA	P	No
35	M	nCHH	*TACR 3*[r]	c.439C>T/ c.824G>A	p.Leu147Phe/ p.Trp275X	AR	absent	absent	absent	NA	P/P	Gianetti *et al.* J Clin Endocrinol Metab, 2010 (19)
36	M	nCHH	*TACR 3* *IGFALS*	c.824G>A/c.824G>A c.125C>T/WT	p.Trp275X/p.Trp275X p.Pro42Leu/WT	AR AR	0.0314[a] 0.0023[a]	absent 0.08[a]	absent absent	NA	P B	Gianetti *et al.* J Clin Endocrinol Metab, 2010 (19) No
37	M	nCHH	*TACR 3* *POLR 3B*	c.1007A>G/c.1007A>G c.971A>G/ WT	p.Gln336Arg/p.Gln336Arg p.Lys324Arg/ WT	AR AR	absent absent	absent absent	absent absent	NA	P PB	No No
38	M	nCHH	*TACR 3*	c.824G>A /WT	p.Trp275X/WT	AR	0.0314[a]	absent	absent	NA	P	Gianetti *et al.* J Clin Endocrinol Metab, 2010 (19)
39	F	nCHH	*TACR 3*	c.824G>A /WT	p.Trp275X/WT	AR	0.0314[a]	absent	absent	NA	P	Gianetti *et al*. J Clin Endocrinol Metab, 2010 (19)
40	M	KS	*PROKR2*	c.115G>A/WT	p.Glu39Lys/WT	AR	absent	absent	absent	NA	PB	No
41	M	KS	*PROKR2* *WDR11*	c.518T>G/WT c.3571G>A/WT	p.Leu173Arg/WT p.Gly1191Ser/WT	AR AD	0.2186[a] 0.0147[a]	0.24[a] 0.16[a]	0.13[a] absent	NA	B VUS	Dodé *et al.* PLoS Genet, 2006 (61) No
42	F	KS	*PROKR2*	c.518T>G/WT	p.Leu173Arg/WT	AR	0.2186[a]	0.24[a]	0.13[a]	NA	B	Dodé *et al.* PLoS Genet, 2006 (61)
43	M	nCHH	*PROK2* *WDR11*	c.163delA/WT c.1066G>A/WT	p.Ile55X/WT p.Val356Ile/WT	AR AD	0.0111[a] 0.1591[a]	absent 0.08[a]	absent absent	NA	P VUS	Pitteloud *et al.* Proc Natl Acad Sci, 2007 (81) No
44	M	nCHH	*PROK2*	c.163delA/WT	p.Ile55X/WT	AR	0.0111[a]	absent	absent	NA	P	Pitteloud *et al.* Proc Natl Acad Sci, 2007 (81)
45	F	KS	*PROK2*	c.297dupT/WT	p.Gly100fs/WT	AR	0.0104[a]	absent	absent	NA	P	Abreu *et al.* J Clin Endocrinol Metab, 2008 (18)
46	M	KS	*PROK2*	c.332C>A/WT	p.Pro111Gln/WT	AR	0.183[a]	0.4[a]	absent	NA	PB	No
47	M	nCHH	*IGSF10*	c.1297delC/WT	p.Gln433fs/WT	AD	0.0079[a]	absent	absent	Positive	P	No
48	M	nCHH	*IGSF10*	c.2126G>T/WT	p.Gly709Val/WT	AD	0.0324[a]	0.24[a]	absent	NA	VUS	No

			SPRY 4	c.776C>T/WT	p.Ser259Phe/WT	AD	absent	absent	absent		PP	No
49	M	nCHH	*IGSF1 0*	c.6830A>G/c.4613C >T	p.Asp2277Gly/ p.Thr1538Ile	AD	0.0176[a] /0.0285[a]	absent	absent/0.08	NA	PP/VUS	No
50	F	nCHH	*IGSF1 0*	c.5405A>T/WT	p.Asp1802Val/WT	AD	0.2723[a]	0.32[a]	absent	NA	B	No
51	M	nCHH	*IGSF1 0*	c.7217C>T/WT	p.Ala2406Val/WT	AD	0.0801[a]	0.24[a]	absent	NA	VUS	No
52	M	nCHH	*GNHR 1*	c.142-2A>C/c.142-2A>C		AR	absent	absent	absent	NA	P	No
53	F	KS	*GNHR 1*	c.141G>C/WT	p.Glu47Asp/WT	AR	0.1492[a]	0.24[a]	0.13[a]	NA	P	No
54	M	nCHH	*FGF8*	c.560_574del/WT	p.187_192del/WT	AD	absent	absent	absent	NA	P	No
55	F	KS	*FGF8*	c.G617A/WT	p.Arg206Gln/WT	AD	absent	absent	absent	NA	P	No
56	M	KS	*IL17R D*	c.878C>T/WT	p.Pro293Leu/WT	AD	0.0568[a]	0.08[a]	0.26[a]	NA	VUS	No
57	M	KS	*IL17R D*	c.2003C>T/WT	p.Ser668Phe/WT	AD	0.0043[a]	0.08[a]	0.13[a]	Negative	B	No
58	M	KS	*POLR 3A*	c.1745G>T/WT	p.Arg582Leu/WT	AR	0.8561[c]	0.99[a]	3.51[a]	NA	B	No
59	F	nCHH	*POLR 3A*	c.1745G>T/WT	p.Arg582Leu/WT	AR	0.8561[c]	0.99[a]	3.51[a]	NA	B	No
			FLRT3	c.821A>G/WT	p.Tyr274Cys/WT	AD	0.0072[a]	absent	absent		VUS	No
60	F	KS	*POLR 3B*	c.1010T>C/WT	p.Val337Ala/WT	AR	0.0004[a]	absent	absent	NA	B	No
			SOX10	deletion exons 1-6		AD	absent	absent	absent		P	No
61	F	nCHH	*SEMA 3A*	c.1923G>C/WT	p.Gln641His/WT	AD	0.1102[a]	0.7[a]	0.13[a]	NA	VUS	No
62	M	nCHH	*SEMA 3A*	c.2197C>T/WT	p.Arg733Cys/WT	AD	0.0065[a]	0.16[a]	absent	NA	VUS	No
63	M	KS	*SEMA 7A*	c.406C>T/WT	p.Arg136Trp/WT	AD	0.0043[a]	absent	absent	Negative	VUS	No
64	M	nCHH	*SEMA 7A*	c.493G>A/WT	p.Glu165Lys/WT	AD	0.0152[a]	0.08[a]	absent	NA	VUS	No
65	M	nCHH	*DMXL 2*	c.5267G>A/WT	p.Arg1756His/WT	AR	0.0004[a]	absent	absent	NA	PB	No
66	M	KS	*DMXL 2*	c.5267G>A/WT	p.Arg1756His/WT	AR	0.0004[a]	absent	absent	NA	PB	No
67	M	nCHH	*DMXL 2*	c.1463C>T/WT	p.Thr488Met/WT	AR	0.0408[a]	0.08[a]	absent	NA	B	No
68	M	KS	*WDR1 1*	c.C1592G/WT	p.Ser531Cys/WT	AD	0.0039[a]	absent	absent	NA	VUS	No
69	M	KS	*WDR1 1*	c.2962G>A/WT	p.Glu988Lys/WT	AD	0.1799[a]	absent	0.39[a]	NA	VUS	No
70	M	KS	*NSMF*	c.721G>A/WT	p.Ala241Thr/WT	?	0.0024[a]	absent	absent	NA	VUS	No

71	F	nCHH	*NSMF*	c.1453G>A/WT	p.Val485Ile/WT	?	0.0039[a]	0.08[a]	absent	NA	VUS	No
72	M	nCHH	*FGF17*	c.196G>A/WT	p.Val66Met/WT	AD	absent	absent	absent	NA	PP	No
73	M	KS	*IGFALS*	c.1195G>A/WT	p.Gly399Arg/WT	AR	0.0257[a]	absent	absent	NA	PB	No
74	F	nCHH	*IGSF1*	c.709C>G/WT	p.Pro237Ala/WT	AD	0.0007[a]	absent	absent	NA	VUS	No
75	F	nCHH	*SOX10* *OTX2*	c.1018G>A/WT c.163G>T/WT	p.Val340Met/WT p.Ala55Ser/WT	AD AD	0.0064[a] 0.0046[a]	absent absent	absent absent	NA	VUS VUS	No No
76	M	KS	*PNPLA6*	c.1340C>T/WT	p.Pro447Leu/WT	AR	0.4756[b]	0.32[a]	0.78[a]	NA	B	No
77	F	nCHH	*RNF216*	c.2353T>C/WT	p.Ser785Pro/WT	AR	0.0012[a]	absent	0.13[a]	NA	PB	No

Dx, diagnosis; MAF, Minor allele frequency; WT, wild type; KS, Kallmann syndrome; nCHH, normosmic congenital hypogonadotropic hypogonadism; P, pathogenic; PP, probably pathogenic; B, benign; PB, probably benign; VUS, variant of uncertain significance
Segregation data: NA, family DNA not available; Positive: healthy family does not have the same variant; Negative: healthy family has the same variant
** In-house database: data from exomic sequencing covering 774 alleles
[a] all heterozygous
[b] five homozygous
[c] eleven homozygous
γ Previously identified by Sanger: patient 1 *FGFR1* p.Arg609X/WT, patient 5 *ANOS1* p.Trp462X[20], patient 6 *PROK2* p.Gly100fs/p.Gly100fs[11], patient 11 *FGFR1* p.Gly97Ser/WT, patient 16 *FGFR1* p.Arg250Trp/WT[9], patient 19 *FGFR1* p.Ala343Val/WT[9], patient 28 *GNRHR* p.Val134Gly/p.Arg262Gln[21], patient 31 *ANOS1* p.Ala30fs[20], patient 32 *ANOS1* c.1062+1G>T[20], patient 35 *TACR3* p.Leu147Phe/p.Trp275X[20].

Table 3. Pathogenic and probably pathogenic variants identified in 43 CHH patients.

Gene	Diagnosis	cDNA genotype	Aminoacid genotype	Allele Frequency AC (%)	GenomAD MAF (%)	ABraOM MAF (%)	In-house database MAF (%)	avsnp144	Prediction sites that were considered pathogenic	GERP
FGFR1	KS	c.83C>T	p.Pro28Leu	1 (0.38)	absent	absent	absent	rs145434725	4/4	4.58
	nCHH	c.1825C>T	p.Arg609X	1 (0.38)	absent	absent	absent	rs121909639	2/4	4.48
	KS	c.289G>A	p.Gly97Ser	1 (0.38)	absent	absent	absent	NA	4/4	5.38
	KS	c.748C>T	p.Arg250Trp	1 (0.38)	absent	absent	absent	NA	4/4	4.98
	nCHH	c.857dupC	p.P286fs	1 (0.38)	absent	absent	absent	NA	NA	NA
	nCHH	c.962_963del	p.Lys321fs	1 (0.38)	0.0004	absent	absent	NA	NA	NA
	KS	c.1028C>T	p.Ala343Val	1 (0.38)	absent	absent	absent	NA	3/4	5.16
	KS	c.2008G>A	p.Glu670Lys	1 (0.38)	absent	absent	absent	rs397515446	4/4	5.9
	KS	c.2070+1G>A		1 (0.38)	0.0406	absent	absent	NA	2/4	5.9
	nCHH	c.2275G>T	p.Glu759X	1 (0.38)	absent	absent	absent	NA	2/4	5.88
	nCHH	c.2300G>A	p.Cys767Tyr	1 (0.38)	absent	absent	absent	NA	4/4	5.33
CHD7	KS	c.7963_7970del	p.G2655fs	1 (0.38)	absent	absent	absent	NA	NA	NA
	KS	c.8068_8076del	p.2690_2692del	1 (0.38)	absent	absent	absent	NA	NA	NA
ANOS1	KS	c.90_100del	p.Ala30fs	1 (0.38)	absent	absent	absent	NA	NA	NA
		c.153A>G	p.Met1Val	1 (0.38)	absent	absent	absent	NA	NA	NA
		c.566_567del	p.E189fs	1 (0.38)	absent	absent	absent	NA	NA	NA
		c.1062+1G>T		1 (0.38)	absent	absent	absent	rs387906427	2/4	NA
		c.1385G>A	p.Trp462X	1 (0.38)	absent	absent	absent	NA	NA	NA
		c.1632_1635del	p.Leu544fs	2 (0.77)	absent	absent	absent	NA	NA	NA
GNRHR	nCHH	c.31C>A; c.30T>A	p.Gln11Lys; p.Asn10Lys	1 (0.38)	0.0135	absent	absent	rs104893843; rs776834867	2/4	0.96; 5.18
	KS/nCHH	c.317A>G	p.Gln106Arg	4 (1.52)	0.2712	0.16	0.13	rs104893836	4/4	6.17
		c.401T>G	p.Val134Gly	1 (0.38)	0.0028	absent	absent	rs188272653	2/4	6.04
	nCHH	c.416G>A	p.Arg139His	2 (0.77)	0.017	absent	absent	rs104893842	2/4	6.17
		c.785G>T	p.Arg262Gln	1 (0.38)	0.1804	absent	absent	rs104893837	4/4	2.78

		c.847T>C	p.Tyr283His	1 (0.38)	0.0008	absent	absent	NA	4/4	5.43
GNHR1	nCHH	c.142-2A>C		1 (0.38)	absent	absent	absent	NA	NA	NA
TACR3	nCHH	c.439C>T	p.Leu147Phe	1 (0.38)	absent	absent	absent	NA	3/4	4.41
		c.824G>A	p.Trp275X	5 (1.9)	0.0314	absent	absent	rs144292455	2/4	5.52
		c.1007A>G	p.Gln336Arg	2 (0.77)	absent	absent	absent	NA	4/4	5.21
TAC3	nCHH	c.209-1G>C		2 (0.77)	0.0108	absent	absent	rs146391497	2/4	4.09
FGF8	nCHH	c.560_574del	p.187_192del	1 (0.38)	absent	absent	absent	NA	NA	NA
	KS	c.617G>A	p.Arg206Gln	1 (0.38)	absent	absent	absent	NA	4/4	3.79
SOX10	KS	deletion exons 1-6		1 (0.38)	absent	absent	absent	NA	NA	NA
PROK2	nCHH	c.163delA/WT	p.Ile55X/WT	1 (0.38)	0.0111	absent	absent	rs554675432	NA	NA
	KS	c.297dupT	p.Gly100fs	1 (0.38)	0.0104	absent	absent	NA	NA	NA
IL17RD	nCHH	c.1608_1611del	p.Glu536fs	1 (0.38)	absent	absent	absent	NA	NA	NA
		c.1697C>T	p.Pro566Leu	2 (0.77)	0.2024 (all in heterozygous)	0.8 (all in heterozygous)	0.39 (all in heterozygous)	rs61742268	4/4	3.73
IGSF10	nCHH	c.1297delC	p.Gln433fs	1 (0.38)	0.0079	absent	absent	rs762330687	NA	NA
		c.6830A>G	p.Asp2277Gly	1 (0.38)	0.0179	absent	absent	rs150554446	4/4	4.03
SPRY4	HHIn	c.776C>T	p.Ser259Phe	1 (0.38)	absent	absent	absent	NA	4/4	4.92
FGF17	nCHH	c.196G>A	p.Val66Met	1 (0.38)	absent	absent	absent	NA	4/4	4.75

KS, Kallmann syndrome; nCHH, normosmic congenital hypogonadotropic hypogonadism; NA, not avaliable; AC, allele count; MAF, Minor allele frequency