

GENETICS IN ENDOCRINOLOGY

Genetic counseling for congenital hypogonadotropic hypogonadism and Kallmann syndrome: new challenges in the era of oligogenism and next-generation sequencing

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Abstract

Congenital hypogonadotropic hypogonadism (CHH) and Kallmann syndrome (KS) are rare, related diseases that prevent normal pubertal development and cause infertility in affected men and women. However, the infertility carries a good prognosis as increasing numbers of patients with CHH/KS are now able to have children through medically assisted procreation. These are genetic diseases that can be transmitted to patients' offspring. Importantly, patients and their families should be informed of this risk and given genetic counseling. CHH and KS are phenotypically and genetically heterogeneous diseases in which the risk of transmission largely depends on the gene(s) responsible(s). Inheritance may be classically Mendelian yet more complex; oligogenic modes of transmission have also been described. The prevalence of oligogenicity has risen dramatically since the advent of massively parallel next-generation sequencing (NGS) in which tens, hundreds or thousands of genes are sequenced at the same time. NGS is medically and economically more efficient and more rapid than traditional Sanger sequencing and is increasingly being used in medical practice. Thus, it seems plausible that oligogenic forms of CHH/KS will be increasingly identified making genetic counseling even more complex. In this context, the main challenge will be to differentiate true oligogenism from situations when several rare variants that do not have a clear phenotypic effect are identified by chance. This review aims to summarize the genetics of CHH/KS and to discuss the challenges of oligogenic transmission and also its role in incomplete penetrance and variable expressivity in a perspective of genetic counseling.

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Invited Author's profile

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Introduction

Congenital hypogonadotropic hypogonadism (CHH, MIM 615267) with normal olfaction (normosmic CHH, or nCHH) or with altered odor perception (anosmia or hyposmia), a form known as Kallmann syndrome (KS, MIM 147950, 244200, 308700, 610628, 612370, 612702), are rare genetic diseases that prevent pubertal development in both males and females (1, 2, 3, 4). They are also responsible for infertility in both sexes (5, 6, 7, 8, 9). The impact of CHH on puberty, sexuality and reproduction result from deficient secretion or action of gonadotropin-releasing hormone (GnRH). This causes inadequate secretion of the pituitary gonadotrophins LH (luteinizing hormone) and FSH (follicle-stimulating hormone), which affects gonadal function starting in fetal life, through adolescence and into adulthood (2, 6). The pathophysiology of CHH is classically divided into two major forms: the first involves abnormal differentiation, development or migration of GnRH neurons during fetal life, preventing their correct anatomical positioning in the hypothalamus – the KS paradigm (10, 11, 12, 13). The other form is due to either signaling abnormalities that involve neuroendocrine factors necessary for GnRH secretion or responsiveness to the stimulatory effects of GnRH on gonadotrope pituitary cells – normosmic paradigm (nCHH) (14, 15, 16, 17).

There is growing evidence, however, that the pathophysiology of CHH may sometimes involve a combination of genetic abnormalities that simultaneously affect both neuronal development and the gonadotropic cascade (18, 19).

A large number of loci underlying CHH/KS have been discovered in the past 30 years (Fig. 1) (2, 19). Notable, the genetic architecture is now known to be far more complex than previously thought. Classic Mendelian transmission was initially proposed as the exclusive model; yet, this is not evident in a significant portion of familial and sporadic cases (2, 19). Thus, genetic counseling is quite complex and sometimes unreliable. One key point to bear in mind is that most infertile patients with CHH and KS have a very good reproductive prognosis (5, 6, 7, 8, 9). The majority of men and women with CHH/KS have the potential to conceive with appropriate hormone treatment and/or assisted reproductive technologies (ART). All CHH/KS patients seeking fertility should have counseling and discussions with their provider/genetic specialist on the risk of transmitting the disease (2). The reliability of genetic counseling for a given patient will depend on the genes involved and the mode of transmission.

Over the past nearly five years, genetic assessment of patients with inherited diseases, including CHH/KS, has increasingly used massively parallel next-generation sequencing (NGS), allowing simultaneous analysis of tens to thousands of genes, depending on whether targeted-exome or whole-exome sequencing is used (2, 20, 21, 22). Consequently, detecting more than one rare but potentially deleterious variants in a given patient (oligogenism or potential oligogenism) is becoming increasingly common (20, 21, 22). This review summarizes the classical modes of transmission for CHH/KS loci reported in the literature and explores the growing role of oligogenism. In the context of CHH/KS, genetic testing is primarily performed to facilitate diagnosis and for informing decision making around fertility. With technologic advances, the field is moving rapidly. Based on the latest literature, this review aims to help health care professionals to provide the most reliable and informed genetic counseling for the patients they care for.

Clinical diagnosis of CHH/KS

In either sex, CHH/KS is usually diagnosed in adolescents and young adults who present with arrested or absent puberty (1, 2, 3, 4). The hormonal diagnosis is based on a concomitantly low (or inappropriately normal) serum level of both pituitary gonadotrophins and sex steroids (1, 2, 3, 4, 23, 24, 25). The rare exception concerns mutations of the genes encoding follicle-stimulating hormone (FSH) and luteinizing hormone (LH) beta subunits (described later). Detailed phenotypic studies, differential diagnoses and hormonal findings in CHH/KS are available in recent reviews (2) and will not be discussed here.

When CHH is suspected, a detailed history and physical examination are needed to identify if there is an abnormal sense of smell (hyposmia, anosmia) indicative of KS or other signs suggestive of a more complex syndrome (2). This step is important not only for establishing the pathophysiological framework, but also at ensuring optimal medical management and ascertaining the phenotypes of other family members. Indeed, a thorough assessment of the family including a 3-generation pedigree is crucial for determining the likely mode of transmission (26). Using a checklist can help facilitate such an assessment (Supplementary Fig. 1, see section on supplementary data given at the end of this article) The pedigree/genogram should distinguish mutations carriers in affected and unaffected family members as well as

Table 1 Main genes associated with in congenital hypogonadotropic hypogonadism, Kallmann syndrome (KS) and related syndromes.

Gene	OMIM	Cytogenetic location	Exon number	Mode of inheritance	Main phenotype	Main non reproductive associated signs
Isolated CHH						
<i>GNRH1</i>	614841	8p21.2	4	AR/oligo*	nCHH/GnRH deficiency	No
<i>GNRHR</i>	146110	4q13.2	3	AR/oligo*	nCHH/GnRH deficiency	No
<i>KISS1</i>	614842	1q32.1	3	AR*	nCHH/GnRH deficiency	No
<i>KISS1R</i>	614837	19p13.3	5	AR*	nCHH/GnRH deficiency	No
<i>TAC3</i>	614839	12q13.3	6	AR*	nCHH/GnRH deficiency	No
<i>TACR3</i>	614840	4q24	5	AR*	nCHH/GnRH deficiency	No
<i>FSHB</i>	136530	11p14.1	3	AR	Dissociated HH/FSH deficiency	No
<i>LHB</i>	152780	19q13.33	3	AR	Dissociated HH/FSH deficiency	No
KS						
<i>ANOS1 (KAL)</i>	300836	Xp22.31	14	X-linked*	KS	MM-S,RA
<i>FGFR1</i>	147950	8p11.23	18	AD/AR/Oligo/de novo*	KS/nCHH	CLP,DA,BA, SHF
<i>FGF8</i>	612702	10q24.32	6	Oligo	KS/nCHH	CLP,DA,BA
<i>FGF17</i>	603725	8p21.3	7	Oligo	KS/nCHH	?
<i>PROK2</i>	610628	3p13	4	AR/AD/Oligo*	KS/nCHH	No**
<i>PROKR2</i>	244200	20p12.3	2	AR/AD/Oligo*	KS/nCHH	No**
<i>CHD7</i>	612370	8q12.2	38	AD/AR/Oligo/de novo	CHARGE/KS/nCHH	CLP,HI,EEA,SCC,C
<i>NSMF (NELF)</i>	614838	9q34.3	16	Oligo	KS/nCHH	?
<i>HS6ST1</i>	614880	2q14.3	2	Oligo ?	KS/nCHH	?
<i>WDR11</i>	614858	10q26.12	29	?	KS/nCHH	?
<i>SEMA3A</i>	614897	7q21.11	17	AD ?/Oligo ?	KS	No
<i>SEMA7A</i>	607961	15q24.1	15	Oligo	KS/CHH	?
<i>SEMA3E</i>	608166	7q21.11	18	Oligo	KS/CHH	?
<i>PLXNA1</i>	601055	3q21.3	33	Oligo?	KS/CHH	?
<i>SOX10</i>	602229	22q13.1	4	AD	Waardenbourg/KS	HI,SCC, ISHH
<i>IL17RD</i>	615267	3p14.3	13	Oligo	KS/CHH	?
<i>FEZF1</i>	613301	7q31.32	6	AR	KS	No
Syndromic HH						
<i>DAX1 (NROB1)</i>	300200	Xp21.2	2	X-linked	CHH/AHC	AI
<i>HESX1</i>	182230	3p14.3	4	AR	C-Hypopit	SOD
<i>LHX4</i>	602146	1q25.2	6	AR	C-Hypopit	Phyp,ST,CM
<i>PROP1</i>	601538	5q35.3	3	AR	C-Hypopit	PMass,Phyp,ST,
<i>LEP</i>	614962	7q32.1	3	AR	Morbid Obesity/HH	
<i>LEPR</i>	614963	1p31.3	18	AR	Morbid Obesity/HH	
<i>PCSK1</i>	162150	5q15	14	AR	Morbid Obesity/HH/AI	ACTHD, HGID
<i>OTUD4</i>	611744	4q31.21	22	AR	HH+Ataxia	PD
<i>RNF216</i>	212840	7p22.1	17	AR	HH+Ataxia	PD
<i>PNPLA6</i>	603197	19p13.2	37	AR	Gordon Holmes/Boucher-Neuhauser	CD
<i>SOX2</i>	184429	3q26.33	1	AR	C-Hypopit	An

ACTHD, ACTH deficiency; AD, autosomal dominant; AHC, adrenal hypoplasia congenita; AI, andenal insufficiency; An, anophthalmia; AR, autosomal recessive; BA, bone abnormalities; C, coloboma; CD, chorioretinal dystrophy; C-Hypopit, Congenital Hypopituitarism; CLP, Cleft lip/palate; CM, Chiari malformation; DA, dental agenesis; EEA, External ear anomalies; HGID, hyperglycemia and insulin deficiency; HH, Hypogonadotropic Hypogonadism; HI, Hearing impairment; ISHH, iris, skin, hair, hypopigmentation; KS, Kallmann syndrome; MM-S, mirror movements-synkinesia; nCHH, normosmic Congenital Hypogonadotropic Hypogonadism; Oligo, oligogenic or potentially oligogenic (see text); Phyp, pituitary hypoplasia; PMass, pituitary masses; RA, renal agenesis; SCD, Semicircular canal dysplasia; SHF, split hand foot malformations; SOD, septo-optic dysplasia; ST, sella turcica abnormalities.

*See also Fig.2; **in subjects with bi-allelic loss of function mutations.

Families with nCHH and mutations in ligands (i.e. *GNRH1*, *KISS1*, *TAC3*) of the above-mentioned receptors are far more rare. Indeed, only 4 nCHH families carrying biallelic *GNRH1* mutations have been reported (50, 51, 52), along with a few cases of nCHH/KS probands with monoallelic mutations and an additional mutation

in another CHH/KS loci (i.e. potential oligogenic transmission – see below) (51, 53, 54). As for *KISS1*, only one, consanguinous, family has been described to date, in which members with nCHH carry mutations of this gene in the homozygous state (55), whereas the heterozygous relatives are healthy. Five familial cases involving *TAC3*

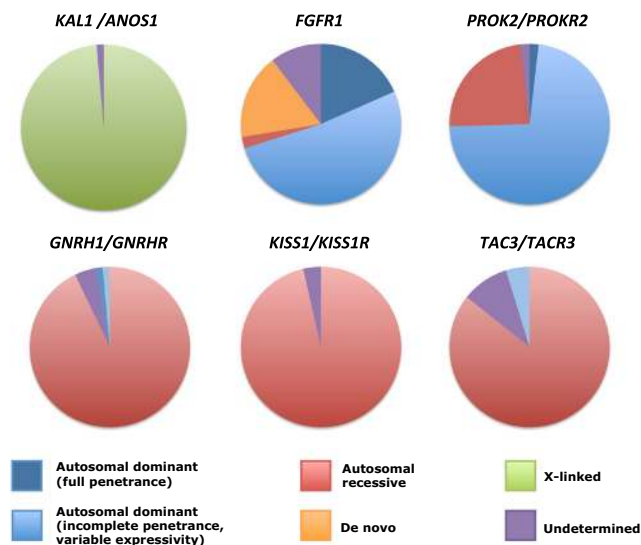


Figure 2

Inheritance patterns of the main CHH/KS genetic forms.

The indicated inheritance patterns have been established on the basis of the analysis of solely well-genotyped families available in the literature (see supplementary references).

The number of analyzed pedigrees is as follows: *KAL1/ANOS1*, $n=144$; *FGFR1*, $n=87$; *PROK2*, $n=10$; *PROKR2*, $n=44$; *GNRH1*, $n=12$; *GNRHR*, $n=63$; *KISS1*, $n=1$; *KISS1R*, $n=27$; *TAC3*, $n=5$; *TACR3*, $n=20$. Only pedigrees in which the genetic mutation was carried by the *propositus* and at least one first-line relative have been considered. *De novo* mutations have been defined only when both parents' genetic status was available. Please note that for *GNRHR*, *GNRH1*, *KISS1R*, *TAC3* and *TACR3* genes, inheritance patterns classified as undetermined or autosomal dominant refer to families in which mutations are carried exclusively in monoallelic state.

mutations have been reported (45, 46, 47, 48, 49). In all these reports, those harboring the *TAC3* variant in a heterozygous state were disease-free (45, 46, 47, 48, 49). Analysis of the genotype/phenotype relationship in these familial cases again shows that transmission takes place in AR manner, with high penetrance when both alleles are mutated (45, 46, 47, 48, 49).

Mutations of the genes coding for the two beta-subunits specific to LH and FSH have also been described. Fewer than 10 hypogonadal patients with *LHB* mutations have been reported to date (56, 57, 58, 59, 60). Men with *LHB* mutations are reported to have absent puberty and/or signs of hypogonadism linked to profound testosterone deficiency, azoospermia and testicular hypotrophy. In one case, treatment with hCG resulted

in normal circulating testosterone and restored fertility (58). Hormonal evaluations show variable gonadotrophin profiles (high or undetectable serum LH) depending on the assay method used (radioimmunoassay with polyclonal antibodies, radioreceptor assay, or sandwich methods with monoclonal antibodies) and the type of mutation. A single female case of *LHB* mutation has been described to date in a consanguineous Brazilian family (57). The patient had normal pubertal development, with first menstruation at age 13 years, but chronic oligomenorrhea with a normal ultrasound aspect of the ovaries and antral follicles (64). Regarding *FSHB* (61, 62, 63, 64, 65, 66), a limited number of cases have also been described, with male or female hypogonadism. Except for a unique case with pubertal delay (62), the very rare male patients with *FSHB* mutations have normal puberty but are infertile with azoospermia and undetectable circulating FSH (63, 64, 66). Affected women have absent or partial puberty and primary amenorrhoea (61, 64, 65). These women also have low serum estradiol, undetectable FSH (as in men) and elevated circulating LH (61, 64, 65). Such a dissociation between the two gonadotropins is strongly evocative of this diagnosis. In all the cases of hypogonadism caused by *FSHB* or *LHB* mutations so far described, the disease is transmitted in AR mode: affected patients have biallelic mutations (homozygotes or composite heterozygotes) and their parents, whether consanguineous or not, are healthy heterozygotes, and unaffected relatives are either mutation free or heterozygous (56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66).

***FEZF1* as an autosomal recessive form of KS**

In 2014, Kotan and coworkers (67), by combining candidate gene screening with autozygosity mapping and whole-exome sequencing, identify a homozygous missense loss-of-function *FEZF1* mutation, and a nonsense *FEZF1* mutation in two independent consanguineous Kurdish families, each with two KS siblings (67). The parents and unaffected siblings were either homozygous for wildtype *FEZF1* or heterozygous for the *FEZF1* variants indicating AR inheritance (67). Notably, no additional phenotypes/signs were observed (67) (Table 1).

To date, despite the analysis of more than 200 KS patients by whole-exome or targeted-exome sequencing, no new cases of KS due to *FEZF1* mutations have been reported (Bouligand & Young Ms in preparation). These mutations thus appear to be exceptionally rare.

PROKR2-associated CHH/KS: clear autosomal recessive inheritance in a subset of patients

Pedigree analysis of CHH/KS cases associated with mutations of *PROKR2* shows that AR transmission is evident in approximately 20% of cases (Fig. 2 and Supplementary References). In such instances, patients with bi-allelic mutations display the KS phenotype while relatives carrying heterozygous mutations in *PROKR2* are unaffected (2, 68, 69). In the literature to date, AR transmission is demonstrated a minority of cases of patients harboring rare variants in *PROKR2*. Other modes of transmission that have been proposed are presented below.

Counseling for families exhibiting AR nCHH/KS

In autosomal recessive (AR) pedigrees, males and females are equally affected, and affected individuals usually exist in sibships with unaffected parents and possibly unaffected brothers and sisters. However, it should not be forgotten that, simple chance dictates that only men will be affected in some familial cases with AR transmission, wrongly pointing to X-linked inheritance (42).

Consanguinity (parents who are cousins) or at least endogamy (both parents belonging to the same small ethnic or geographic population, as observed in some island communities and mountain villages) strongly supports autosomal recessive inheritance (44, 50, 55, 67). The majority of these patients carry mutations in the homozygous state. It should be remembered, however, that consanguinity/endogamy is not mandatory in CHH with AR transmission. Indeed, many patients with CHH carrying biallelic mutations in the composite heterozygous state do not have consanguinous parents (14, 15, 17).

In a familial context of AR inheritance (Table 1), prospective parents who are heterozygous mutation carriers will be advised that there is a 25% chance that their future offspring will be affected. When a CHH propositus expressed the wish to have children, genetic testing of the partner may help to refine the risk of recurrence (2, 26).

X-linked recessive CHH with high penetrance

KAL1/ANOS1

The first *locus* to be discovered to underlie KS was identified in 1991 (35, 36, 37, 38). Initially, it was called *ADMLX* (adhesion molecule-like, X-linked) (35) or *KALIG-1/KALIG1* (36), then *KAL1* (12, 69), and finally *ANOS1* in the most recent nomenclature (2, 70; <https://www.omim.org/entry/300836>).

KAL1/ANOS1 is located on the short arm of the X chromosome in region Xp22.3, consistent with X-linked transmission (2, 12, 35, 36, 37, 38, 69, 70). It comprises 14 exons and encodes a 680-amino-acid protein (12, 69). *KAL1/ANOS1* mutations reported to date consist mainly of nucleotide deletions or insertions that result in sequence frameshifts or in the introduction of premature stop codons. To date, a minority of the *KAL1/ANOS1* mutations associated with X-linked KS are amino acid substitutions (missense mutations) (12, 69, 70, 71, 72).

In the 144 families described to date (Fig. 2, and Supplementary references), almost all the patients are men with hemizygous *KAL1/ANOS1* mutations. In familial forms, heterozygous women are always asymptomatic carriers (2, 12, 69, 70, 71, 72). However, one series identified 10 women harboring variants. One woman with KS was found to carry a rare *KAL1/ANOS1* variant in a biallelic state that was predicted to be deleterious (4). The other nine had heterozygous variants, and these sometimes co-existed with mutations of other genes linked to CHH/KS, pointing to possible oligogenism (see below).

In the reported cases of KS associated with *KAL1/ANOS1* mutations, the penetrance is near-complete as almost all patients have both CHH and anosmia (more rarely hyposmia) (12, 69, 70, 71, 72, 73). Other signs associated with this genetic form, such as mirror movements (also called bimanual synkinesis) and renal agenesis, are frequent, but they do not always cosegregate with the mutation identified in a given family (69, 70, 71, 72, 73, 74, 75, 76), for reasons that are still not clear. Other, rarer associated signs such as deafness (35, 36, 37, 38, 69, 70, 71, 72, 73, 74, 75, 76) and vas deferens agenesis, have been described in men with *KAL1/ANOS1* mutations (69, 70, 71, 72, 73, 74), but their inconsistent presence remains unexplained. Some patients with X-linked KS have large deletions in chromosome region Xp22.3, removing several adjacent genes. In these patients, other clinical signs have been reported (ichthyosis, mental retardation or dwarfism), which may or may not be associated with one another and which are part of a so called contiguous gene syndrome (12, 35, 36, 37, 38, 75, 76).

DAX1/NROB1

DAX1 was one of the first genes discovered to be linked to CHH (77, 78). *DAX1* mutations are associated with a syndromic form in which CHH is associated with primary adrenal failure due to congenital adrenal hypoplasia (CAH) (OMIM: 300200). *DAX1*, also called *NROB1* according

to recent nomenclature, codes for an orphan receptor composed of 470 amino acids and expressed in the adrenal cortex, the gonads, the ventro-median nucleus of the hypothalamus and the pituitary gland (79, 80). *NROB1* has a key role in the development of the gonads and adrenal cortex. *DAX1/NROB1* is located on the short arm of chromosome X, in region Xp21.2 (77, 78). Logically, CAH associated with CHH linked to *DAX1/NROB1* mutations affects only males (hemizygotes), whereas heterozygous female carriers are free of CHH and CAH (79, 80). A single exception has been reported, in a family in which women with biallelic mutations were affected (81). In men with mutations of this gene, adrenal insufficiency generally occurs in the neonatal period or early childhood (79, 80). Penetrance in men carrying mutations of this gene is near-complete, based on dozens of published cases (79, 80). There is, however, a degree of phenotypic variability in affected men, even within the same family: cases of severe adrenal insufficiency of neonatal onset have been described, other relatives with the same mutation having developed adrenal insufficiency later in life (79, 80, 82, 83). CHH linked to *DAX1/NROB1* mutations may result from impairment of both the hypothalamic and the pituitary components of the gonadotrope axis (79, 80, 81, 82, 83) but, whereas the pituitary involvement is clear and reproducible (no pituitary increase in response to prolonged pulsatile GnRH administration) (82, 83, 84, 85), the hypothalamic involvement can only be indirectly supported (79, 80, 81, 82, 83, 84, 85).

One peculiarity of this genetic form is that it has been virtually impossible to trigger spermatogenesis by prolonged gonadotropin stimulation in the large number of men with azoospermic CAH (32, 82, 86). These men, independent of their congenital gonadotropin deficiency, appear to have specific testicular impairment (87, 88, 89) of the Sertoli cell that express *NROB1* (89).

Cryptorchidism has been described in several patients with *DAX1/NROB1* mutations (84, 89, 90, 91), but it may be less frequent than that in men born with CHH linked to *KAL1/ANOS1* mutations (2, 73, 74, 92). The severity of CHH in men with *DAX1/NROB1* mutations can differ within the same family (79, 80, 82, 88, 93). Some *DAX1/NROB1* mutated patients may even have spontaneous puberty and varying degrees of altered spermatogenesis (79, 80, 82, 94).

Counseling for X-linked forms

As mentioned earlier, only males are affected by *ANOS1/KAL1* and *DAX1/NROB1* mutations, inherited from

unaffected female carriers (2, 26). One requirement for X-linked inheritance is a lack of male-to-male transmission in a given pedigree. In typical X-linked recessive pedigrees, carrier females have a 50% chance that their male children will have KS, while 50% of their female offspring will carry the mutated gene. All female children of affected male patients will be obligate carriers, and all male offspring will be free of the genetic defect. These data highlight the importance of screening unaffected female carriers when a propositus is diagnosed with KS and an *ANOS1/KAL1* mutation. The age at which female carriers should be screened for the mutation is controversial. In our center, we take into account the opinion of the girls' parents, whose written consent is required for genetic analysis. We advise them that screening is best done before the age of puberty. We propose a specific appointment for genetic counseling in order to address the issue raised by the familial genetic disease, with an endocrinologist, clinical geneticist, the parents, and the obligate carrier daughter. We explain the clinical features that may be transmitted in this genetic form: CHH with a high frequency of cryptorchidism and micropenis (92) and a possibility of renal agenesis and synkinesia (73, 74) if the mutation lies in *ANOS1/KAL1*. In the case of *DAX1/NROB1* mutation, we explain the consequences of primary adrenal failure and the severity of the associated infertility (32, 82, 84, 86, 91).

FGFR1 autosomal dominant (AD) form and the challenges of incomplete penetrance and variable expressivity

FGFR1 was the first gene to be identified as an autosomal dominant (AD) form of KS (Fig. 1) (2, 10, 12, 95). The initial publication reported the KS patients with *FGFR1* mutations in the heterozygous state, some of which (frameshift or nonsense mutations) appeared from the outset to be loss-of-function mutations, as later confirmed (95, 96, 97). More than 140 *FGFR1* mutations have been reported to date and are of variable types (nonsense, missense, frameshift and splicing mutations) (Supplementary references). Deletions are exceptionally infrequent (12, 98, 99) and typically involve contiguous genes carried away by microdeletions in the 8p11.2 region. Such patients are reported to have a more complex syndromic clinical picture, with spherocytosis (100, 101).

While the initial pedigrees described were suggestive of AD transmission (Fig. 2), some individuals with *FGFR1* mutations do not manifest KS, which is why penetrance was considered to be incomplete (2, 95, 96). The second

finding was that not all mutated subjects in a given family had both main signs of KS but that some had isolated anosmia or simple transient pubertal delay (92, 95, 96, 102, 103, 104). This discordance between the two main signs of KS was subsequently confirmed by numerous independent teams (Fig. 2) (2). An important finding reported by both Pitteloud and coworkers and Xu and coworkers was that *FGFR1* mutations could cause not only KS but also normosmic form as verified by olfactory testing, even in several members of a given family (96, 105, 106). The existence of normosmic forms of CHH linked to *FGFR1* mutations has since been confirmed by others (107, 108, 109).

Notably, it is necessary to highlight the relatively frequent occurrence of *de novo* mutations, as demonstrated by negative *FGFR1* analysis in both parents of mutated proband (Fig. 2). These *FGFR1* neomutations explain a number of sporadic cases of KS and nCHH (2, 69).

Regarding the reproductive phenotype, one difference between KS linked to mutations in *FGFR1* and to *KALI/ANOS1* lies in the clinical severity of CHH. In men with *KALI/ANOS1* mutations, not only is the penetrance of CHH almost complete but, on average, the pre- and postnatal gonadotropin deficit is also more severe (i.e. higher frequency of micropenis, cryptorchidism and complete absence of pubertal development (6, 92, 104). In contrast, in men with *FGFR1* mutations, the degree of gonadotropin deficiency is more variable, ranging from profound, to partial puberty and even to reversal (2, 25, 92, 104).

The non-reproductive signs first linked to *FGFR1* mutations include bone abnormalities (2, 12, 74, 95, 96, 102, 103), dental agenesis (2, 12, 95, 110), cleft lip and cleft palate (2, 12, 69, 74, 95, 109). The presence of all these abnormalities has since been confirmed by several independent teams, but is variably present. Taking all reported nCHH/KS patients with *FGFR1* mutations into account, their prevalence is not clearly established. In addition, co-segregation of these associated signs is very often imperfect in families with nCHH/KS and *FGFR1* mutations (2, 96, 103, 109), thus complicating genetic counseling. Cleft palate and/or cleft lip can sometimes be severe (110, 111, 112) but is fortunately absent in most reported cases (2, 12, 69, 95, 96, 102, 103, 104, 105, 106).

Some clinical signs that are very common in men with KS linked to *KALI/ANOS1* mutations, such as mirror movements, have also been reported in some KS patients with *FGFR1* mutations, but their prevalence is much lower (69, 95, 103). To our knowledge, no cases of renal agenesis associated with *FGFR1* mutations have

been reported (as of november 2017), even though this associated malformation has been reported in autosomal cases with unknown genetic defect (74).

Some patients harboring mutations in *FGFR1* have a syndromic presentation. Hartsfield's syndrome appears to represent the most severe extreme of the phenotypic spectrum associated with *FGFR1* mutations (113, 114). This syndrome includes holoprosencephaly and ectrodactyly, with or without hare lip/cleft palate and CHH (113, 114). The clinical severity may be explained either by biallelic *FGFR1* mutations or by loss-of-function mutations with a dominant negative effect (113, 114). Another severe syndromic form of CHH/KS very commonly associated with *FGFR1* mutations includes split hand/foot malformations (115). In such cases, the CHH is usually severe, and biallelic *FGFR1* mutations have also been reported.

Other genetic forms of KS with predominantly autosomal dominant transmission

CHD7

CHD7 (chromodomain helicase DNA-binding protein 7, a gene located in chromosome region 8q12.1, MIM 608892) was first discovered as a genetic cause of the CHARGE syndrome (coloboma, heart defects, atresia choanae, retarded growth and development, genital hypoplasia, ear anomalies/deafness) (116) (MIM 214800). In 2005, Pinto and coworkers (117) showed that the genital hypoplasia of the CHARGE syndrome was actually linked to CHH. The same authors showed that CHARGE patients had olfactory-bulb agenesis, meaning that this complex syndrome included the two main signs of KS (117). Later, Kim and coworkers showed that patients with *CHD7* mutations could also have a minor/paucisymptomatic form of this syndrome, along with a KS phenotype or even nCHH, without the other classical clinical signs of the CHARGE syndrome listed above (118, 119). More recent work confirmed the existence of isolated KS in subjects harboring *CHD7* mutations and showed that, in these forms, the mutations are usually of the missense type with partial loss of function, whereas truncating mutations (nonsense, frameshift and splice mutations) are far more frequent in the traditional CHARGE syndrome (120, 121). Phenotypic analysis of families with members carrying deleterious *CHD7* missense mutations showed that, as with *FGFR1* mutations, there is very broad phenotypic

variability: patients bearing the same mutation could have KS, nCHH or isolated anosmia (120, 121). Similar to *FGFR1*, pedigree analysis reveals broad phenotypic variability as patients carrying the identical mutation may present with KS, nCHH or isolated anosmia. In some families, maternal or paternal transmission of mutations has been observed without any history of prior treatment for infertility consistent with autosomal dominant transmission with variable penetrance of CHH and anosmia and/or signs of CHARGE syndrome (120, 121). *CHD7* *de novo* mutations also seem highly prevalent in CHH/KS patients (119, 120).

CHD7 and *FGFR1* appear to be the genes most frequently mutated in autosomal forms of CHH/KS (120, 121, and Bouligand *et al.* Ms in preparation). There is still some controversy regarding the ability of *CHD7* mutations causing CHH with normal olfaction. Some teams have reported *CHD7* mutations only in subjects with KS, and others in patients with both KS and nCHH (118, 119, 120, 121). This discrepancy may reflect either recruitment bias or to the use of differing methods of evaluating olfaction (simple questioning or formal testing and olfactometry or olfactory bulbs MRI). Another important point is that some individuals initially considered to have isolated KS actually had signs of the CHARGE syndrome and that the initial clinical diagnosis of KS was in fact incorrect, owing to insufficiently thorough initial clinical examination (122).

Signs frequently associated in patients with *CHD7* mutations include deafness and outer-ear abnormalities, hare lip/cleft palate and cardiac abnormalities (120, 121, 122, 123). The frequency of these associated, non-reproductive signs has not been systematically estimated in patients with KS/nCHH. It may have been underestimated, owing to inadequately exhaustive clinical examination (122, 123). It is therefore essential to seek these disorders in KS/CHH propositi, in order to optimize genetic counseling.

SOX10

SOX10 (MIM 602229) belongs to the SOX transcription factor family, whose members are involved in a number of multiorgan developmental processes. It encodes for a transcription factor expressed by GnRH neuron precursors. A role of *SOX10* in Kallmann syndrome was recently demonstrated by Pingault and coworkers (124). *SOX10* mutations associated with the KS phenotype are mainly missense loss-of-function or frameshift mutations present in the heterozygous state. To date, relatively few cases of KS linked to *SOX10* mutations have been described

(124, 125, 126, 127, 128, 129). However, their number may well increase rapidly, simply because this gene will be more frequently analyzed in patients with KS/nCHH as next-generation sequencing methods are adopted (see below) (129). At the phenotypic level, this genetic form of KS is characterized by a high prevalence of neurogenic deafness associated with semi-circular canal defects similar to those seen in CHH patients with *CHD7* mutations (124, 125, 126, 130). Other noteworthy clinical signs in these patients with *SOX10* mutations include abnormal iris pigmentation (124, 126, 127) and wisps/patches of white hair. In the few KS/CHH pedigrees so far described, transmission of *SOX10* mutations is autosomal dominant but again, as with *FGFR1* and *CHD7*, the penetrance of the various clinical signs is variable even among related individuals carrying the same *SOX10* mutation (124, 125, 126, 127, 128, 129, 130).

Counseling for AD forms

Classically, autosomal dominant (AD) pedigrees display a 'vertical' inheritance pattern that contrasts with the 'horizontal' pattern characteristic of AR families (26). If we exclude *de novo* mutations of *FGFR1* or *CHD7* (described earlier), typical AD inheritance is usually associated with more than one affected generation with members exhibiting a CHH/KS phenotype; males and females are equally affected and both female-to-male and male-to-male transmission may be observed (Fig. 2). One important point is that in families with AD inheritance of KS, penetrance and expressivity can vary both for the main signs (CHH and impaired sense of smell) and associated signs (2, 95, 96, 118, 119, 120, 121, 124, 125, 126). This means that members of a family harboring the identical same CHH/KS mutation may have either a complex form with many associated signs or isolated CHH/KS, isolated nCHH or isolated anosmia, transient delayed puberty and no potentially evident phenotype (95). Because of this incomplete penetrance, some pedigrees may be suggestive of AR transmission (95).

In pedigrees indicating AD transmission, patients and their families should be informed that the risk of transmitting the mutation is 50% (26, 69, 74, 131), but often, the phenotype and severity are much more difficult to predict with accuracy. In a family that has variable phenotypes associated with the same mutation, patients and their relatives may be able to better understand this variability. In contrast, it is more difficult to explain the variable penetrance and expression resulting from a possible AD inheritance of a *de novo* mutation

(131, 132, 133). As mentioned, *de novo* mutations of *FGFR1* and *CHD7* are not uncommon (2, 12, 69, 119, 120, 121, 133 and Bouligand Young unpublished results). In some cases, putative *de novo* mutations are in fact discovered to be inherited yet parents are negative due to mosaicism (132). In the context of an incomplete family phenotype, it is difficult to predict the risk of transmission of the respective disease features. The literature can be useful for recurrent mutations, but care should be taken due to the modifying effects of different familial genetic background, thus altering the phenotypic expression of a recurrent deleterious rare variant. Genetic counseling will also take into account the isolated nature of the mutation or, on the contrary, its association with other rare CHH/KS gene variants that would be inherited from asymptomatic parents and could point to oligogenism (see below and Fig. 3). The incomplete penetrance and variable expressivity observed in pedigrees with these CHH/KS

genes with apparent AD transmission greatly complicates genetic counseling. One potential explanation for these observed discrepancies may be the fact that rare variants (oligogenism) in multiple loci may be contributing to the observed phenotype.

Genetic forms of CHH/KS with complex, multiple or indeterminate modes of transmission

PROK2/PROKR2

PROK2 codes for a peptide containing 81 amino acids, called prokinectin 2, while *PROKR2* codes for its type 2, G-protein-coupled receptor (134, 135, 136, 137, 138). The first mutations of the *PROK2/PROKR2* genes were reported by Dodé and coworkers in 2006, in patients of both

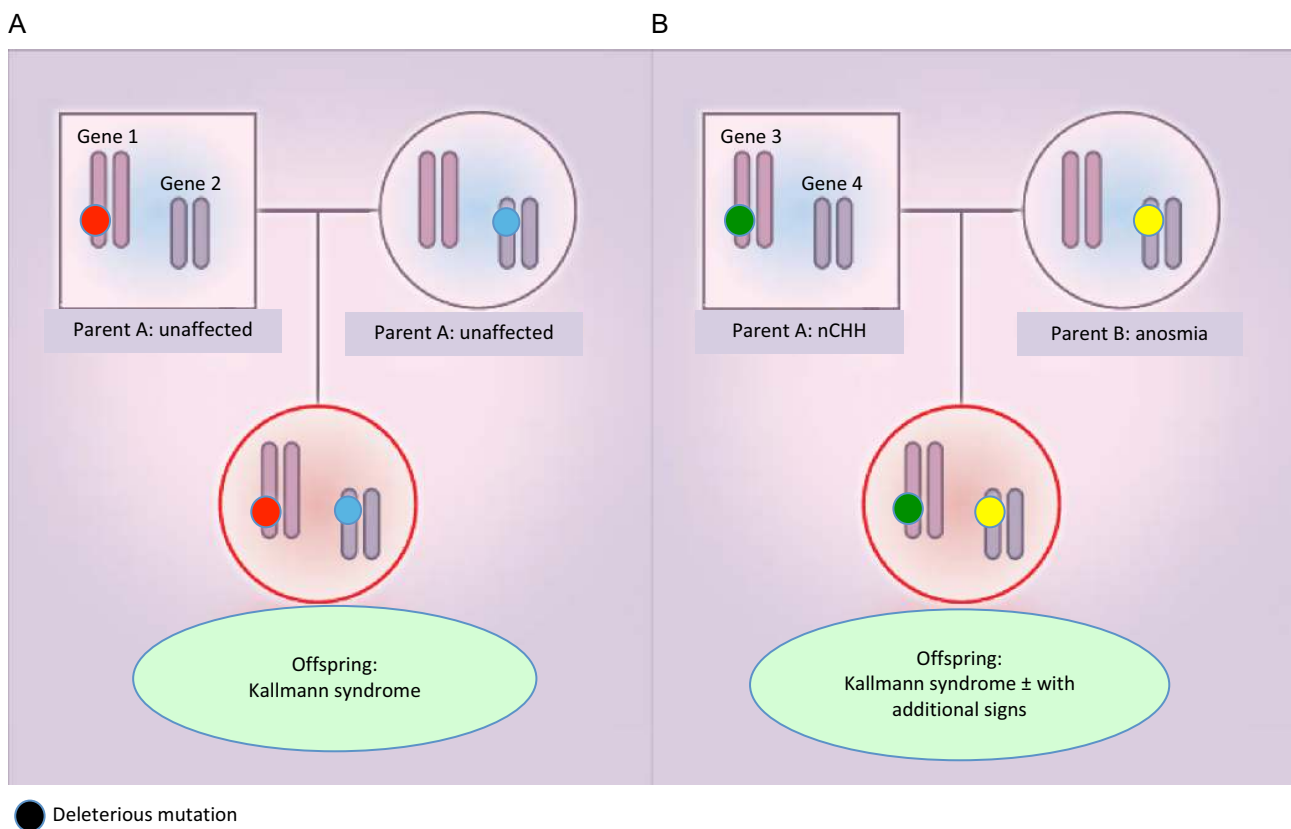


Figure 3

Model of nonmendelian (digenic) CHH/KS inheritance affecting either penetrance or expressivity. (A) Parents carrying mutations in only one gene are unaffected. Only double heterozygotes carrying mutations in both genes 1 and 2 are affected by the disease. (B) Parents carrying a mutation in only one gene (3 or 4) present only part of the Kallmann phenotype. Only double heterozygotes carrying mutations in both genes 3 and 4 are affected by full Kallmann syndrome (± associated with non olfactory-non reproductive phenotypes). See published examples in (2).

sexes with KS or nCHH. In this initial publication, the authors describe familial cases with pedigrees suggestive of autosomal dominant transmission with variable penetrance, as well as sporadic cases (Fig. 2) (134).

PROKR2 and *PROK2* mutations have since been reported in a number of patients with either KS or nCHH (134, 135, 136, 137, 138, 139, 140). *PROKR2* mutations (mainly missense but also nonsense and frameshift mutations) are significantly much more prevalent than rare variants in *PROK2*, which are carried in the heterozygous, homozygous or compound heterozygous state (134, 135, 136, 137, 138, 139, 140, 141). Many of these *PROKR2* mutations are predicted to have deleterious effects or have been shown to alter receptor signaling or trafficking *in vitro* (135, 138, 142, 143). Nearly a dozen *PROK2* mutations (missense or frameshift) have been reported in a small number of KS/CHH patients, carried in the simple heterozygous and biallelic states (134, 135, 136, 137, 138, 139, 140, 141, 142, 143).

Interestingly, whereas only mice with homozygous knockout of the orthologous genes *prok2* and *prokr2* exhibit a phenotype reminiscent of KS (135, 144), biallelic mutations in *PROKR2* or *PROK2* (i.e. demonstrated autosomal recessive transmission – see above) have been observed in a minority of KS/CHH of patients – approximately 20% of pedigrees (Fig. 2 and Supplementary references). Most patients are in fact heterozygotes, and thus, the mode of transmission seems to be sometimes autosomal dominant and sometimes potentially oligogenic (Fig. 2) (134, 135, 136, 137, 138, 139, 140, 141, 143). In this complex genetic context, prior *in vitro* studies co-expressing wild-type and mutant receptors did not observe an effect on wild-type receptor signaling activity (142, 143). These observations argued against a dominant negative effect of monoallelic *PROKR2* mutations in affected patients. However, very recent studies contradict this prior work. Some *PROKR2* deleterious mutants co-transfected *in vitro* with the normal receptor, have a dominant negative effect (138). These data provide the first molecular basis for an autosomal dominant mode of transmission of mutations in *PROKR2* (138).

That being said, we must recognize that in a number of patients, a dominant negative effect of *PROKR2* mutations carried in the heterozygous state does not seem to account for the observed CHH/KS phenotype (68, 138, 142). It is possible that, in the many patients with *PROK2/PROKR2* mutations carried in the simple heterozygous (monoallelic) state, the CHH/KS phenotype could result from synergistic effects of several deleterious genetic events at different loci, i.e. oligogenism (68, 134, 138)

(see below). Finally, given the significant prevalence of some potentially deleterious *PROKR2* rare variants in the general population (134, 137 and <http://exac.broadinstitute.org/gene/ENSG00000101292>) we cannot exclude, that in some CHH/KS patients carrying *PROKR2* heterozygous mutations (sporadic or familial cases), these rare variants may be present by chance, without having a determining role in the appearance or modulation of the CHH/KS phenotype. Given uncertainties to predict phenotype in offspring, it is advisable that in the presence of a patient carrying a heterozygous *PROKR2* mutation, the approach to genetic counseling should be extremely cautious if the pedigree does not clearly demonstrate a specific mode of inheritance. Additional difficult cases are those of pedigrees where *PROK2* or *PROKR2* deleterious rare variants are carried in the heterozygous state by several related subjects expressing the CHH/KS phenotype over several generations even if the penetrance is incomplete (68, 138). We think it is fair to point out a risk of transmission to the patient while recognizing the difficulty in quantifying it.

Attempts to identify associated non-reproductive, non-olfactory manifestations in CHH/KS patients harboring mutations of *PROKR2* or *PROK2* have been made by a number of groups. In fact, additional clinical anomalies frequently reported in patients with *KAL1*, *FGFR1*, *CHD7* or *SOX10* mutations are very rare in *PROK2/PROKR2*-mutated patients, if we exclude additional mutations in the above-mentioned genes. Thus, no cases of renal agenesis or hare lip/cleft palate have been reported, and only exceptional cases with bimanual synkinesia, hearing loss and hypodontia (12, 69, 134, 135, 136, 137, 138, 139, 140). Moreover, some additional disorders, each observed in very few patients, may represent fortuitous associations; these include obesity, diabetes mellitus, psychomotor disorders and depression (136, 137, 138). Particular cases worthy of note include the association of *PROKR2* mutations with hypopituitarism, with or without morphological abnormalities of the pituitary stalk or in context of septo-optic dysplasia (145, 146, 147). In these cases, an oligogenic architecture (146, 148, 149, 150) (see below) or anomalies at two different loci (151) might explain the complex phenotypes.

In fact, published data indicate that in the majority of nCHH and KS patients carrying *PROK2/PROKR2* mutations KS or nCHH is isolated, i.e. without non-reproductive and non-olfactory phenotypes. This is rather reassuring for candidate patients seeking to benefit from medically assisted reproduction, because of the rarity of additional disorders that might affect their offspring's

health (e.g. renal agenesis) or quality of life (hare lip or cleft palate, hearing loss).

CHH/KS associated with mutations in genes involved in the *FGFR1* signaling pathways

In 2008, five years after the discovery of *FGFR1* as a KS responsible gene, Falardeau and coworkers (152), using a candidate-gene approach, reported for the first time in male and female KS/CHH patients, mutations in *FGF8*, a gene encoding a key *FGFR1* ligand. At the same time, Chung and coworkers, using *Fgf8* hypomorphic mice, demonstrated that FGF8 signaling through *FGFR1* was required for the emergence of GnRH neurons in the medial-ventral olfactory epithelium (153). In the initial report by Falardeau, heterozygous mutations affected all four FGF8 splice isoforms (FGF8a, FGF8b, FGF8e and FGF8f), while two others only affected FGF8e and FGF8f (152). The authors also demonstrated that the two mutated FGF8b and FGF8f ligands exhibited decreased biological activity *in vitro*. Since then, few additional patients harboring *FGF8* mutations have been reported (154). The majority of affected CHH/KS patients carry *FGF8* mutations in the heterozygous state (152, 153, 154). Several reported pedigrees suggest an autosomal dominant mode of inheritance, yet probable oligogenism has also been proposed in others cases; this possibility has been reinforced by animal models (155). Patients with KS or CHH who harbor mutations in *FGF8* also display additional phenotypes including sensorineural deafness, cleft lip and cleft palate, flat nasal bridge, camptodactyly and hyperlaxity (2, 152, 153, 154).

The fact that *KAL1*, *FGFR1* and *FGF8* encode components of the FGF pathway led investigators to search for additional CHH/KS candidate genes involved in FGF modulation. First, Tornberg and coworkers reported loss-of-function mutations of heparan sulfate 6-O-sulfotransferase 1 (*HS6ST1*) in families with CHH, suggesting a role of this gene in GnRH neuron development or function (156). These results were consistent with previous findings in *C. elegans*, which showed that heparan 6-O-sulfation was required for FGF8-mediated *FGFR1* signaling.

Secondly, Miraoui and coworkers investigated whether or not CHH patients harbored mutations in members of the so-called FGF8 synexpression group and validated the ability of a bioinformatics algorithm based on protein-interacting data to identify candidate genes (109). This *in silico* approach also encompasses sequence analysis, homology, expression, structure and function. In this way,

the authors identified five additional CHH/KS candidate genes, namely *FGF17*, *IL17RD*, *DUSP6*, *SPRY4* and *FLRT3*. They also demonstrated that the *FGF17* and *IL17RD* mutations affected protein function *in vitro*. To validate the candidate genes, they sequenced 386 unrelated CHH/KS individuals and 155 controls and found a number of mutations in the patients. Interestingly, the authors found that *IL17RD* mutations were only present in KS patients and that they were strongly associated with hearing loss (109). Recently, beta klotho (*KLB*) which signals through *FGFR1* has been linked with CHH/KS and has expanded the role of *FGFR1* signaling in the pathogenesis of CHH/KS (157).

KS and mutations in genes involved in the semaphorin pathway

In 2011, Cariboni and coworkers demonstrated that *Sema3A*-knockout mice exhibited abnormal olfactory bulb development and also defective GnRH neuron migration, leading to a hypogonadal phenotype (158). *SEMA3A* belongs to the class 3 semaphorins, a family of secreted proteins that modulate axonal guidance and neuronal migration by binding to neuropilins (NRP1 and NRP2) and plexins. In 2012, Young and coworkers reported that heterozygous deletion of 11 of the 17 *SEMA3A* exons, detected with a pangenomic approach (CGH array, see below), cosegregated with a KS phenotype in a family with several affected members, strongly suggesting the involvement of this gene in the development of the olfactory system and in the migration of GnRH neurons in humans (159). The relevance of this first report was reinforced by work from Hanchate and coworkers (160) who screened 386 KS patients for *SEMA3A* mutations and identified missense mutations in 24 cases and a small frameshift deletion in one case. As in the seminal paper, all the mutations reported by Hanchate were present in the heterozygous state and sometimes associated with mutations in other CHH/KS genes (see oligogenism below). Most of the missense mutations reported by Hanchate and coworkers resulted in impaired secretion of semaphorin-3A by transfected COS-7 cells or in reduced signaling activity of the mutated semaphorin-3A protein in the GN11 cell line derived from embryonic GnRH cells, suggesting that these mutations could have a pathogenic effect (160). It is known that class 3 semaphorins signal predominantly through transmembrane receptors called plexins, and particularly through plexin-A1. In keeping with the above-mentioned role of *Sema3A*, Marcos and coworkers recently reported that the embryonic phenotype

of *Plxn1*^{-/-} mutant mice, which lack plexin-A1, is similar to that of human KS fetuses (161). Pathohistological analyses revealed abnormal development of the peripheral olfactory system and defective embryonic migration of GnRH neurons to the hypothalamic brain region, resulting in reduced fertility in adult males but not females. The same authors screened 250 patients with full KS for the presence of *PLXNA1* mutations and identified different non-synonymous mutations in 15 patients, all in the heterozygous state (161). These rare variants were predicted *in silico* to affect plexin-A1 stability or signaling, and functional assays confirmed the deleterious impact of some identified variants. These results suggested that inadequate *PlxnA1* signaling could be involved in the abnormal olfactory and reproductive phenotypes of some KS patients. However, the facts that *PLXNA1* mutations were only found in the heterozygous state in these KS patients and that they were associated with only a partial deleterious effect points to a probable oligogenic form. Using a combination of exome sequencing and computational modeling, Cariboni and coworkers identified a shared point mutation in semaphorin 3E (*SEMA3E*) in 2 brothers with KS identifying *SEMA3E* as an essential gene for GnRH neuron development (162).

NELF/NSMF

NELF (nasal embryonic LHRH factor) is currently called NSMF (Nmda receptor synaptonuclear signaling and neuronal migration factor) (MIM 608137) in the HGNC nomenclature. NELF was first found to be expressed in mouse migratory GnRH neurons and to encode a guidance molecule for olfactory axon projections; it was also thought to play a role in the migration of GnRH neurons (163). It naturally became a KS candidate gene (164). *NELF/NSMF* maps to 9q34.3 and contains 16 exons with a 1590-bp open reading frame encoding a 530-amino-acid protein. The role of *NELF/NSMF* in the pathophysiology of CHH/KS is still controversial (165, 166). On the one hand, a first biallelic knockout mouse model exhibited only a subtle phenotype in females and no reproductive phenotype in males, whereas *NELF/NSMF* is an autosomal gene in both mice and humans (165). Another, more recent knock-out model showed no KS-like phenotype in either males or females (166): Spilker and coworkers found no indications of hyposmia or hypogonadotropic hypogonadism in either males or females. The differences between these two murine models may be linked either to more or less thorough phenotypic analysis (144, 167) or to non-identical genetic backgrounds. In humans,

several publications have suggested a link between *NELF/NSMF* mutations and KS. So far, a few different mutations have been identified in CHH patients, always in the heterozygous state and usually co-existing with mutations in other validated or candidate genes (164, 167, 168, 169). There is no solid evidence at this time suggesting that *NELF/NSMF* mutation alone can cause the KS phenotype. However, it might contribute to the phenotype by acting in concert with other CHH genes and/or modulate the severity of the phenotype in a context of oligogenism (169) (see below). Pangenomic analyses of CHH/KS patients and their relatives may help to elucidate these questions.

WDR11

WDR11 (MIM 606417), also called *BRWD2*, is a member of the WD-repeat-containing protein family. This autosomal gene contains 29 exons and is located in chromosome region 10q26. Mutations in *WDR11* were first reported in CHH/KS by Kim and coworkers (170). The locus was found in a KS patient, near a breakpoint of a balanced translocation between chromosomes 10 and 12 (170). An heterozygous c.3450T>G transversion in *WDR11* exon 28, resulting in a p.Phe1150Leu (F1150L) substitution at a highly conserved residue near the C terminus (169) were also identified in a man with incomplete nCHH and in an unrelated woman with complete nCHH. In the same report, Kim also described, in a Turkish man with complete CHH, bilateral cryptorchidism and a normal sense of smell, a heterozygous c.1303G>A transition in *WDR11* exon 10, resulting in an p.Ala435Thr substitution at a highly conserved residue within the sixth WD domain. Finally, these authors described in a KS man, a c.2070T>A transversion in exon 16 of this gene, resulting in a p.His690Gln substitution at a highly conserved residue within the ninth WD domain. None of these *WDR11* rare variants were found in a population of more than 400 Caucasian controls (170).

Since this initial report, other *WDR11* variants affecting highly conserved amino acids have been identified by means of NGS in series of patients with either CHH (171) or unexpected reproductive phenotypes within the spectrum of disorders of sexual development that are different from CHH phenotypes (172). In addition, Choucair and coworkers, using chromosomal microarray analysis, described the case of a male patient of Lebanese origin who had a *de novo* interstitial 4.5-Mb deletion of the 10q26.1 region that includes *WDR11* and another 23 genes, of which two (*BAG3* and *FGFR2*) were found to

be disease related (173). Interestingly, the male proband had micropenis and cryptorchidism (compatible with CHH); yet, gonadotropin deficiency was not mentioned in this report. Additional clinical features present in this patient included growth and psychomotor retardation, microcephaly and flat feet, in keeping with a contiguous gene syndrome (173).

The emergence of oligogenism in CHH/KS

The existence of an inherited disease with digenic transmission was first reported in 1994 by Kajiwara and coworkers (174). These authors described three families with retinitis pigmentosa in which only double heterozygotes carrying both *ROM1* and *peripherin/RDS* gene mutations were affected by the disease. They challenged classical Mendelian transmission and suggested that synergy between two deleterious genetic events in a given individual could result in a disease phenotype. The same authors also predicted that this type of oligogenic architecture would exist in other hereditary diseases (174). For their part, Beales and coworkers showed that oligogenic transmission was involved in the Bardet-Biedl syndrome (175). They reported that, in some families, a combination of three mutant alleles at two different loci, including *BBS1* (trialelic inheritance), was necessary for the disease onset. They proposed that *BBS1* in fact participates in complex non-Mendelian inheritance and that, in different families, *BBS1* mutations interact genetically with mutations at each of the other known *BBS* genes to cause the disease phenotype. This questioning of classical Mendelian transmission in certain genetic diseases is elegantly summarized in the review of Badano and Katsanis (176). This type of oligogenic architecture has also recently been demonstrated in non-syndromic forms of primary ovarian insufficiency (POI), a condition that can also affect both puberty and fertility (177, 178).

With regard to CHH/KS, in 2006 Dodé and coworkers described the first KS patient carrying mutations in two known responsible genes, namely *PROKR2* and *KAL1/ANOS1* (134). The authors raised for the first time the possibility of digenism in this pathological context. Their hypothesis was significantly reinforced a year later by Pitteloud and coworkers (169), who reported two families with KS/CHH and mutations in two known responsible genes. In one of these families, certain individuals carried mutations of a single gene (*FGFR1* or *NELF*), while others carried mutations in both these genes. In the other family, the relatives carried inactivating mutations of *GNRHR* in the mono- or biallelic state, sometimes together with a

deleterious *FGFR1* mutation. Interestingly, the patients' phenotypes were variable in these two families, the clinical syndrome being more severe or more complex in subjects carrying both *FGFR1* and *NELF* mutations. The existence and prevalence of oligogenism or potential oligogenism was first systematically evaluated in 2010 by Sykiotis and coworkers in a large series of CHH/KS patients (179). The authors used Sanger sequencing to screen for mutations in 8 of the genes known at that time to be associated with CHH/KS (*KAL1/ANOS1*, *FGFR1*, *FGF8*, *PROK2*, *PROKR2*, *GNRHR* and *GPR54/KISS1R*). They showed that 2.5% of the CHH/KS patients carried mutations in two or more of these genes. Later, analysis was expanded to include to 7 additional genes involved in *FGFR1* signaling (*FGF171*, *FGF18*, *IL17RD*, *DUSP6*, *SPRY2*, *SPRY4* and *FLRT3*), identifying the prevalence of oligogenism (and potential oligogenism) at nearly 7% (24 of 350 CHH/KS probands bore rare variants in at least two loci) (109). This publication contained additional evidence indicating a real role of oligogenism in the phenotypic variability observed among patients belonging to the same family (109). The concept of an additive or synergistic gene–gene interaction was recently further reinforced by Hong and coworkers (113). As the known loci account for roughly 50% of cases, is it plausible to imagine that as more loci are identified that the rate of certain/potential oligogenicity will continue to rise.

The existence of oligogenism in CHH patients has largely been either suggested or demonstrated over the past ten years by several independent teams (109, 134, 136, 148, 149, 150). Its prevalence, which will probably vary according to the more or less stringent criteria used either to select genes for sequencing or identified variants, appears to be significantly higher in more recent studies, in which the exons of the dozens of genes so far implicated in CHH/KS were analyzed simultaneously by means of massively parallel sequencing (157) (see below).

The main challenge to demonstrating oligogenic transmission in CHH/KS will be to differentiate true oligogenism (as defined above) from the increasingly frequent situation wherein several rare, potentially deleterious variants are incidentally identified in a patient – yet without a clearly demonstrable impact or significantly altered phenotype. In such cases, effective genetic counseling will demand a collectively identified and defined 'minimum consensus criteria' for oligogenism. First-line criteria should certainly include consideration of the co-segregation of combined rare variants with specific or modified phenotype(s) (174, 175, 176). Evidence from *in silico* and *in vitro* studies demonstrating the deleterious

nature of each rare variant on the respective encoded protein is another important criterion. An additional challenge relates to impact that individual mutations have on the CHH/KS phenotype – as this may be altered by variants in other loci. Thus, phenotype may vary based on the particular genes involved in the pathology of CHH/KS. For some genes, the pathological implication is very strong, e.g. *ANOS1* (formerly *KALI*), *FGFR1*. For other loci, typically discovered more recently, the link is less clear. Animal knockout models that induce phenotypes paralleling the human disease strengthen conclusions regarding the implication of certain genes in CHH/KS (e.g. *PROK2/PROKR2* and *SEMA3A*). However, evidence supporting involvement in CHH/KS is less clear or unavailable for many loci (19). Additionally, the reliable determination of oligogenic transmission is hindered by the fact that the mode of transmission is yet to be firmly established for many genes potentially involved in the CHH/KS phenotype (2, 19) (Table 1). Thus, for patients harboring mutations in genes with relatively weak evidence and/or an uncertain mode of transmission, it is difficult to predict if CHH/KS will be transmitted to offspring of affected parents.

Limitations of Sanger sequencing

Since the discovery of the first genes responsible for CHH/KS in the early 1990s (Fig. 1), genetic analyses of CHH patients have mainly been based on Sanger sequencing, a method in which the exons and intron–exon junctions of each gene are analyzed separately. Sanger sequencing is laborious, slow and very costly when large numbers of genes need to be analyzed (Table 2), which has gradually become the rule in this setting (Fig. 1 and Table 1). With Sanger sequencing, when analysis of a first gene shows no deleterious genetic event capable of explaining the phenotype, it was necessary to analyze a second gene, then a third and so on. Initially (before 2005), Sanger sequencing was acceptable because relatively few genes were known to cause CHH/KS (Fig. 1). These limitations help to explain why, prior to 2005, about two-thirds of patients had no known genetic cause (12, 69). Additionally, there are analytical biases as screening was often not performed in cases where the patient's phenotype did not match the gene of interest. This was especially the case when the gene in question was large (more than 20 exons). This analytical bias long excluded from genetic analyses those patients with borderline clinical forms that were nevertheless associated with mutations in a particular large gene. The best example is *CHD7* analysis

Table 2 Approximative cost^a of Sanger vs next-generation sequencing (NGS) of a CHH/KS gene^b.

Sanger sequencing of <i>CHD7</i> gene NM_017780	
Coding DNA (kilobases)	9
Number of amplicons	18
Overall cost of the entire <i>CHD7</i> coding regions analysis by Sanger sequencing	1214 €
Sanger: cost for 500 base pairs	68 €
Overall cost of sequencing of 100 CHH/KS validated and candidate genes by targeted exome	2200 €
Number of 'equivalent' amplicon for the 250 kb NGS targeted exome panel	500
NGS targeted exome: cost for 500 base pair (250 kilobases NGS panel)	4€
Cost ratio Sanger/NGS	15

The example of *CHD7*.

^aIn France according to of price scale fixing by French National Health Universal Insurance.

^bTargeted exome^m NGS approach performed at Bicêtre Hospital that includes a panel of around 100 validated or candidate CHH/KS genes.

in patients with KS or nCHH and no clinical signs of the CHARGE syndrome (see above). Another bias is that when a potentially deleterious rare variant was found in a validated or candidate gene, the analysis was halted because a likely 'genetic explanation' for the phenotype had been found (i.e. pre-oligogenicity period). These attitudes, combined with the limited number of known responsible genes, probably explain why it took until 2006 for the first case of digenism to be discovered (134).

New methods for CHH/KS genetic diagnosis: pangenomic approaches

Advent of next-generation massive parallel sequencing

Since 2010, genetic analyses based on so-called next-generation sequencing (NGS), such as whole-exome sequencing (WES) (20, 21, 22, 180, 181, 182), have gradually entered medical practice, especially in the past five years. WES allows the simultaneous sequencing of nearly 20 000 genes (20, 21, 22, 180). The technical aspects of this molecular genetics approach have been extensively described in several recent excellent reviews (180, 181, 182) and will not be dealt with here, apart from emphasizing the need for appropriate bioinformatic algorithms (filters/pipelines) to process and classify the enormous amount of data thus generated by NGS. In the context of CHH/KS, an important consideration is that WES targets only about 1% of the genome, represented by the exons that encode protein sequences. The resulting

limitation is that WES, unlike whole-genome sequencing (WGS), does not allow the sequencing of intronic or regulatory regions, which could also have a significant role in a given disease (180, 181). In addition, WES coverage of intron–exon junctions is variable, depending on the specific design features of each technique. In some genes, WES may not properly capture these exon/intron boundary regions, where mutations may be of critical importance in the phenotype of CHH by causing splicing defects (180, 47). Further, some genetic abnormalities are poorly detected by WES, such as large rearrangements (deletions, translocations, duplications, chromosomal abnormalities) (20, 21, 22, 159, 180, 181). In CHH/KS, WES currently allows the analysis of all the coding portions of known responsible genes (Table 1). In addition to speed, a primary advantage of WES is its diagnostic yield. Indeed, the rate of detecting a mutation involved in the disease (positivity rate) exceeds 30%. Importantly, the detection rate will depend on the number of genes actually analyzed by the bioinformatics workflow and on the degree of coverage (total or only partial gene sequencing). The prevalence of rare variants will also depend on the choice of bioinformatic filter used to select them (178).

In clinical practice, the use of WES may lead to the detection of known mutations in a hereditary disease (recurrent mutations), new pathogenic variants in a previously validated gene or potentially deleterious rare variants in new genes (162, 178, 180, 181, 182). With WES, although several thousand genes are sequenced, bioinformatics analysis generally focuses on validated or candidate genes first, and then on genes coding for proteins involved in biological systems potentially relevant to the pathophysiology of the disease (178). But one of the main advantages of this method, without *a priori* assumptions, is that it can reveal mutations in unexpected genes, provided there are informative families (with large numbers of affected and unaffected members) available to ensure significant co-segregation between the rare variant(s) and the phenotype (180, 181, 182, 183). In the ‘targeted exome’ approach, only a panel of tens or hundreds of genes previously chosen for their relevance to the disease are sequenced (184). The targeted exome method, focusing on a disease or group of diseases, is most often used for diagnostic testing in reference centers. (17, 176, 184, 185, 187)

A recent major medical breakthrough is that WES and targeted ES significantly limit the above-mentioned analytical biases observed with the Sanger method. Hypothesis-free analyses have revealed attenuated forms of certain genetic diseases that were classically considered

to have an obligatorily complex phenotype, thus leading to a broadening of their clinical spectra and a less dogmatic view of their genotype–phenotype relationships (20, 21, 22, 180, 181, 182, 185). Further, by analyzing tens or hundreds of genes is that the probability of finding several deleterious or potentially deleterious genetic variants in a given patient is significantly increased. This helps to explain the progressive increase in rates of observed oligogenism rise in CHH/KS or in other reproductive disorders (20, 21, 22, 177, 178, 184, 186). Another recent breakthrough provided by WES is the demonstration that some complex or severe phenotypes can in fact result from a combination of several diseases in the same patient – so-called overlapping syndromes – caused by simultaneous mutations separately on several genes, being responsible for different diseases in a ‘blended’ fashion (20, 21, 22, 151, 185, 186). Similarly, NGS may help to understand the inconsistent combinations of certain clinical signs in familial or sporadic CHH/KS. We may thus be able to explain why CHH/KS subjects sharing an identical penetrating mutation exhibit different phenotypes, ranging from minor (isolated CHH) to complex and severe (2, 185).

One of the major challenges introduced by NGS is the increasingly frequent detection of multiple rare variants affecting one or more genes whose involvement in a given disease is well demonstrated, possible, uncertain or unlikely (see above). Indeed, this raises systematic questions regarding the possible impact on the protein function and in particular, the relationship with the observed phenotype (i.e. causal or not). To help clarify these issues, a number of criteria must be analyzed using filters/pipelines to classify and categorize the identified variants. Rare variants may be classified as pathogenic because they either alter protein function or are very likely to be involved in the phenotype (i.e. co-segregation in a pedigree, previously associated with a particular phenotype). At the other end of the spectrum, variants may be classified as benign if they are without functional consequence and/or are unlikely related to the phenotype (180, 188). Categorizing many variants in between these two extremes can be challenging as their impact is less clear. Clinical genetics societies have attempted to reach consensus on this matter to assist clinicians in interpreting such variants (188, 189). The harmonized language has established standardized terminology for variants including ‘pathogenic’, ‘likely pathogenic’, ‘uncertain significance’, ‘likely benign’ and ‘benign’ (188). Classification is determined by set criteria including population variant frequency in control subjects or

databases (e.g. ExAC, <http://exac.broadinstitute.org>), *in silico* prediction algorithms (demonstrating a deleterious effect on the protein), *in vitro* functional studies (when available) and co-segregation with the phenotype in a pedigree.

Comparative genomic hybridization CGH array

Another pangenomic technique developed in parallel to WES is CGH array (comparative genomic hybridization array) (CGH). This approach can screen the entire genome for copy number variants (CNVs) in a single run (75, 76, 159). This method is widely used as a complementary procedure for molecular cytogenetic analysis. Indeed, CGH arrays detect submicroscopic deletions with higher resolution than conventional G-banding chromosome analysis. CGH array is complementary to WES, in that it detects chromosomal micro-rearrangements (deletions and duplications) that are not reliably detected by WES (159, 180, 181, 182). CGH array-based studies have effectively been employed for KS gene discovery in a family with *SEMA3A* deletions that co-segregate with the KS phenotype – and which had not previously been detected by Sanger sequencing (159 and J Young, unpublished results). CGH array can also detect the deletions of previously known genes in patients with isolated KS or a contiguous gene syndrome (75, 76, 159, 173, 187).

From clinical diagnosis of CHH/KS to genetic counseling

As noted, diagnostic evaluation necessitates a thorough clinical examination and history- (see check-list in [Supplementary Table 1](#)). The first consultation is quite lengthy but detailing a 3-generation pedigree can continue over subsequent consultations thus providing the patient time to inquire with relatives including medical issues (i.e. infertility) and specific phenotypes. It must be borne in mind that the history is usually based on patient statements and that errors or omissions may undermine the analysis of co-segregation. To facilitate the largest possible number of relatives should be individually questioned and examined. Given the cost-efficiency of exome analysis ([Table 2](#)), many centers and diagnostic laboratories have moved to using a targeted exome approach. In addition, many patients who have previously undergone Sanger sequencing of selected CHH genes are now being systematically re-analyzed using NGS thus enabling the discovery of rare, potentially deleterious variants in patients who previously had no

genetic diagnosis (188). Targeted exome analysis has also been used to detect additional deleterious rare variants in patients in whom Sanger sequencing had previously revealed a deleterious mutation. Thus, in keeping with the recent literature, the numbers of CHH/KS patients with a genetic diagnosis and with potential or certain oligogenic transmission have increased markedly in our center (Bouligand *et al.*, manuscript in preparation).

Genetic counseling and mode of inheritance

Genetic counseling should be raised at the very first consultation, which usually takes place during adolescence. Depending on the mode of transmission suspected – Mendelian or oligogenic (see below) – the medical advice will be more or less categorical. The patient and his/her family should be informed that genetic counseling can evolve with the discovery of new genetic abnormalities. In our experience, genetic counseling becomes most important when reproductive decisions are undertaken and when they envisage having children, usually around the ages of 25–35. These discussions typically evolve over time and need several encounters – particularly in the context of fertility-inducing treatments. Certainly, cascade screening for other family members may not be acceptable for some families, and this should be handled in a sensitive manner yet with an even presentation of identified familial risk. One aspect of uncertainty relates to the possibility of reversibility (25). To date, no clear predictors (clinical or genetic) have been identified and so appropriate expectation should be set for patients. Another key element meriting discussion is that the known loci only account for roughly half of cases. Thus, it is equally likely that a patient may opt for genetic screening yet will not obtain a result of a rare variant in a identified CHH/KS gene. This may be mitigated in some instances as hormone profiling of neonates during the mini-puberty (first 6-month of life) can biochemically determine if CHH/KS has been transmitted to the offspring (6, 131). If this is planned, adequate coordination with pediatricians should be put in place to ensure this critical window is not missed (2, 6, 131). Therapeutically, effort should be made to elicit the patients' values during these encounters, provide accurate data and support or enable them to make informed decisions that are in line with their value system. Given the complexity of the genetic architecture, reproductive decisions and family dynamics an interprofessional approach can be useful for a comprehensive counseling.

Oligogenic inheritance

As discussed earlier, the term oligogenic transmission implies that the phenotype only emerges, or is only significantly magnified, when more than one *CHH/KS* gene is mutated (174, 175, 176, 177). In other words, before diagnosing oligogenism, it is necessary to show that the phenotype and/or expression of associated signs cosegregate(s) with a greater oligogenic load than other members of the same family who are either unaffected or have only an attenuated form. Figure 3 shows a model of oligogenic transmission that could be observed in CHH/KS (2). Genetic analysis of the *propositus* and relatives (ideally in three generations), at a minimum by targeted exome sequencing or, preferably, by WES, is the best way of suspecting or establishing oligogenic transmission. The probability that non-relatives free of CHH/KS will carry one or several putative mutations responsible for this type of disease is low, given the scarcity of CHH/KS mutations in the general population (109, 178 and <http://exac.broadinstitute.org>). This could lead to 'dilution' of the oligogenic load and make CHH/KS recurrence in the offspring less likely. Another precondition for reliable genetic counseling of patients with CHH/KS and oligogenic transmission is to determine the contribution of each variant carried by affected subjects. Establishing this type of penetrance hierarchy is not always easy: it implies fairly exhaustive phenotypic and genotypic analysis of the affected and unaffected members of the probands' family (ideally in three generations) and studies of the phenotypic impact of each possibly deleterious variant when present in isolation. Unfortunately, this information is not always available.

Another point to consider is the mode of transmission (not always known) of each rare variant carried by the patient.

One critical unsettled issue for genetic counseling is the distinction between real oligogenism, as discussed earlier, and the existence of a 'transient mutation' in a family with a monogenic form of CHH/KS with classical Mendelian transmission but an incidental mutation of another CHH/KS gene that does not cosegregate with the phenotype nor influence its severity or associated signs. To address this diagnostic issue, it is once again necessary to compare the detailed phenotypes of the different family members, and the segregation of relevant rare variants. It will require, as before, rigorous and exhaustive phenotypic evaluation in the largest possible number of family members (ideally in three generations) and broader knowledge of the genes involved in the CHH/KS

phenotype. One practical issue that affects the reliability of genetic counseling is that, despite repeated attempts to collect them, phenotypic and/or genetic familial data may remain elusive.

For the CHH/KS specialist or geneticist performing genetic counseling, an important consideration is how to communicate results of genetic analysis when several, rare, potentially deleterious variants are identified that may cause or modify the CHH/KS phenotype. This is not a simple matter and currently remains a point of debate (189, 190). Certainly, cultural and national ethical-legal aspects are major considerations as regulations vary across countries. Another element of consideration is the context in which the testing was performed, i.e. in the course of hospital-based care or in a research setting. In certain European countries like France, one is obliged to comply with a patient request for full disclosure of genetic test results in situations of care-based/diagnostic testing. That being said, it is our opinion that specialist clinicians and geneticists play a key part in deciphering results (188, 189). They play an important part in evaluating whether or not the weight of evidence supports a significant role for a particular defect (in a particular gene) in contributing to the diagnosis and potential transmission to offspring (188, 189). One can certainly communicate that NGS may identify variants in other genes that are potentially involved in the disease; yet, the current state of the science is insufficient to implicate them in the pathogenesis of the condition.

Conclusion

CHH/KS are rare, genetically transmissible diseases with high phenotypic and genetic heterogeneity. Given their familial nature and the availability of effective medical treatment for infertility, genetic counseling is an essential part of these patients' care. In some cases, the disease exhibits classical Mendelian inheritance with autosomal recessive, autosomal dominant or X-linked transmission. However, in many cases, the CHH/KS phenotype and/or associated signs are not consistent with these modes of transmission, and it emerges that both the penetrance and the phenotypic variability are oligogenic in nature. This new, complex genetic architecture is increasingly recognized by concurrent analysis of tens or hundreds of genes with next-generation sequencing techniques. This modified genetic landscape of CHH/KS has major implications for these patients' essential genetic counseling. The main challenge now, and in the future,

is to differentiate true oligogenism from rare variants fortuitously identified in a patient/family that neither have significant impact on the observed phenotype nor the clinical variability of CHH/KS.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/EJE-17-0749>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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