Congenital Hypogonadotropic Hypogonadism and Constitutional Delay of Growth and Puberty Have Distinct Genetic Architectures

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**Abstract**

Objective: Congenital hypogonadotropic hypogonadism (CHH) and constitutional delay of growth and puberty (CDGP) represent rare and common forms of GnRH deficiency, respectively. Both CDGP and CHH present with delayed puberty, and the distinction between these two entities during early adolescence is challenging. More than 30 genes have been implicated with CHH, while the genetic basis of CDGP is poorly understood.

Design: We characterized and compared the genetic architectures of CHH and CDGP, to test the hypothesis of a shared genetic basis between these disorders.

Methods: Exome sequencing data was used to identify rare variants in known genes in CHH (n=116), CDGP (n=72), and control cohorts (n=36,874 ExAC and n=405 CoLaus).

Results: Mutations in at least one CHH gene were found in 51% of CHH probands, which is significantly higher than in CDGP (7%, \(p=7.6\times10^{-11}\)) or controls (18%, \(p=5.5\times10^{-12}\)). Similarly, oligogenicity (defined as mutations in more than one gene) was common in CHH patients (15%) relative to CDGP (1.4%, \(p=0.002\)) and controls (2%, \(p=6.4\times10^{-7}\)).

Conclusions: Our data suggests that CDGP and CHH have distinct genetic profiles, and this finding may facilitate the differential diagnosis in patients presenting with delayed puberty.

**Introduction**

Congenital hypogonadotropic hypogonadism (CHH [MIM: 146110]) is a rare disorder affecting approximately 1 in 4,000 births\(^1\). It is caused by GnRH deficiency, and subsequently results in altered activation of the hypothalamic-pituitary-gonadal (HPG) axis that controls sexual maturation and fertility. Clinically, CHH presents as absent/incomplete puberty and
infertility. It is characterized by isolated low sex steroids in the setting of low (or inappropriately normal) serum gonadotropins in the absence of other hypothalamo-pituitary defects. Clinically, CHH is a heterogeneous disorder. In the presence of anosmia (the inability to smell) in approximately 50% of CHH probands, the condition is termed Kallmann Syndrome (KS [MIM: 308700]). Other associated phenotypes such as hearing loss, synkinesia, renal agenesis, ataxia, and cleft lip/palate are also observed with variable frequency\(^2\). Interestingly, a higher than expected proportion (10%) of family members of CHH probands report a history of delayed puberty\(^3\). Moreover, reversal of hypogonadotropic hypogonadism in CHH patients after discontinuing hormone therapy also points to a clinical overlap between the two entities\(^4\). Therefore, congenital delay of growth and puberty (CDGP), also termed self-limited delayed puberty, and CHH appear to be part of the same clinical spectrum--one being classically described as transient (CDGP) and the other as permanent (CHH)\(^3\). In contrast to CHH, CDGP is a common disease, observed in 2-2.5% of the population\(^5\).

Since the initial genetic report implicating KAL1 (now ANOS1)\(^6,7\), the genetics of CHH has been widely studied. Similar to its diverse clinical presentation, the genetic architecture of CHH is also heterogeneous, with several modes of inheritance having been described including autosomal dominant, autosomal recessive, X-linked and de novo. Mutations in more than 30 genes have been shown to cause CHH\(^2\), however they only account for approximately 35% of cases\(^8\). Defects in genes involved in GnRH neuron development and olfactory system usually result in KS, whereas mutations in genes involved in GnRH secretion or homeostasis result in normosmic CHH (nCHH). Interestingly, clinical overlap between KS and nCHH has been reported, with a disease spectrum rather than a binary classification for normosmic and anosmic\(^9\).
In parallel, genetic overlap between KS and nCHH also exists, with several genes mutated in both subgroups\(^2\).

Although long thought to be a monogenic disorder, frequent observations of incomplete penetrance and variable expressivity within and across families suggested this model was insufficient to fully explain the observed phenotypes in CHH. Indeed, previous work by our team and others has shown that oligogenic inheritance (i.e. more than one gene mutated in the same individual) can at least partially explain some of these phenomena\(^8,10\). Synergistic effects between CHH genes have been also described \textit{in vitro} (e.g. FGF8/FGFR1)\(^11\) and \textit{in vivo} (e.g. KISS1/KISS1R)\(^12\). Oligogenicity has been proposed in heterogeneous genetic disorders such as Bardet-Biedl syndrome (BBS)\(^13\) and retinitis pigmentosa\(^14\). In addition, oligogenicity is also proposed for other endocrine diseases such as premature ovarian failure\(^15,16\) with the constellation of more than one gene mutated.

Pubertal timing is a highly heritable trait as up to 50-80% of the variance is explained by genetic factors\(^17\). Consistently, CDGP runs in families with complex inheritance pattern\(^18\), but in contrast to CHH, little is known about the genetics of CDGP. A recent study identified mutations with low frequencies (MAF<2.5%) in IGSF10 in 13% of CDGP probands. IGSF10 is a large protein that is part of the immunoglobulin superfamily, and appears to have a developmental role in GnRH neuron migration\(^19\). In addition, genome-wide association studies (GWAS) evaluating common and rare variants in the timing of puberty identified significant associations with hundreds of loci, including regions near or within ANOS1, TACR3, LEPR, and PCSK1 — four known CHH genes. Taken together, these loci account for <3% of the variance in age of puberty onset\(^20,21\). In view of the possible overlap between the pathophysiology of delayed puberty and conditions of GnRH deficiency, few studies have searched for mutations in CHH genes in CDGP.
cohorts. A homozygous partial loss-of-function mutation in \textit{GNRHR} was found in two brothers, one with CDGP and one with CHH\textsuperscript{22}. Of 50 CDGP patients investigated for mutations in \textit{TAC3} and \textit{TACR3}, only one mutation in a single patient was found in the latter gene\textsuperscript{23}. However, a recent study screening 21 CHH genes in a CDGP cohort (n=56) found potentially pathogenic mutations in 14\% of patients\textsuperscript{3}. Recently, low frequency (MAF <2.5\%) potentially pathogenic variants in \textit{IGSF10} were found in 10\% of CHH patients\textsuperscript{19}, suggesting the hypothesis of a partial genetic overlap between CHH and CDGP.

Currently, the differential diagnosis between CHH and CDGP at early adolescence remains challenging, as both conditions present with isolated delay in puberty. Further, there are no specific biochemical markers to accurately differentiate these two disorders\textsuperscript{24}. In the current study, we explored the genetic architecture of both CHH and CDGP, and investigated whether genetic testing could assist in the differential diagnosis. We also characterized the genetic overlap between KS and nCHH using a comprehensive screening of all CHH genes, and defined the mutational spectrum of CHH genes in the control population.

**Subjects and Methods**

**Patient and control cohorts**

The study cohort includes 116 CHH probands of European descent (n=61 KS, n=55 nCHH) with a 2:1 male-to-female ratio consistent with previous reports of male predominance\textsuperscript{1}. The diagnosis of CHH was determined by 1) absent or partial puberty by 17 years\textsuperscript{25} 2) low/normal serum gonadotropin levels in the setting of low serum testosterone/estradiol levels, 3) otherwise normal anterior pituitary function, and 4) normal imaging of the hypothalamic-pituitary area\textsuperscript{25}. 

7
Olfaction was assessed by self-report and/or formal testing\(^9\) using the UPSIT or Sniffin’ Stick tests. When possible, family members were recruited for clinical and genetic studies.

The delayed puberty cohort consists of 72 unrelated probands with CDGP of primarily Finnish European origin and has been previously described in detail\(^26\). All patients met the diagnostic criteria for CDGP, defined as 1) onset of Tanner genital stage II two SDs later than population average (i.e. in boys testicular volume >3 ml after 13.5 years of age and in girls Tanner breast stage II after 13.0 years of age)\(^27\). Medical history, clinical examination, and routine laboratory tests were performed to exclude chronic illnesses, and the diagnosis of CHH was ruled out by spontaneous pubertal development at follow-up. All patients were followed until near-full pubertal development was attained (i.e., Tanner stage 4).

Ethnically-matched controls (non-Finnish European (NFE) and Finnish European (FIN)) from the Exome Aggregation Consortium (ExAC)\(^28\) were used for individual variant and gene mutation frequencies. Oligogenicity was assessed using the “Cohorte Lausannoise” (CoLaus) control population, consisting of 405 participants of mixed European origin, phenotyped as described in Firmann \textit{et al.}\(^29\). This population-based cohort was assembled as part of a cardiovascular risk study, and therefore has a typical distribution of pubertal age relative to the general population. The ages of the cohort participants are 35-75 years old (mean 51 +/- 11 years).

**DNA extraction and sequencing**

DNA was extracted from peripheral blood leukocytes using the PureGene kit (QIAGEN, Hilden, Germany). Exome sequencing was performed on CHH and CDGP cohorts using the SureSelect V2 or V5 probes (Agilent, Santa Clara, USA) or the Nimblegen SeqCap EZ Exome
V2 (Roche, Basel, Switzerland), and sequenced on the HiSeq 2000 platform (Illumina, San Diego, USA) at either BGI (BGI, Shenzen, PRC) or Otogenetics (Otogenetics Corp., Atlanta, USA). Exome sequencing on CoLaus DNA was performed at the Wellcome Trust Sanger Institute (WTSI) as part of a partnership between the Institute, the CoLaus principal investigators and the Quantitative Sciences dept. of GlaxoSmithKline (GSK, Brentford, UK).

**Definition of genes to be screened**

“CHH genes” are those which met the following criteria: 1) identified as CHH genes in Boehm *et al.*\(^2\), 2) had publications demonstrating loss-of-function variants, 3) had been demonstrated to be expressed in organs/tissues relevant for GnRH biology, and 4) covered by the exome capture probes. Twenty-four genes met these criteria: *ANOS1, SEMA3A, FGF8, FGF17, SOX10, IL17RD, AXL, FGFR1, CHD7, HS6ST1, PCSK1, LEP, LEPR, FEZF1, NSMF, PROKR2, WDR11, PROK2, GNRHI, GNRHR, KISS1, KISS1R, TAC3*, and *TACR3*. In addition, we screened the *IGSF10* gene, recently implicated in CDGP and CHH\(^19\).

**Bioinformatics analysis and downstream variants filtering**

Exome sequences from CHH probands, CDGP probands, and CoLaus controls were analyzed following the Genome Analysis Toolkit (GATK) Best Practices\(^30\). The computations were performed at the Vital-IT Center for High-Performance Computing of the Swiss Institute of Bioinformatics. Variants called with a genotype quality (GQ) <50 were excluded. The complete set of CHH gene variants from the ExAC database was downloaded from the ftp site (ftp://ftp.broadinstitute.org/pub/ExAC_release/release0.3). Annotation was performed using SnpEff\(^31\) version 4.0. Intronic variants within +/- 6 bp of exonic boundaries and predicted to
affect splicing by MaxEnt$^{32}$ with a wild-type vs. mutated site change of +/- 20% were retained, as well as inframe/frameshift indels, stop-gain, and missense variants. Protein-truncating variants (PTVs) were defined as frameshift, stop-gain and splice variants$^{28}$.

For the purpose of this study, we define as mutations 1) rare (MAF <1%) PTVs, 2) rare missense variants predicted to be damaging to protein function by at least one in silico algorithm (SIFT$^{33}$ or PolyPhen-2$^{34}$), and 3) loss-of-function variants based on in vitro studies, regardless of in silico predictions.

**Statistical analyses**

Statistics for individual and oligogenic variants in cases vs. controls were performed using a two-tailed Fisher’s exact test. Gene-based allele frequencies in ExAC were calculated dividing the sum of alternate allele counts in ethnically-matched samples with the average of alleles inspected. Gene-collapsed rare variant association (RVA) tests in cases vs. controls were calculated using mutated allele frequencies in a two-tailed Fisher’s exact test. Statistical significance in gene-based RVA tests was defined using Bonferroni correction, dividing nominal significance (0.05) with the number of tests performed (i.e., genes analyzed, n=25); hence, the cutoff to determine significance was set at $p=0.002$.

**Results**

**CHH genes are mutated in 51% of CHH probands but only in 7% in CDGP probands**

Exome sequencing was performed on 116 CHH probands, and 59 (51%) harbored mutations in 20 of the 25 genes evaluated (Fig. 1A, Supplementary Table 1). No mutations were identified in NSMF, FEZF1, PCSK1, LEP and LEPR. Nearly two thirds of familial CHH
probands carried mutations in CHH genes (27/44, 61%), while the frequency in sporadic probands was lower (32/72, 44%) (Supplementary Figure 2).

*FGFR1* and *CHD7* were the most frequently mutated genes in CHH probands (Fig. 1A), and both were statistically enriched for mutations compared to ExAC NFE controls (Table 1, Supplementary Figure 1). All of the identified *FGFR1* and *CHD7* mutations were present in a heterozygous state (Supplementary Table 2). In addition, a significant enrichment of mutations was observed for *SOX10*, with a prevalence of 4% in CHH patients (Table 1, Supplementary Table 1).

Exome sequencing identified 7% (n=5) of CDGP probands harboring mutations in the known CHH genes, all of which are heterozygous (Fig. 1D, Supplementary Table 3). Thus, the genetic profile of the CDGP cohort more closely resembles the controls (both ExAC Finnish and non-Finnish controls) rather than CHH probands. Among the six identified mutations, there were five missense and one intronic change predicted to affect splicing. Three mutations were private compared to 3,307 Finnish ExAC controls. Only one CDGP proband harbored two mutated genes (oligogenicity) (1.4%, p=0.002 vs. CHH), a similar rate as observed in controls (Fig. 3, Supplementary Table 2). Clinically, this CDGP patient had spontaneous puberty at 14.3 years and achieved normal adult testicular volume and testosterone levels over the subsequent 2.4 years, thereby excluding a diagnosis of CHH.

**Prevalence of putative *IGSF10* mutations in CHH is similar to CDGP**

We found a large number of CHH patients (19/116, 16.4%) harboring putative *IGSF10* mutations compared to CDGP (8/72, 11.1%) (Fig. 1A-D). Our data did not show an enrichment for mutations in our cohorts as similar frequencies were seen in controls (Fig. 1A-D).
KS and nCHH show both exclusive and shared genetic architectures

We examined the mutational spectrum relative to the two subgroups of CHH — KS (n=61) and nCHH (n=55). Among KS, FGFR1 and CHD7 were the most frequently mutated genes, and together with SOX10 are significantly enriched when compared to controls (Fig. 1B, Table 1). This finding is even more robust when evaluating the KS subgroup alone. Similarly, FGF8 showed a prevalence of 1.6% in KS yet this association was not evident in the CHH cohort as a whole. Mutations in ANOS1, SEMA3A, FGF17 and FGF8 were only found in KS.

Among normosmic (nCHH), FGFR1 and CHD7 were also the most frequently mutated genes. Mutations in GNRHR and TACR3 were only found in nCHH (7% and 5%, respectively) (Fig. 1C). Further, FGFR1, KISS1, GNRHR and TACR3 were significantly enriched in nCHH cases compared to ExAC NFE controls (Table 1).

In addition to FGFR1 and CHD7, six other CHH genes (SOX10, IL17RD, AXL, HS6ST1, PROKR2 and WDR11) were mutated in both KS and nCHH (Fig. 1B-C). This represents an increased genetic overlap in comparison to prior report\textsuperscript{2}. Overall, these results indicate both exclusive and shared genetic architectures for both KS and nCHH.

nCHH probands are enriched with biallelic mutations

Biallelic mutations (i.e. homozygous or compound heterozygous changes in the same gene) were found exclusively in nCHH (6/55, 11%), and were not seen in KS (p=0.01), CDGP (p=0.006), or in CoLaus (p=2.3x10\textsuperscript{-6}) (Fig. 2). Furthermore, 4/15 (27%) genes mutated in nCHH (GNRHR, GNRH1, PROKR2, PROK2, TACR3) only exhibited biallelic mutations, consistent with their recessive mode of inheritance. (Fig. 2C, Supplementary Table 1).
Oligogenicity is a common factor in CHH patients

Oligogenicity was present in 17/116 (15%) of CHH probands (Fig. 3) – a higher frequency than previous reports of 2.5%-7%\textsuperscript{8,10}. A significantly lower rate of oligogenicity was observed in controls (CoLaus: 2%, \( p=6.4\times10^{-7} \)).

Additionally, although monogenic inheritance was more frequent in KS (46%) compared to nCHH (25%, \( p=0.03 \)), CDGP (6%, \( p=3.7\times10^{-8} \)) and CoLaus (16%, \( p=4.6\times10^{-7} \)), similar frequencies of oligogenicity were identified in both KS (13%) and nCHH (16%) (Fig. 3, Supplementary Table 3).

Among the 20 mutated genes identified in CHH patients, 84% (n=16 genes) participated in oligogenicity (Fig. 2A). Of these 16 genes, mutations in \textit{IL17RD}, \textit{HS6ST1}, \textit{KISS1R}, and \textit{TAC3} occurred exclusively in an oligogenic manner. \textit{ANOS1}, \textit{FGF17}, \textit{KISS1} and \textit{PROK2} were the only genes exclusively showing monogenic inheritance (Fig. 2A).

Among possible gene combinations, \textit{FGFR1} with \textit{CHD7} was the most frequent pair interaction (n=4), followed by \textit{FGFR1/IL17RD} and \textit{CHD7/HS6ST1} (n=2) (Fig. 4A). One KS proband (Fig. 4B, Pedigree 1) carrying mutations in both \textit{CHD7} and \textit{FGFR1}, had two daughters after receiving fertility treatment. One of them carried both mutations and was eventually diagnosed with KS, while the second unaffected daughter did not harbor either of the two changes. In Pedigree 2, we identified three mutated genes (\textit{FGFR1}, \textit{CHD7} and \textit{SOX10}) in a KS proband. His KS brother showed overlapping \textit{FGFR1} and \textit{SOX10} mutations. As there were no phenotypic differences between the proband and his sibling, the \textit{CHD7} mutation is likely not critical in the etiology of KS for this pedigree. Last, we identified a KS proband (Fig. 4B, Pedigree 3) harboring an \textit{IL17RD} mutation inherited from his anosmic mother, and a \textit{de novo}
FGFR1 mutation. We did not identify any CHH gene mutations in the anosmic father or the unaffected brother.

The majority of mutations in CHH probands are private

When assessing the mutations identified in CHH probands, more than half of them (38/68, 56%) were not found in the ExAC NFE controls (n=33,370), and therefore are private.

In total, we identified 1,492 putative mutations in ExAC NFE controls and 80 mutations in 72/405 (18%) CoLaus controls. However, the majority of mutations in CoLaus (89%, \( p=6.6\times10^{-4} \)) (Fig. 2E) occurred in a monoallelic pattern. Given the variant-based (rather than sample-based) nature of the ExAC database, the allelic patterns in these controls could not be assessed.

Protein-truncating variants are enriched in CHH probands

Protein-truncating variants (PTVs) are defined as changes predicted to severely disrupt genes, i.e. splicing, frameshift and stop gain variants. A large fraction of the discovered mutations in CHH probands were PTVs (20/68, 29%), while the frequency was significantly lower (5%) in ExAC NFE controls (\( p=1.0\times10^{-9} \)). Overall, 18% (n=21) of patients in our cohort harbored at least one PTV in the known CHH genes. Specifically, the CHH cohort was enriched for splice variants in FGFR1 (2.6%, \( p=1.7\times10^{-4} \)), and for frameshift/stop gain variants in FGFR1 (8%, \( p=6.9\times10^{-13} \)), SOX10 (1.7%, \( p=1.2\times10^{-5} \)), and TACR3 (1.7%, \( p=4.9\times10^{-5} \)) when compared to ExAC NFE.

We observed that 80% of PTVs in CHH were in genes with a high constraint to these type of variants (i.e., probability of being loss-of-function intolerant – \( pLi>0.9 \))\(^{28} \), a higher
frequency than in ExAC controls ($p=0.002$). Conversely, the majority of PTVs in ExAC (60%) were present in PTV-tolerant genes ($pLi<0.1$) (Supplementary Figure 4). Furthermore, a large majority of PTVs found in CHH probands (16/20, 80%) are heterozygous and occur in CHH genes where mutations are inherited in an autosomal dominant mode. Moreover, all of the PTVs identified in CHH probands likely result in haploinsufficiency, as they do not lie within the last exon (or in last 50 bp of penultimate exon), and are therefore likely to be subjected to nonsense-mediated decay (NMD)\textsuperscript{35}.

**Discussion**

CDGP and CHH are part of a continuum of GnRH deficiency, ranging from transiently delayed to a complete absence of puberty. However, it is challenging to make a clinical distinction between CHH and CDGP, in adolescents presenting with delayed puberty. In this study, we investigated the genetic overlap between these two conditions focusing on rare variants in known CHH genes and \textit{IGSF10}, a gene recently identified in CDGP. CHH and CDGP differ in terms of the number of patients harboring mutations in individual CHH genes (51% vs. 7%), as well as the overall mutational load in CHH genes (oligogenicity). In both instances, the CDGP probands more closely resembled the control cohort. We also observed similar frequencies of putative \textit{IGSF10} mutations in CDGP and CHH probands, although higher than previously reported\textsuperscript{19}. It is important to note, however, that the previous study by Howard \textit{et al.} utilized a different filtering strategy to identify low-frequency variants, specifically focusing on variants with MAF of <2.5% — a level consistent with the frequency of CDGP. Notably, the present study focused on variants with MAF<1.0% given the rarity of the CHH phenotype, Thus, it is not surprising that different results would be achieved. This would suggest that variants with MAF
1.0-2.5% may contribute more strongly to the CDGP phenotype. Indeed, the most frequent \textit{IGSF10} variant in the CDGP cohort (p.Glu161Lys) has a MAF of 2.0% in the Finnish population. In the current study the variants identified have not been functionally validated nor has segregation with trait within pedigree been analysed, both of which were used to identify definitive pathogenic variants in the previous study by Howard \textit{et al.} Furthermore, the lack of an association of \textit{IGSF10} mutations with CDGP or CHH in the current study may reflect a limitation of rare variant burden testing. It is possible that in a very large gene such as \textit{IGSF10} there may be a large number of non-causal variants or both protective and deleterious variants, and the proportion of these may vary between different populations. In summary, the current data shows that the genetic profile of the Finnish CDGP patients, while enriched for rare putative pathogenic variants in \textit{IGSF10} as compared to ethnically-matched controls, closely resemble the profile of both ExAC Finnish and non-Finnish control cohorts with respect to known CHH genes.

Recent GWAS studies have identified hundreds of loci associated with puberty onset in the general population\textsuperscript{20, 21}, with several signals lying close to or within CHH genes suggestive of a genetic overlap between CHH and CDGP. However, GWAS signals may result from intergenic, intronic, promoter, or other regulatory changes that are not detected by exome sequencing. Therefore, our results in CHH and CDGP patients could have missed pathogenic mutations in regulatory regions. Notably, a genome-wide significant signal in the coding sequence was reported in \textit{TACR3} (p.Trp275*), a mutation identified in nCHH in this report as well as in previous studies\textsuperscript{8, 36, 37}. Although prior GWAS studies have not identified an association for its ligand \textit{TAC3}, we identify mutations in \textit{TAC3} in both CHH and CDGP cohorts. Further, \textit{TAC3} mutations were previously reported in CHH as well as CDGP\textsuperscript{3}. Combined, these
data implicate the neurokinin B pathway in both CHH and CDGP. We propose that larger studies examining pathways rather than individual genes identified by GWAS are required to further elucidate the genetic overlap between CHH and CDGP.

Using whole exome sequencing to examine a larger number of CHH genes in our study, we identified mutations in 51% of CHH cases. This is increased in relation to prior reports of 31%\textsuperscript{10} and 35%\textsuperscript{8} respectively. Our data is mostly consistent with a recent publication by Francou et al.\textsuperscript{38} evaluated a large cohort of nCHH patients of European descent for pathogenic variants in \textit{KISS1R}, \textit{GNRHR}, \textit{TAC3}, \textit{KISS1}, \textit{TAC3}, and \textit{GNRH1}.

We report a genetic overlap between KS and nCHH. Using a gene-collapsed rare variant association study (RVAS) on the entire CHH cohort, we found significant associations for \textit{FGFR1}, \textit{CHD7}, and \textit{SOX10}. Separating CHH into KS and nCHH, the burden test remained significant for \textit{FGFR1} in both subgroups while \textit{CHD7} and \textit{SOX10} were significant only for KS. Notably, significant association appears for \textit{FGF8} in KS while \textit{GNRHR}, \textit{TAC3}, \textit{KISS1} showed association only in nCHH. A significant enrichment of rare variants in the \textit{RNF216} gene was recently shown in patients with CHH and cerebellar ataxia\textsuperscript{39}. In contrast, no enrichment in \textit{KISS1} rare variants was detected in 1,025 CHH patients, without respect to the phenotypic subgroups\textsuperscript{12}. These data point towards the importance of phenotypic clustering to identify novel associated genes\textsuperscript{8, 40}. Finally, our results show that such burden tests might miss associations in important genes like \textit{KISS1R}, because of the low frequency of rare variants in both patient and control population.

Oligogenicity occurs in our study in 15% of CHH cases as compared to 2.5% and 7% in previous reports\textsuperscript{8, 10} using nearly identical strategies for variant classification. This increase is due in part to the increased number of CHH genes screened using exome sequencing. Although
this study does not provide molecular evidences of oligogenic interactions, previous studies demonstrated that oligogenicity is a critical factor in CHH pathogenesis\textsuperscript{8,11,41}. Recent guidelines from the American College of Medical Genetics aid in the identification of pathogenic variants in a clinical setting\textsuperscript{42}. While these guidelines are suited only for monogenic disorders, they do provide a structured framework from which to evaluate variants. Using these guidelines, all ACMG pathogenic or likely pathogenic variants were also classified as pathogenic in the current study (Supplementary Table S2). However, a large number of pathogenic variants detected in the current study were classified as unknown significance using ACMG guidelines. This is primarily due to the weight assigned to (1) familial segregation that is not applicable in the setting of oligogenicity and (ii) detection of \textit{de novo} that was not possible in this study as parental DNA was not available for most probands.

The combination of mutations in both \textit{FGFR1} and \textit{CHD7} occurred most frequently (n=4 probands). These two genes might play coordinated roles during GnRH neuron development and migration as \textit{CHD7} regulates the transcription of Fgf8, a major ligand for \textit{FGFR1} in GnRH neuron ontogeny\textsuperscript{11}. Moreover, both \textit{FGFR1} and \textit{CHD7} are expressed in relevant tissues for CHH, such as the olfactory bulb and hypothalamus\textsuperscript{43}. A previous report also suggested functional interactions between these genes, as CHH patients with mutations in \textit{FGFR1} and \textit{CHD7} exhibit overlaps in associated phenotypes (cleft lip/palate, coloboma or ear anomalies)\textsuperscript{44}.

One notable exception to oligogenicity was \textit{ANOS1} – which was inherited in an exclusively monoallelic fashion due to its X-linked recessive mode of inheritance and high penetrance. Mutations in other genes such as \textit{TAC3}, \textit{KISS1}, \textit{PROK2}, and \textit{PROKR2} were primarily biallelic, and oligogenic interactions were not observed — likely due to their recessive mode of inheritance. Interestingly, the frequency of monogenic inheritance in KS was
significantly higher than in nCHH. To date, it is unclear whether this difference is due to distinct genetic architecture or that “missing” partners in an oligogenic inheritance for KS have yet to be discovered.

We discovered putatively pathogenic mutations in CHH genes in up to 17% of controls, which at first glance seems counterintuitive. Importantly, oligogenicity was only rarely found in controls (2%), further supporting the oligogenic model of CHH pathogenesis. Additionally, many of the putative heterozygous mutations in controls were found in genes with an autosomal recessive inheritance, which would explain the lack of obvious reproductive phenotypes among controls. Further, CHH and controls differ markedly for protein-truncating variants (PTVs) (29% versus 5%, respectively), and the PTVs in controls were less likely to be pathogenic.

This study expands our understanding of the genetic architecture of both CHH and CDGP, and highlights the very large proportion of cases of CDGP that are not explained by mutations in known genes. Further, the genetic profiles of CHH and CDGP appear to be distinct with respect to the 25 CHH genes studied here, with the understanding that ethnic differences between groups (European vs Finnish) could contribute to this finding. This observation may facilitate differential diagnosis of CHH and CDGP in early adolescence when a clear and early diagnosis is critical to initiate timely induction of puberty in patients with CHH. A genetic test resulting in 1) more than one CHH gene mutated (oligogenicity), 2) hemizygous ANOS1 mutations in male patients, or 3) biallelic mutations in genes associated with autosomal recessive inheritance would favor a diagnosis of CHH. Additional comprehensive studies in larger cohorts may enable genetic testing to inform a more precise differential diagnosis in the clinical setting.
Disclosure

Ethics approval and consent to participate
This study was approved by the ethics committee of the University of Lausanne. All participants provided written informed consent prior to study participation. The study protocol was approved by the Ethics Committee for Paediatrics, Adolescent Medicine and Psychiatry, Hospital District of Helsinki and Uusimaa (and extended to encompass Kuopio, Tampere and Turku University Hospitals) (570/E7/2003). UK ethical approval was granted by the London-Chelsea NRES committee (13/LO/0257). The study was conducted in accordance with the guidelines of The Declaration of Helsinki.

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

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Author contributions

NP and LD designed the research project. DC, JSA, NP, SRH, CX, GP, FS, AS and LD analyzed the data. JM, CC, JL, XL, HY, JZ prepared and sequenced DNA. JSA and DC managed the project. SRH, CX, SS, LF, LM, PMB, CDG, ADP, WSD, JMF, MH, MLM, JL, CF, AN, FPH, DP, VP, SP, RQ, GS, DA, DK, SabS, OTI, and ODEX team provided for samples DNA and phenotyping. DC, JSA, CX, and NP wrote and prepared the original draft. AAD, GPS, BJS, JSA, NP, CX, LD, SRH reviewed and edited the manuscript.

References

1. Mitchell AL, Dwyer A, Pitteloud N & Quinton R. Genetic basis and variable phenotypic expression of Kallmann syndrome: towards a unifying theory. Trends Endocrinol Metab 2011 22 249-258.
2. Boehm U, Bouloux PM, Dattani MT, de Roux N, Dode C, Dunkel L, Dwyer AA, Giacobini P, Hardelin JP, Juul A, Maghnie M, Pitteloud N, Prevot V, Raivio T, Tena-Sempere M, Quinton R & Young J. Expert consensus document: European Consensus
Statement on congenital hypogonadotropic hypogonadism--pathogenesis, diagnosis and treatment. Nat Rev Endocrinol 2015 11 547-564.

3. Zhu J, Choa RE, Guo MH, Plummer L, Buck C, Palmert MR, Hirschhorn JN, Seminara SB & Chan YM. A shared genetic basis for self-limited delayed puberty and idiopathic hypogonadotropic hypogonadism. J Clin Endocrinol Metab 2015 100 E646-654.

4. Raivio T, Falardeau J, Dwyer A, Quinton R, Hayes FJ, Hughes VA, Cole LW, Pearce SH, Lee H, Boepple P, Crowley WF, Jr. & Pitteloud N. Reversal of idiopathic hypogonadotropic hypogonadism. N Engl J Med 2007 357 863-873.

5. Waldstreicher J, Seminara SB, Jameson JL, Geyer A, Nachtigall LB, Boepple PA, Holmes LB & Crowley WF, Jr. The genetic and clinical heterogeneity of gonadotropin-releasing hormone deficiency in the human. J Clin Endocrinol Metab 1996 81 4388-4395.

6. Legouis R, Hardelin JP, Levilliers J, Claverie JM, Compain S, Wunderle V, Millasseau P, Le Paslier D, Cohen D, Caterina D & et al. The candidate gene for the X-linked Kallmann syndrome encodes a protein related to adhesion molecules. Cell 1991 67 423-435.

7. Franco B, Guioli S, Pragliola A, Incerti B, Bardoni B, Tonlorenzi R, Carrozzo R, Maestrini E, Pieretti M, Taillon-Miller P, Brown CJ, Willard HF, Lawrence C, Graziella Persico M, Camerino G & Ballabio A. A gene deleted in Kallmann's syndrome shares homology with neural cell adhesion and axonal path-finding molecules. Nature 1991 353 529-536.

8. Miroufi H, Dwyer AA, Sykiotis GP, Plummer L, Chung W, Feng B, Beenken A, Clarke J, Pers TH, Dworzynski P, Keefe K, Niedziela M, Raivio T, Crowley WF, Jr., Seminara SB, Quinton R, Hughes VA, Kumanov P, Young J, Yialamas MA, Hall JE, Van Vliet G, Chanoine JP, Rubenstein J, Mohammadi M, Tsai PS, Sidis Y, Lage K & Pitteloud N. Mutations in FGF17, IL17RD, DUSP6, SPRY4, and FLRT3 are identified in individuals with congenital hypogonadotropic hypogonadism. Am J Hum Genet 2013 92 725-743.

9. Lewkowitz-Shpuntoff HM, Hughes VA, Plummer L, Au MG, Doty RL, Seminara SB, Chan YM, Pitteloud N, Crowley WF, Jr. & Balasubramanian R. Olfactory phenotypic spectrum in idiopathic hypogonadotropic hypogonadism: pathophysiological and genetic implications. J Clin Endocrinol Metab 2012 97 E136-144.

10. Sykiotis GP, Plummer L, Hughes VA, Au M, Durrani S, Nayak-Young S, Dwyer AA, Quinton R, Hall JE, Gusella JF, Seminara SB, Crowley WF, Jr. & Pitteloud N. Oligogenic basis of isolated gonadotropin-releasing hormone deficiency. Proc Natl Acad Sci U S A 2010 107 15140-15144.

11. Falardeau J, Chung WC, Beenken A, Raivio T, Plummer L, Sidis Y, Jacobson-Dickman EE, Eliseenкова AV, Ma J, Dwyer A, Quinton R, Na S, Hall JE, Huot C, Alois N, Pearce SH, Cole LW, Hughes V, Mohammadi M, Tsai P & Pitteloud N. Decreased FGF8 signaling causes deficiency of gonadotropin-releasing hormone in humans and mice. J Clin Invest 2008 118 2822-2831.

12. Chan YM, Broder-Fingert S, Paraschos S, Lapatto R, Au M, Hughes V, Bianco SD, Min L, Plummer L, Cerrato F, De Guillebon A, Wu IH, Wahab F, Dwyer A, Kirsch S, Quinton R, Cheetham T, Ozata M, Ten S, Chanoine JP, Pitteloud N, Martin KA, Schifffmann R, Van der Kamp HJ, Nader S, Hall JE, Kaiser UB & Seminara SB. GnRH-deficient phenotypes in humans and mice with heterozygous variants in KISS1/Kiss1. J Clin Endocrinol Metab 2011 96 E1771-1781.
13. Beales PL, Badano JL, Ross AJ, Ansley SJ, Hoskins BE, Kirsten B, Mein CA, Froguel P, Scambler PJ, Lewis RA, Lupski JR & Katsanis N. Genetic interaction of BBS1 mutations with alleles at other BBS loci can result in non-Mendelian Bardet-Biedl syndrome. *Am J Hum Genet* 2003 72 1187-1199.

14. Kajiwara K, Berson EL & Dryja TP. Digenic retinitis pigmentosa due to mutations at the unlinked peripherin/RDS and ROM1 loci. *Science* 1994 264 1604-1608.

15. Bouilly J, Beau I, Barraud S, Bernard V, Azibi K, Fagart J, Fèvre A, Todeschini AL, Veitia RA, Beldjord C, Delemer B, Dode C, Young J & Binart N. Identification of Multiple Gene Mutations Accounts for a new Genetic Architecture of Primary Ovarian Insufficiency. *J Clin Endocrinol Metab* 2016 101 4541-4550.

16. Patino LC, Beau I, Carlosama C, Buitrago JC, Gonzalez R, Suarez CF, Patarroyo MA, Delemer B, Young J, Binart N & Laisse P. New mutations in non-syndromic primary ovarian insufficiency patients identified via whole-exome sequencing. *Hum Reprod* 2017 1-9.

17. Gajdos ZK, Hirschhorn JN & Palmert MR. What controls the timing of puberty? An update on progress from genetic investigation. *Curr Opin Endocrinol Diabetes Obes* 2009 16 16-24.

18. Wehkalampi K, Widen E, Laine T, Palotie A & Dunkel L. Patterns of inheritance of constitutional delay of growth and puberty in families of adolescent girls and boys referred to specialist pediatric care. *J Clin Endocrinol Metab* 2008 93 723-728.

19. Howard SR, Guasti L, Ruiz-Babot G, Mancini A, David A, Storr HL, Metherell LA, Sternberg MJ, Cabrera CP, Warren HR, Barnes MR, Quinton R, de Roux N, Young J, Guiochon-Mantel A, Wehkalam K, Andre V, Gothilf Y, Cariboni A & Dunkel L. IGSF10 mutations dysregulate gonadotropin-releasing hormone neuronal migration resulting in delayed puberty. *EMBO Mol Med* 2016 8 626-642.

20. Perry JR, Day F, Elks CE, Sulem P, Thompson DJ, Ferreira T, He C, Chasman DI, Esko T, Thorleifsson G, Albrecht E, Ang WQ, Corre T, Cousminer DL, Feenstra B, Franceschini N, Ganna A, Johnson AD, Kjellqvist S, Lunetta KL, McMahon G, Nolte IM, Paternoster L, Porcu E, Smith AV, Stolk L, Teumer A, Tsennikova N, Tikkanen E, Ulivi S, Wagner EK, Amin N, Bierut L, Byrne EM, Hottenga J, Koller D, Mangino M, Pers TH, Yerges-Armstrong LM, Hua Zhao J, Andrus IL, Anton-Culver H, Atsma F, Bandinelli S, Beckmann MW, Benitez J, Blomqvist C, Bojesen SE, Bollka J, Bonanni B, Brauch H, Brenner H, Buring JE, Chang-Claude J, Chanock S, Chen J, Chenevix-Trench G, Colle J, Couch FJ, Couper D, Coviello AD, Cox A, Czene K, D'Adamo A P, Davey Smith G, De Vivo I, Demereth EW, Dennis J, Devilee P, Dieffenbach AK, Dunning AM, Eiriksdottir G, Eriksson JG, Fasching PA, Ferrucci L, Flesch-Janys D, Flyger H, Foroud T, Franke L, Garcia ME, Garcia-Closas M, Geller F, de Geus EE, Giles GG, Gudbjartsson DF, Gudnason V, Guenel P, Guo S, Hall P, Hamann U, Haring R, Hartman CA, Heath AC, Hofman A, Hoving MJ, Hopper JL, Hu FB, Hunter DJ, Karasik D, Kiel DP, Knight JA, Kosma VM, Kutilik Z, Lai S, Lambrechts D, Lindblom A, Magi R, Magnusson PK, Mannermaa A, Martin NG, Masson G, McArdle PF, McArdle WL, Melbye M, Michailidou K, Mihailov E, Milani L, Milne RL, Nevanlimna H, Neven P, Nohr EA, Oldshinkel AJ, Oostra BA, Polatotic A, Peacock M, Pedersen NL, Peterlengo P, Peto J, Pharoah PD, Postma DS, Pouta A, Pylkas K, Radice P, Ring S, Rivadeneira F, Robino A, Rose LM, Rudolph A, Salomaa V, Sanna S, Schlessinger D, Schmidt MK, Southey MC, Sovio U, Stampfer MJ, Stockl D, Storniolo AM, Timpson
NJ, Tyrer J, Visser JA, Vollenweider P, Volzke H, Waeb G, Waldenberger M, Wallaschowskii H, Wang Q, Willemenget S, Winqvist R, Wollfennbuttel BH, Wright MJ, Australian Ovarian Cancer S, Network G, kConFab, LifeLines Cohort S, InterAct C, Early Growth Genetics C, Boomsma DI, Econs MJ, Khaw KT, Loos RJ, McCarthy MI, Montgomery GW, Rice JP, Streten EA, Thorsteinsdottir U, van Duijn CM, Alizadeh BZ, Bergmann S, Boerwinkle E, Boyd HA, Crisponi L, Gasparini P, Gieger C, Harris TB, Ingelsson E, Jarvelin MR, Kraft P, Lawlor D, Metspalu A, Pennell CE, Ridker PM, Snieder H, Sorensen TI, Spector TD, Strachan DP, Uitterlinden AG, Wareham NJ, Widen E, Zygmunt M, Murray A, Easton DF, Stefansson K, Murabito JM & Ong KK. Parent-of-origin-specific allelic associations among 106 genomic loci for age at menarche. *Nature* 2014 **514** 92-97.

21. Lunetta KL, Day FR, Sulem P, Ruth KS, Tung JY, Hinds DA, Esko T, Elks CE, Altmaier E, He C, Huffman JE, Mihailov E, Porcu E, Robino A, Rose LM, Schick UM, Stolk L, Teumer A, Thompson DJ, Traglia M, Wang CA, Yerges-Armstrong LM, Antoniou AC, Barbieri C, Coviello AD, Cucca F, Demerath EW, Dunning AM, Gandon I, Grove ML, Gudbjartsson DF, Hocking LJ, Hofman A, Huang J, Jackson RD, Karasik D, Kriebel J, Lange EM, Lange LA, Langenberg C, Li X, Luan J, Magi R, Morrison AC, Padmanabhan S, Pirie A, Polasek O, Porteous D, Reiner AP, Rivadeneira F, Rudan I, Sala CF, Schlessinger D, Scott RA, Stockl D, Visser JA, Volker U, Vozei D, Wilson JG, Zygmunt M, Consortium EP-I, Generation S, Boerwinkle E, Buring JE, Crisponi L, Easton DF, Hayward C, Hu FB, Liu S, Metspalu A, Pennell CE, Ridker PM, Strauch K, Streten EA, Tonio C, Uitterlinden AG, Ulivi S, Volzke H, Wareham NJ, Wellons M, Franceschini N, Chasman DI, Thorsteinsdottir U, Murala M, Ong KK & Perry JR. Rare coding variants and X-linked loci associated with age at menarche. *Nat Commun* 2015 **6** 7756.

22. Lin L, Conway GS, Hill NR, Dattani MT, Hindmarsh PC & Achermann JC. A homozygous R262Q mutation in the gonadotropin-releasing hormone receptor presenting as constitutional delay of growth and puberty with subsequent borderline oligospermia. *J Clin Endocrinol Metab* 2006 **91** 5117-5121.

23. Tusset C, Noel SD, Trarbach EB, Silveira LF, Jorge AA, Brito VN, Cukier P, Seminara SB, Mendonca BB, Kaiser UB & Latronico AC. Mutational analysis of TAC3 and TACR3 genes in patients with idiopathic central pubertal disorders. *Arq Bras Endocrinol Metabol* 2012 **56** 646-652.

24. Harrington J & Palmert MR. Clinical review: Distinguishing constitutional delay of growth and puberty from isolated hypogonadotropic hypogonadism: critical appraisal of available diagnosti studies. *J Clin Endocrinol Metab* 2012 **97** 3056-3067.

25. Pitteloud N, Hayes FJ, Boepple PA, DeCruz S, Seminara SB, MacLaughlin DT & Crowley WF, Jr. The role of prior pubertal development, biocheical markers of testicular maturation, and genetics in elucidating the phenotypic heterogeneity of idiopathic hypogonadotropic hypogonadism. *J Clin Endocrinol Metab* 2002 **87** 152-160.

26. Wehkalamri K, Widen E, Laine T, Palotie A & Aon L. Association of the timing of puberty with a chromosome 2 locus. *J Clin Endocrinol Metab* 2008 **93** 4833-4839.

27. Palmert MR & Dunkel L. Clinical practice. Delayed puberty. *N Engl J Med* 2012 **366** 443-453.

28. Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, O'Donnell-Luria AH, Ware JS, Hill AJ, Cummings BB, Tukiainen T, Birnbaum DP, Kosmicki JA, Duncan
LE, Estrada K, Zhao F, Zou J, Pierce-Hoffman E, Berghout J, Cooper DN, Deflaux N, DePristo M, Do R, Flannick J, Fromer M, Gauthier L, Goldstein J, Gupta N, Howrigan D, Kiezun A, Kurki MI, Moonshine AL, Natarajan P, Orozco L, Peloso GM, Poplin R, Rivas MA, Ruano-Rubio V, Rose SA, Ruderfer DM, Shakir K, Stenson PD, Stevens C, Thomas BP, Tiao G, Tusie-Luna MT, Weisburd B, Won HH, Yu D, Altshuler DM, Ardissino D, Boehnke M, Danesh J, Donnelly S, Elousa R, Florez JC, Gabriel SB, Getz G, Glatt SJ, Hultman CM, Kathiresan S, Laakso M, McCarroll S, McCarthy MI, McGovern D, McPherson R, Neale BM, Palotie A, Purcell SM, Saleheen D, Scharf JM, Sklar P, Sullivan PF, Tuomilehto J, Tsuang MT, Watkins HC, Wilson JG, Daly MJ, MacArthur DG & Exome Aggregation C. Analysis of protein-coding genetic variation in 60,706 humans. Nature 2016 536 285-291.

29. Firmann M, Mayor V, Vidal PM, Bochud M, Pecoud A, Hayoz D, Paccaud F, Preisig M, Song KS, Yuan X, Danoff TM, Stirnadel HA, Waterworth D, Mooser V, Waeger G & Vollenweider P. The CoLaus study: a population-based study to investigate the epidemiology and genetic determinants of cardiovascular risk factors and metabolic syndrome. BMC Cardiovasc Disord 2008 8 6.

30. DePristo MA, Banks E, Poplin R, Garmella KV, Maguire JR, Hartl C, Philippakis AA, del Angel G, Rivas MA, Hanna M, McKenna A, Fennell TJ, Kernytsky AM, Sivachenko AY, Cibulskis K, Gabriel SB, Altshuler D & Daly MJ. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet 2011 43 491-498.

31. Cingolani P, Platts A, Wang le L, Coon M, Nguyen T, Wang L, Land SJ, Lu X & Ruden DM. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. Fly (Austin) 2012 6 80-92.

32. Yeo G & Burge CB. Maximum entropy modeling of short sequence motifs with applications to RNA splicing signals. J Comput Biol 2004 11 377-394.

33. Ng PC & Henikoff S. SIFT: Predicting amino acid changes that affect protein function. Nucleic Acids Res 2003 31 3812-3814.

34. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS & Sunyaev SR. A method and server for predicting damaging missense mutations. Nat Methods 2010 7 248-249.

35. Nagy E & Maquat LE. A rule for termination-codon position within intron-containing genes: when nonsense affects RNA abundance. Trends Biochem Sci 1998 23 198-199.

36. Gianetti E, Tusset C, Noel SD, Au MG, Dwyer AA, Hughes VA, Abreu AP, Carroll J, Trarbach E, Silveira LF, Costa EM, de Mendonca BB, de Castro M, Lofrano A, Hall JE, Bolu E, Ozata M, Quinton R, Amory JK, Stewart SE, Arlt W, Cole TR, Crowley WF, Kaiser UB, Latronico AC & Seminara SB. TAC3/TACR3 mutations reveal preferential activation of gonadotropin-releasing hormone release by neurokinin B in neonatal life followed by reversal in adulthood. J Clin Endocrinol Metab 2010 95 2857-2867.

37. Xu N, Kim HG, Bhagavath B, Cho SG, Lee JH, Ha K, Meliciani I, Wenzel W, Podolsky RH, Chorich LP, Stackhouse KA, Grove AM, Odom LN, Ozata M, Bick DP, Sherins RJ, Kim SH, Cameron RS & Layman LC. Nasal embryonic LHRH factor (NELF) mutations in patients with normosmic hypogonadotropic hypogonadism and Kallmann syndrome. Fertil Steril 2011 95 1613-1620 e1611-e1617.
38. Francou B, Paul C, Amazit L, Cartes A, Bouvattier C, Albarel F, Maiter D, Chanson P, Trabado S, Brailly-Tabard S, Brue T, Guiochon-Mantel A, Young J & Bouligand J. Prevalence of KISS1 Receptor mutations in a series of 603 patients with normosmic congenital hypogonadotrophic hypogonadism and characterization of novel mutations: a single-centre study. *Hum Reprod* 2016 **31** 1363-1374.

39. Margolin DH, Kousi M, Chan YM, Lim ET, Schmahmann JD, Hadjiavassiliou M, Hall JE, Adam I, Dwyer A, Plummer L, Aldrin SV, O'Rourke J, Kirby A, Lage K, Milunsky A, Milunsky JM, Chan J, Hedley-Whyte ET, Daly MJ, Katsanis N & Seminara SB. Ataxia, dementia, and hypogonadotropism caused by disordered ubiquitination. *N Engl J Med* 2013 **368** 1992-2003.

40. Villanueva C, Jacobson-Dickman E, Xu C, Manouvrier S, Dwyer AA, Sykiotis GP, Beenken A, Liu Y, Tommiska J, Hu Y, Tiosano D, Gerard M, Leger J, Drouin-Garraud V, Lefebvre H, Polak M, Carel JC, Phan-Hug F, Hauschild M, Plummer L, Rey JP, Raivio T, Bouloux P, Sidis Y, Mohammadi M, de Roux N & Pitteloud N. Congenital hypogonadotropic hypogonadism with split hand/foot malformation: a clinical entity with a high frequency of FGFR1 mutations. *Genet Med* 2015 **17** 651-659.

41. Dode C, Teixeira L, Levilliers J, Fouveaut C, Bouchard P, Kottler ML, Lespinasse J, Lienhardt-Roussie A, Mathieu M, Moerman A, Morgan G, Murat A, Toublanc JE, Wolczynski S, Delpech M, Petit C, Young J & Hardelin JP. Kallmann syndrome: mutations in the genes encoding prokineticin-2 and prokineticin receptor-2. *PLoS Genet* 2006 **2** e175.

42. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL & Committee ALQA. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* 2015 **17** 405-424.

43. Sanlaville D, Etchevers HC, Gonzales M, Martinovic J, Clement-Ziza M, Delezoide AL, Aubry MC, Pelet A, Chemouny S, Crraud C, Audollent S, Esclupavit C, Goudefroye G, Ozilou C, Fredouille C, Joye N, Morichon-Delvallez N, Dumez Y, Weissenbach J, Munnich A, Amiel J, Encha-Razavi F, Lyonnet S, Vekemans M & Attie-Bitach T. Phenotypic spectrum of CHARGE syndrome in fetuses with CHD7 truncating mutations correlates with expression during human development. *J Med Genet* 2006 **43** 211-217.

44. Hardelin JP & Dode C. The complex genetics of Kallmann syndrome: KAL1, FGFR1, FGF8, PROKR2, PROK2, et al. *Sex Dev* 2008 **2** 181-193.
Figure legends

Fig. 1. KS and nCHH display both shared and specific genetic patterns, and CDGP is not characterized by genetic overlap with CHH.

Histograms showing CHH genes and IGSF10 mutational prevalence in (A) CHH, (B) KS, (C) nCHH, (D) CDGP, (E) CoLaus, and (F) ExAC Finnish (FIN) cohorts. The prevalence of probands with variants in each gene are noted in black for nonsynonymous (i.e. missense and inframe InDels), white for splicing, and grey for nonsense (i.e. frameshift and stop gained) variants.

Fig. 2. The majority of CHH genes are inherited in a oligogenic fashion in CHH probands, a trend not observed in CDGP probands and CoLaus controls.

Histograms showing CHH genes mutational prevalence in (A) CHH, (B) KS, (C) nCHH, (D) CDGP and (E) CoLaus screened individuals. Each bar contains the frequency of each gene inheritance: monoallelic (grey), biallelic (yellow) or oligogenic (red).

Fig. 3. Oligogenicity is a common factor in CHH, and it is not found in CDGP. Histogram showing the frequency of KS (red), nCHH (yellow), CHH (orange), CoLaus (blue) and CDGP (green) individuals having no rare variants in CHH genes, one gene mutated or at least two genes mutated (oligogenicity). Differences between KS, nCHH and CHH vs. CoLaus controls were analyzed via a two-sided Fisher’s exact test. p < 0.05 was considered significant. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001. Not significant differences among KS, nCHH and CHH vs. CoLaus are not displayed.
Fig. 4. *FGFR1* and *CHD7* are frequently inherited in digenic fashion among CHH probands.

(A) Matrix showing shading-coded frequencies CHH genes digenic combinations.

(B) Available pedigrees of CHH probands displaying oligogenic inheritance. Circles denote females; squares denote males; arrows depict probands; WT denotes wild-type.
Table 1. CHH known genes mutated allele frequencies in CHH, KS, nCHH, CDGP probands and CoLaus, 1000 Genomes and ExAC European controls.

| Phenotype reported | CHH RVA test | KS RVA test | nCHH RVA test | CDGP | CoLaus | 1000 Genomes EUR | ExAC FIN | ExAC NFE |
|--------------------|--------------|-------------|---------------|------|--------|--------------------|----------|---------|
| ANOS1              | KS           | 0.9%        | ns            | vs. CoLaus | vs. 1000 Genomes EUR | vs. ExAC NFE | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles |
|                    | SEMA3A       | 1.3%        | ns            | vs. CoLaus | vs. 1000 Genomes EUR | vs. ExAC NFE | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles |
|                    | FGF8         | 0.9%        | ns            | vs. CoLaus | vs. 1000 Genomes EUR | vs. ExAC NFE | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles |
|                    | FGF17        | 0.4%        | ns            | vs. CoLaus | vs. 1000 Genomes EUR | vs. ExAC NFE | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles |
|                    | SOX10        | 2.2%        | ns            | vs. CoLaus | vs. 1000 Genomes EUR | vs. ExAC NFE | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles |
|                    | IL17RD       | 1.3%        | ns            | vs. CoLaus | vs. 1000 Genomes EUR | vs. ExAC NFE | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles |
|                    | AXL          | 1.7%        | ns            | vs. CoLaus | vs. 1000 Genomes EUR | vs. ExAC NFE | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles |
|                    | FGF1R1       | 7.8%        | 3.8E-07       | 8.5E-14     | 9.8% | 1.8E-08    | 2.1E-07    | 6.6E-11  | 5.5% | ns | vs. CoLaus | vs. ExAC NFE | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles |
|                    | CHD7         | 6.9%        | 1.6E-07       | 2.9E-04     | 2.6E-05  | 9.0% | 1.4E-09    | 1.3E-04    | 4.2E-05  | 4.5% | ns | vs. CoLaus | vs. ExAC NFE | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles |
|                    | HS6ST1       | 0.9%        | ns            | vs. CoLaus | vs. 1000 Genomes EUR | vs. ExAC NFE | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles |
|                    | PROKR2       | 3.0%        | ns            | vs. CoLaus | vs. 1000 Genomes EUR | vs. ExAC NFE | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles |
|                    | WDR11        | 0.9%        | ns            | vs. CoLaus | vs. 1000 Genomes EUR | vs. ExAC NFE | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles |
|                    | PROK2        | 0.9%        | ns            | vs. CoLaus | vs. 1000 Genomes EUR | vs. ExAC NFE | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles |
|                    | GNRHR        | 1.3%        | ns            | vs. CoLaus | vs. 1000 Genomes EUR | vs. ExAC NFE | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles |
|                    | GNRHR        | 3.0%        | ns            | vs. CoLaus | vs. 1000 Genomes EUR | vs. ExAC NFE | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles |
|                    | KISS1        | 0.9%        | ns            | vs. CoLaus | vs. 1000 Genomes EUR | vs. ExAC NFE | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles |
|                    | KISS1R       | 0.4%        | ns            | vs. CoLaus | vs. 1000 Genomes EUR | vs. ExAC NFE | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles |
|                    | TAC3         | 0.4%        | ns            | vs. CoLaus | vs. 1000 Genomes EUR | vs. ExAC NFE | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles |
|                    | TAC3R        | 2.2%        | ns            | vs. CoLaus | vs. 1000 Genomes EUR | vs. ExAC NFE | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles | % mutated alleles |

CHH, congenital hypogonadotropic hypogonadism; KS, Kallmann syndrome; nCHH, normosmic congenital hypogonadotropic hypogonadism; CDGP, constitutional delay of growth and puberty; ns, not significant. Rare variant association (RVA) test was performed via a two-sided Fisher’s exact test. Association with p ≤ 0.002 (in bold) were considered significant after Bonferroni correction.
Figure 4

A

B

Pedigree 1

FGFR1
CHD7
WT/WT
WT/WT
P369A/WT

c.1430+1delG/WT

Pedigree 2

FGFR1
CHD7
WT/WT
WT/WT

SOX10

Y654*WT
L260V/VT
D64V/WT

Y654*WT
WT/WT
D64V/WT

Pedigree 3

FGFR1
IL17RD
WT/WT
WT/WT

G1348R/WT
Y370C/WT

WT/WT
WT/WT

CHH
anemia