University of Modena and Reggio Emilia

International Doctorate School in Clinical and Experimental Medicine

XXVI Cycle

GENETIC BACKGROUND OF CENTRAL HYPOGONADOTROPIC HYPOGONADISM

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1. Introduction

1.1. Hypothalamic-Pituitary-Gonadal axis

The physiological function of the human hypothalamic-pituitary-gonadal (HPG) axis is based on the pulsatile release of hypothalamic gonadotropin-releasing hormone (GnRH) (Bonomi et al., 2012). In vertebrates, the decapeptide GnRH regulates the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which govern the onset of puberty, gametogenesis, and estrous cycling, from anterior pituitary gonadotropes (Messina and Giacobini, 2013). Indeed GnRH, bound to its native high-affinity seven-transmembrane gonadotropin-releasing hormone receptor (GnRHR) on the cell surface of the gonadotrope, stimulates signaling cascades resulting in the production of the gonadotropins LH and FSH, which exert their effects on the ovaries and testes, leading to steroidogenesis and gametogenesis and highlighting their critical role in reproductive function (Thompson and Kaiser, 2013) (Figure 1).

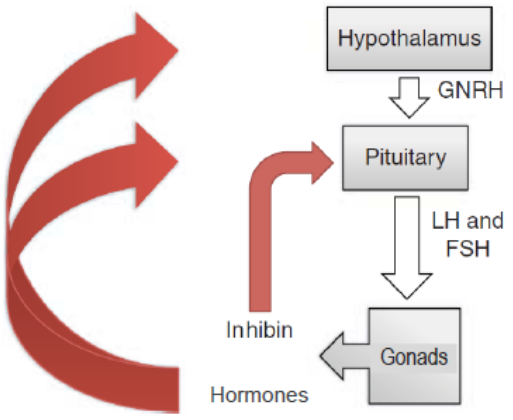

Figure 1. Schematic representation of the HPG axis. The hormone GNRH, produced in the hypothalamus, stimulates the release of the gonadotropins, LH and FSH, from the pituitary. The gonadotropins exert their action on the gonads, stimulating the production of sexual hormones which regulate the HPG axis through the negative feedback. (Zawatski and Lee, 2013).

During postnatal life, GnRH – secreting neurons are integral members of the HPG axis. However, during embryonic development, these cells originate from an extracerebral region, namely the nasal placode, and migrate to the hypothalamus, closed to olfactory-vomeronasal nerves (VNNs) (Messina and Giacobini, 2013) (Figure 2). This migration process can be divided into four specific stages, where several candidate proteins are involved in the movement and appropriate targeting of GnRH neuronal population. The first stage consists in the initiation of GnRH neuron migration, modulated by adhesion molecules (PSA-NCAM, b3GnT1 and KAL1), guidance molecules (EphA5 and NELF), neurotransmitters (GABA and CCK), growth factors (FGF8 and its receptor FGFR1), G protein-coupled receptors (PROK2/PROKR2) and transcription factors (Ebf2). The second stage consists in the movement of VNN and GnRH neurons into the forebrain carried out by Netrin/DCC, semaphorins, plexins and Reelin. The third stage regards the neurons movement towards the hypothalamus, thanks to different proteins such as growth factors (HGF), Tyrosine-Kinase receptors (Axl and Tyro3), Chemokine attractants (SDF-1 and CXCR4) and transcription factors (Nhlh2). Finally, in the latter stage neuronal migration stops and GnRH neurons detach from their axonal guides and disperse in the hypothalamus thanks to the action of KISS1 and its receptor KISS1R (Wierman et al., 2011) (Figure 2, 3).

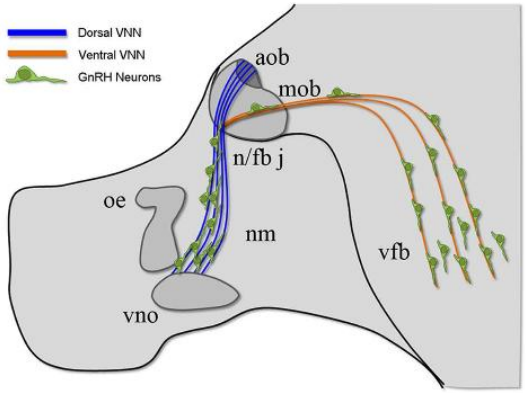

Figure 2. Embryonic migration of the GnRH neurons in the mouse. Schematic representation of the head of a mouse embryo at E14.5, depicting the scaffold of vomeronasal/terminal nerve fibers along which GnRH cells migrate from the nose to the ventral forebrain region. Abbreviations: VNN, vomeronasal nerve; cx, cerebral cortex; ob, olfactory bulb; nm, nasal mesenchyme; oe, olfactory epithelium; vno, vomeronasal organ; n/fb j, nasal/forebrain junction; aob, accessory olfactory bulb; mob, main olfactory bulb; vfb, ventral forebrain (Messina and Giacobini, 2013).

The correct development and coordinated function of the GnRH-secreting neurons and the gonadotropes is essential for the correct activation of the gonads during foetal life and the neonatal period (the so-called 'minipuberty'). After a dormant phase during infancy and childhood, HPG activity is resumed at the time of the puberty and throughout the adult reproductive age (Bonomi et al., 2012).

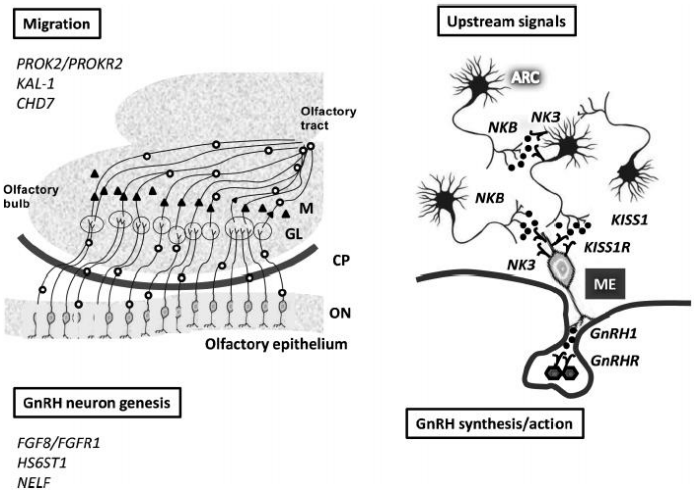

Figure 3. Schematic representation of the genes implicated in HPG activation. In each step, the different genes whose genetic variants disrupt the HPG axis activity are indicated. ARC, arcuate nucleus; CP, cribriform plate; GL, glomerulus; HPG, hypothalamic–pituitary–gonadal;M,mitral cell;ME,median eminence; ON, olfactory neurons (Bonomi et al., 2012).

1.2. Puberty

It is known that the age of onset and length of puberty in several species are influenced by a variety of factors such as genetic, nutrition, physical and social environment (Magnusson and Ljungvall, 2013). In human males, after birth, the HPG axis is active until approximately 6 months of life, with gonadotropin and sex steroid concentrations peaking between 4 and 12 weeks of life ('mini-puberty' of infancy). After this period, GnRH pulsatility reduces and the HPG axis becomes relatively quiescent throughout childhood. Puberty encompasses both gonadarche and adrenarche. Onset of puberty is characterized by pulsatile GnRH release from the hypothalamus that stimulates, after onset of gonadarche (the earliest detectable changes of puberty), pituitary LH secretion, which in turn drives testosterone production by testicular Leydig cells (von Oettingen et al., 2012; Zawatski and Lee, 2013). GnRH also increases FSH secretion, which promotes maturation of the seminiferous tubules and spermatogonia. (Zawatski and Lee, 2013). Adrenarche is defined as the time in the human life history, at about 6 – 7 years of age, when the adrenal androgens, DHEA, DHEAS and androstenedione, produced by adrenal zona reticularis, begin to increase in concentration in the circulation (Hornsby, 2012; Zawatski and Lee, 2013). These adrenal androgens are responsible for the mid-childhood growth spurt. Adrenarche continues up to approximately 14 years of age in both sexes and it is associated with the appearance of apocrine body odor and acne or oily skin as well as axillary and pubic hair growth (pubarche) (Kim et al., 2008a; von Oettingen et al., 2012). Several hormones play an important role in the onset of puberty-related physical changes such as growth hormone (GH), insulin growth factor-1 (IGF-1), in addition to estrogens, in boys and girls (de Water et al., 2013). In males the initial pubertal event is testicular growth, which

usually begins at about 10.5 years of age when the testes are at least 2.5 cm in any dimension (Kim et al., 2008a). Pubarche frequently starts simultaneously with testicular development, while axillary hair growth occurs at about the time of peak height velocity (about 14 years of age) (Petak et al., 2002). Delayed puberty is usually defined as the absence of signs of puberty by age 14 in boys (Kim et al., 2008a).

In females, during childhood the ovarian cycle, controlled by HPG axis, is quiescent (Nader, 2013). Adrenarche (6–8years of age), that begins with the adrenal androgen production, occurs first, clinically evident as pubic and axillary hair growth (Kim et al., 2008a; Nader, 2013). Adrenarche is followed by gonadarche and during this period the nocturnal LH pulse occurs, leading to ovarian production of testosterone and progesterone. Gonadal steroids facilitate the pubertal growth spurt and breast development (thelarche), that generally begins about age 8 – 9 years, leading to menarche (Kim et al., 2008a; Nader, 2013). A growth spurt occurs at 12 years of age, and subsequently, the first menstrual cycle occurs (menarche) (Kim et al., 2008a). Menarche, induced by follicle growth and estradiol production, occurs with the initial shedding of the endometrium, resulting in withdrawal or breakthrough bleeding. Female puberty ends when women ovulate and repeatedly ovulatory cycles ensure reproductive competence (Nader, 2013; Nussey and Whitehead, 2001).

The genetic control of puberty is still not clear but many studies contributed to reveal the key role of several genes in the onset of puberty as the KISS1/KISS1R system (Choi and Yoo, 2013). *KISS1* encodes several kisspeptins, which, after binding to the G-protein coupled receptor KISS1R, may stimulate the release of GnRH from the forebrain stimulating the secretion of gonadotropins from the pituitary (Choi and Yoo, 2013). Kiss1

neurons, in the arcuate nucleus (ARC) (Figure 3) are plausible generators of GnRH pulses through a system of pulsatile kisspeptin release controlled by the action of neurokinin B (NKB)/NK3 system (Figure 3) and dynorphin A that are co expressed in Kiss1 neurons (Bonomi et al., 2012; Choi and Yoo, 2013). However, it is not yet known whether the KISS1/KISS1R system is the initial trigger of puberty or whether it acts as a downstream effector of other regulatory factors (Choi and Yoo, 2013).

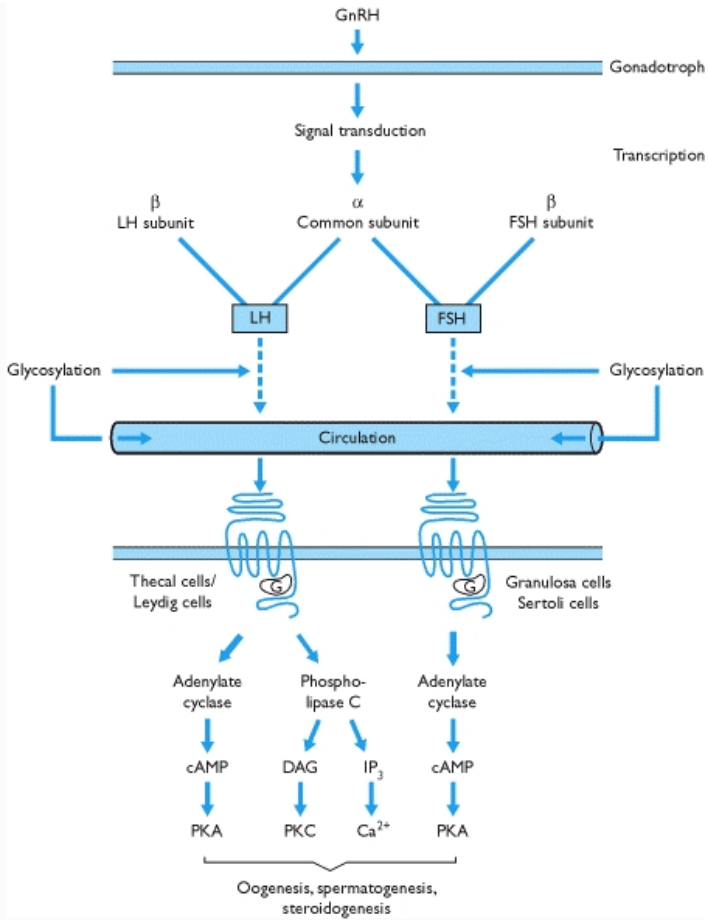

Figure 4. Simplified diagram of the human gonadotropins action. GnRH stimulates transcription of the genes coding for the common α and specific β subunits of LH and FSH. Glycosylation of the proteins occurs in the pituitary and may be modified in the circulation. Glycosylation sites and the charge of different LH and FSH isoforms alter their biological potency. They act on gonadal cells via typical G-protein linked receptors. Abbreviations: PKA/C, protein kinase A/C; DAG, diacylglycerol; PI3, inositol 1, 4, 5-trisphosphate (Nussey and Whitehead, 2001).

1.3. Central Hypogonadotropic Hypogonadism (CHH)

The HPG axis has a critical function in permitting sexual reproduction through the regulation of gametogenesis and through the secretion of sex hormones, conferring secondary sexual characteristics (Fathi and Luo, 2013).

Central Hypogonadotropic Hypogonadism (CHH) is characterized by delayed or absent sexual development and infertility associated with inappropriately low gonadotropins (LH and FSH) and sex steroids (testosterone or estradiol) levels in the absence of anatomical or functional abnormalities of the HPG axis (Bianco and Kaiser, 2009; Shekhar, 2012). In patients with normal levels of circulating gonadotropins, LH and FSH are secreted in a non pulsatile manner and are ineffective at the target level (Bonomi et al., 2012). CHH is a rare disease and its incidence is 1:8000 females and 1:4000 males (Bonomi et al., 2012). The disease associated with a normal sense of smell, in 40% of CHH patients (Shekhar, 2012), is also called normosmic Idiopathic Central Hypogonadism (nICH) or normosmic Hypogonadotropic Hypogonadism (nHH) for some authors, while, the defective sense of smell, hyposmia or anosmia, associated with hypogonadotropic hypogonadism, in 60% of patients (Shekhar, 2012), is called Kallmann Syndrome (KS), which is explained by the common embryonic origins and developmental pathways of GnRH and olfactory neurons (Bianco and Kaiser, 2009; Bonomi et al., 2012; Fathi and Luo, 2013; Sykiotis et al., 2010). CHH is either congenital or acquired, and it can also be isolated (generally classified as idiopathic, ICH) or combined with other pituitary hormone defects (Bonomi et al., 2012). Male patients affected by CHH frequently present a defective androgenisation and growth at a peripubertal age, but micropenis and cryptorchidism may already be evident in the neonatal period, indicating a defective activation of HPG during prenatal development. Female patients generally show primary amenorrhea

and growth retardation. Additional anosmia, midline and/or kidney defects may be present, and they can be linked to specific modes of inheritance (Bonomi et al., 2012; Brioude et al., 2010).

In the past two decades, hypogonadotropic hypogonadism was considered an irreversible disease, to treat with a long-life hormonal exposure. By contrast, it is well known that a small proportions of male patients, up to 10%, after exposure to androgens therapy, may undergo reversal of hypogonadism (Laitinen et al., 2012). In the literature, patients affected by KS or normosmic HH with mutations in fibroblast growth factor receptor 1 (*FGFR1*), *KAL1*, *GNRHR*, *CHD7* genes or with still unknown genetic defects presented a reversible phenotype, after therapy (Kulshreshtha et al., 2013; Pitteloud et al., 2005, 2001; Raivio et al., 2007; Ribeiro et al., 2007). In these patients a spontaneous recovery of LH pulsatile secretion occurred together with normalization of the testosterone level, after therapy suspension (Raivio et al., 2007). Although the precise mechanism of reversal of hypogonadotropic hypogonadism is unclear, plasticity of the GnRH-producing neurons in adulthood could be involved (Raivio et al., 2007). The ability of the nervous system to adapt in response to the environment, called plasticity, is a striking feature of the vertebrate brain. Even though, neurogenesis in humans occurs primarily during embryonic and early postnatal stages, multipotential progenitor cells in the subcortical white matter of the adult human brain have been identified as having the potential to replace neuronal lineages (Nunes et al., 2003). Furthermore, the neurons in the olfactory epithelium and in the dentate gyrus of the hippocampus are generated throughout life (Altman, 1969; Gage, 2000; Quinton et al., 1997) and their generation appears to be modulated by sex steroids (MacLusky et al., 2006). The current hypothesis, explaining reversal of hypogonadotropic hypogonadism, consists in the action

of sex steroids enhancing the plasticity of the neuronal network producing GnRH in the adult human brain, (Raivio et al., 2007).

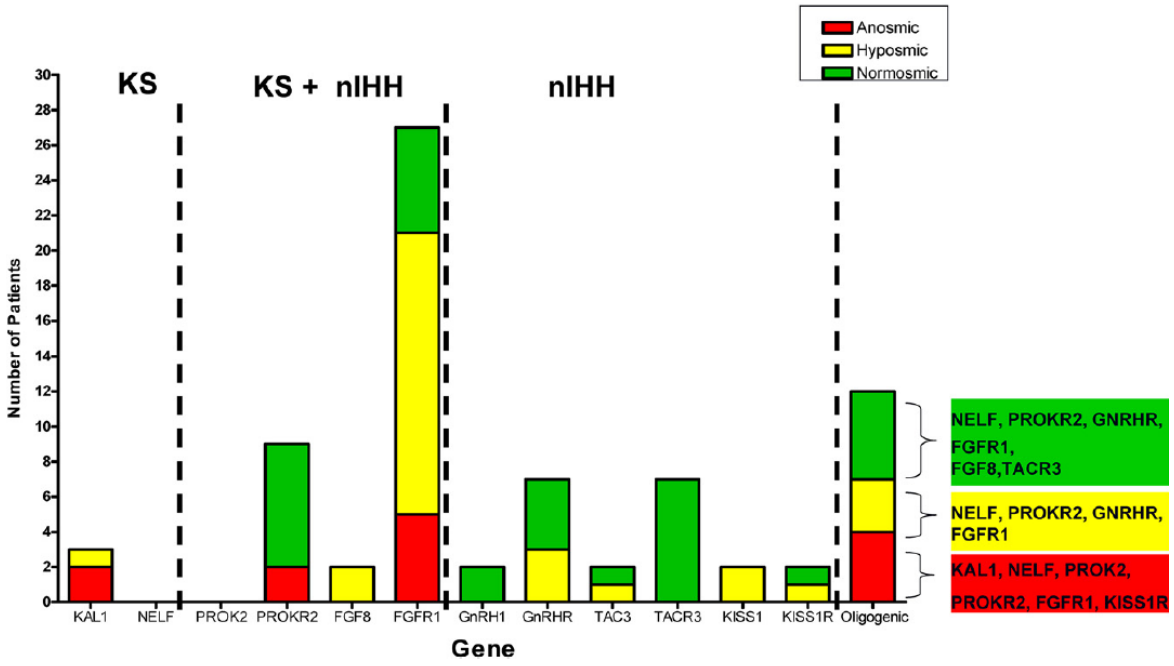

FIG. 5. Pathophysiological overlap between Kallmann syndrome and normosmic HH. Numbers of IHH subjects with protein altering mutations in each gene are shown with their respective olfactory function. The last column represents the number of subjects with oligogenic changes and the individual genes with protein-altering variants found within each olfactory phenotype (Lewkowitz-Shpuntoff et al., 2012).

1.4. Diagnosis and treatment of CHH

Usually the diagnosis of congenital CHH is performed during the second or third decade of life, when the patients manifest delayed pubertal onset, absent or poorly developed secondary sexual characteristics, primary amenorrhea, or infertility. The diagnosis may be suspected before puberty when boys present micropenis and/or unilateral or bilateral cryptorchidism and in the presence of other associated congenital abnormalities, such as midline defects (Han and Bouloux, 2010). In contrast, newborn girls have no obvious abnormal characteristics suggesting a congenital CHH diagnosis. Adult-onset CHH is characterized by secondary amenorrhea, decreased libido, infertility, and

osteoporosis, in women; symptoms of decreased libido, lack of morning erection, erectile dysfunction, inability to perform vigorous activity, depression, fatigue and infertility, in men (Silveira and Latronico, 2013).

The measurement of morning total testosterone is strongly recommended in the initial diagnosis test (Bhasin et al., 2010), specially the measurement of free or bioavailable testosterone levels (Lucas et al., 1991). Although widely used, the practical value of the GnRH test has been questionable because this test does not provide extra diagnostic information compared to baseline gonadotropin levels (Silveira and Latronico, 2013). The pituitary function can be first evaluated by basal hormonal levels (measured by ultrasensitive assays). Thyroid function should be assessed by TSH combined with free T4. IGF-I can be used to evaluate the somatotropic axis, whereas secondary adrenal deficiency can be assessed by measuring a morning cortisol and ACTH. Anosmia can be diagnosed by questioning the patient and by olfactometry as the University of Pennsylvania Smell Identification Test, useful to determine a normal or partially defective olfaction (Silveira and Latronico, 2013).

Magnetic resonance imaging (MRI) of the hypothalamus-pituitary region is very useful in the management of CHH because MRI can demonstrate a malformation or tumors. Renal ultrasound examination is usually recommended to patients with syndromic CHH, such as Kallmann syndrome. The genetic study is usually the last step in the CHH investigation and complete clinical characterization could certainly help in gene selection (Silveira and Latronico, 2013). Bone mineral density of the lumbar spine, femoral neck, and hip is recommended at the initial diagnosis of CHH and after 1 to 2 years of sex steroid therapy in hypogonadal patients with osteoporosis or low trauma fracture (Bhasin et al., 2010).

The aims of therapy for hypogonadal adolescents or young adults are the induction and maintenance of normal puberty and induction of fertility.

Testosterone therapy is recommended in adult men with symptomatic androgen deficiency to improve sexual function and to increase muscle mass and strength. Testosterone is the primary useful treatment to induce and maintain secondary sexual characteristics and sexual function in affected men, but it does not restore fertility (Silveira and Latronico, 2013). Several testosterone formulations are currently available such as intramuscular injections of long-acting testosterone esters, gel formulations or testosterone patches applied nightly (Bhasin et al., 2010). When fertility is desired, gonadotropin therapy is necessary to induce spermatogenesis in affected males. The common gonadotropin therapy combines human chorionic gonadotropin (hCG) and follicle stimulating hormone (FSH). Side effects of gonadotropin treatment include gynecomastia, and the induction of antibodies to hCG, impairing the response to hCG in the future (Delemarre et al., 2008; Thau et al., 1988).

1.5. Genetics of CHH

A small percentage of patients (approximately 4%) shows a chromosomal rearrangement as cause of CHH or KS (Bhagavath et al., 2006) (Table 1), but the majority of hypogonadic patients harbours a mutation in a single or more genes.

Table 1. Chromosomal rearrangements in CHH reported in literature.
(Bhagavath et al., 2006; Kim et al., 2008a).

Chromosomal rearrangement	Phenotype	Author
t(7;12)(q22,q24)	KS	Best et al., 1991 [178]
X;Y	KS	Guioli et al., 1992 [179]
t(3; 9)(9;12)(q13.2;q21.2p13;q15)	KS	Casamassima et al., 1993 [180]
t(13;16)(q14.11;q24)	IHH	Kikuchi et al., 1993 [181]
t(4;12)(q25;q24.2)	hypogonadism?	Elbistan et al., 1994 [182]
t(1;10)(q44;q26)	KS	Schinzel et al., 1995 [183]
46,XY,inv(3)(q24q26.32),t(3;13;18)(q26.32;q21.2;q12.2)	KS	Kroisel et al., 2000 [184]
del8p11.2	KS; spherocytosis	Vermeulen et al., 2002 [185]
46,XY,inv(10)(p15.2q11.22).ish inv(10)(p15.2q21.3)(p15×3), (q21×3)(p15conq21×2)(X)(p11.4q11.2)	IHH	Helszer et al., 2003 [186]
46,XY,t(7;8)(p12.3;p11.2)	IHH	Talaban et al., 2005 [187]
46,XY,t(10;12)(q26.3;q13.1)	IHH	Kim et al., 2005 [188]
46,XY,mos t(3;12)(p13;p13)[18]/46,XY[3]	KS	Bhagavath et al., 2006 [15]
46,XY/46,X,inv(Y)(p11.2q11.2)	IHH and cerebellar ataxia	Bhagavath et al., 2006 [15]
	IHH	Bhagavath et al., 2006 [15]

According to conventional Mendelian inheritance, a monogenic disease is caused by one dominant or two recessive allelic variants in the responsible gene (Sykiotis et al., 2010). In most monogenic disorders, such as retinitis pigmentosa (Kajiwara et al., 1994) and nephronopthisis (Hoefele et al., 2007), there is no perfect correspondence between genotype and phenotype and the coined concepts of incomplete penetrance and variable expressivity describe this mismatch, not providing any explanation about molecular mechanisms.

Isolated GnRH deficiency, caused by defects in the secretion or action of hypothalamic GnRH, is one of the rare genetic diseases originally thought to be strictly monogenic but the numerous studies about this disorder led to the discovery of several new loci (22 genes) (Table 2) with key roles for the developmental and neuroendocrine control of human reproduction (Bonomi et al., 2012; Fathi and Luo, 2013; Sykiotis et al., 2010).

The proteins, encoded by the genes involved so far in this pathology, have been grouped, according to their function, in three functional categories: Development and migration of GnRH neurons, regulation of GnRH secretion

and GnRH action (Bianco and Kaiser, 2009; Bonomi et al., 2012; Fathi and Luo, 2013; Kim and Layman, 2011) (Table 2).

Although multiple mutations have been identified in each gene, no mutations have been identified in the majority of patients (>60%), signifying that yet more disease loci remain to be discover (Sykiotis et al., 2010).

Table 2. Genes involved in normosmc/anosmic CHH.
KS: Kallmann Syndrome; nHH: Normosmic hypogonadotropic hypogonadism; XR: X-linked recessive; AR: Autosomic recesive; AD: Autosomic dominant; UNK: Unknown inheritance.

Genes	Location	Structure (exons)	Inheritance	Phenotype
KAL1	Xp22.3	14	XR	KS
FGFR1 (KAL2)	8p12	18	AD	KS
FGF8 (KAL6)	10q24	6	AD	nHH
PROK2 (KAL4)	3p13	4	AR	KS
PROKR2 (KAL3)	20p12.3	2	AD, AR	KS, nHH
CHD7 (KAL5)	8q12.2	38	AD	CHARGE, KS, nHH
NELF	9q34.3	15	Digenic	KS
GNRH1	8p21-p11.2	4	AR	nHH
GNRHR	4q21.2	3	AR	nHH
KISS1	1q32	3	AR	nHH
KISS1R (GPR54)	19p13.3	5	AR	nHH
LEP	7q31.3	3	AR	nHH
LEPR	1p31	20	AR	nHH
TAC3	12q13-q21	7	AR	nHH
TACR3	4q25	5	AR	nHH
PCSK1	5q15-q21	14	AR	nHH
WDR11	10q26	29	AR	KS, nHH
HS6ST1	2q21	2	Not Mendelian	KS, nHH
SEMA3A	7p12.1	17	AD	KS
LHB	19q13.32	3	AR	nHH
FSHB	11p13	3	AR	nHH
NDN	15q11.2-q12	1	UNK	KS, Preder-Willi

1.5.1 *KAL1*

The *KAL1* gene is located in the short arm of chromosome X (Xp22.3), spans 203 kb of genomic sequence, and contains 14 exons, encoding the extracellular matrix glycoprotein anosmin-1, which appears to be involved in migration of GnRH and olfactory neurons during embryologic development (Zhang et al., 2013). Hypogonadism in KS, due to GnRH deficiency, probably results from a failure of the embryonic neuronal migration, and the defective sense of smell is related to the hypoplasia or aplasia of the olfactory bulbs and tracts (Basaran et al., 2013). Anosmin-1 is a secreted protein containing a cysteine-rich region, a highly conserved four disulfide core whey acid protein (WAP) domain, four fibronectin type III (FNIII) repeats (including FNIII-1, FNIII-2, FNIII-3, and FNIII-4 repeat), and a C-terminal histidine-rich region (Zhang et al., 2013) (Figure 6).

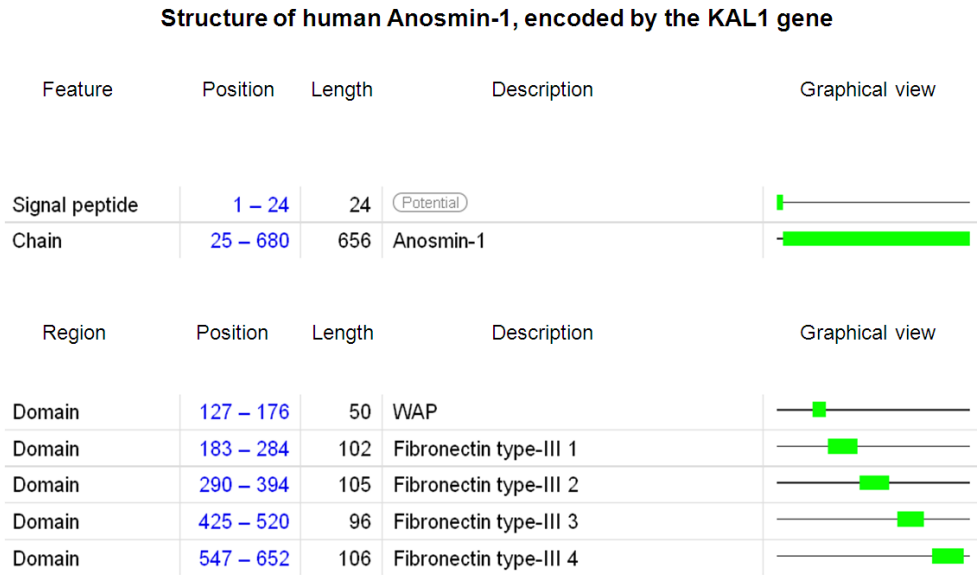

Figure 6. In this picture all five domains of the human anosmin-1 are shown (www.uniprot.org).

Mutations in the *KAL1* gene were first described in males with X-linked recessive KS (Franco et al., 1991). So far, in the literature, over 60 different

mutations such as deletions, missense, frameshift (Dodé and Hardelin, 2009) and gross rearrangements (Basaran et al., 2013; Montenegro et al., 2013) were described. About half mutations fall in WAP domain and in FNIII repeats suggesting their important role in the correct function of anosmin-1 (Trarbach et al., 2007). All *KAL1* mutations account for 33 to 70% of familial cases of KS and 3.1 to 27.8% of apparently sporadic forms of known KS, so far (Basaran et al., 2013; Zhang et al., 2013).

1.5.2 *FGFR1* (*KAL2*) and *FGF8* (*KAL6*)

FGFR1, encoding fibroblast growth factor receptor-1, is a member of the receptor tyrosine kinase superfamily (Figure 7). FGF signaling controls cell proliferation, migration, differentiation, survival, and plays essential roles in various processes of embryonic development. In the presence of heparin sulphate proteoglycans (HSPG), FGF8, (fibroblast growth factor-8), binds with high affinity to FGFR1 and induces receptor dimerisation, and its activation (Hardelin and Dodé, 2008; Koika et al., 2013). The FGFR1 is involved in gastrulation, organ specification, patterning of many tissues, including the brain, as well as the development of the olfactory system (Koika et al., 2013).

Both FGFR1 and FGF8 can cause an autosomal dominant form (KAL2) of KS. The majority of mutations in FGFR1 or FGF8 are missense mutations. Notably, about 30% of the FGFR1 variants found are de novo mutations (Dodé and Hardelin, 2009). In KS patients, skeletal anomalies such as syndactyly, polydactyly, or camptodactyly were exclusively seen in presence of FGFR1/FGF8 mutations (Layman, 2013).

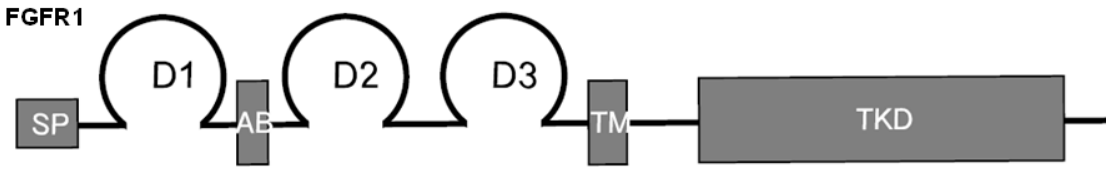

Figure 7. Schematic presentation of FGFR1 protein.
SP = signal peptide, D1–3 = immunoglobulin domains 1–3, AB = acidic domain, TM = transmembrane domain, TKD = tyrosine kinase domain (Koika et al., 2013).

1.5.3 *PROK2* (*KAL4*) and *PROKR2* (*KAL3*)

The *PROK2* gene encodes the protein prokinetecin 2, an 81-amino acid peptide that signals via the G protein-coupled product of the *PROKR2* gene (Topaloglu and Kotan, 2010) (Figure 7). The amino-terminal domain of prokineticin 2 contains a sequence of six amino acid residues (AVITGA), which is conserved in all mammalian and non-mammalian orthologs. Mutations involving this hexapeptide result in the loss of agonist activity on prokineticin receptors. Prokineticins can bind to two different G protein-coupled receptors, PROKR1 and PROKR2, sharing about 85 % sequence identity. They have a central core, formed by seven transmembrane domains (TM1–TM7), and connected by intracellular and extracellular loops. PROKR1 is mainly expressed in peripheral tissues, including endocrine glands whereas PROKR2 shows relatively localized distribution in the central nervous system, in particular in the subventricular zone (SVZ) and in the olfactory bulbs (Dodé and Rondard, 2013). It has been shown that this ligand-receptor system is essential for normal olfactory bulb and reproductive system development in mice and in patients with CHH. Homozygous mutant mice, that do not exhibit the transmembrane receptor Prokr2, show abnormal development of the olfactory bulb and severe atrophy of the reproductive system, but no significant abnormalities were observed in the heterozygous mice (Basaran et al., 2013). The knockout models for both ligand (Prok2) and

receptor (Prokr2), revealed a role in olfactory bulb morphogenesis and sexual maturation, indicating *PROK*2 and *PROKR2* as strong candidate genes for human GnRH deficiency (Libri et al., 2013). Mutations described within the *PROK2/PROKR2* system account for less than 10 % of subjects with KS and normosmic HH (Basaran et al., 2013). Patients with *PROK2* or *PROKR2* mutations have considerable phenotypic variability, ranging from Kallmann syndrome to normosmic HH. A variety of accompanying clinical features including fibrous dysplasia, synkinesia, and epilepsy have been reported in patients with *PROK2* or *PROKR2* mutations. Male patients carrying biallelic mutations in *PROK2* or *PROKR2* have a less variable and on average a more severe reproductive phenotype than patients carrying monoallelic mutations in these genes. Non-reproductive, non-olfactory clinical anomalies associated with Kallmann syndrome seem to be restricted to patients with monoallelic mutations (Topaloglu and Kotan, 2010).

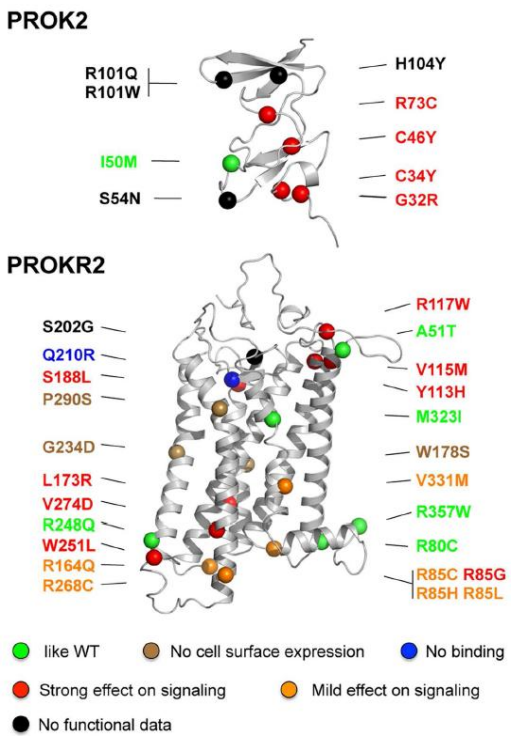

Figure 8. Structural models of prokineticin 2 and Prokr2 and recent missense mutations detected by Dodè and Rondard (2013).
The mutations are classified as similar to wild-type (green), absence of the receptor at the cell surface(brown), absence of ligand-binding (blue), strong or mild effect on signalling (red and orange, respectively) and functional data not available (black).

1.5.4 *CHD7* (*KAL5*)

The chromodomain helicase DNA binding protein 7 (*CHD7*), on chromosome 8, encodes a chromatin-remodeling factor belonging to a family of nine CHD proteins and having in common the ability to utilize ATP hydrolysis to alter nucleosome structure (Kim et al., 2008b; Topaloglu and Kotan, 2010). The large 2997 amino acid CHD7 protein contains two important chromodomains at its N terminus, (Figure 8). Chromodomains have been thought to mediate chromatin interactions and were found to interact with DNA, RNA, and histone targets (Kim et al., 2008b).

Heterozygous mutations in *CHD7* are found in more than 60% of the patients with typical CHARGE syndrome, a multisystem autosomal-dominant or

sporadic disorder including coloboma, heart anomalies, choanal atresia, retardation, genital and ear anomalies (Jongmans et al., 2009). In the literature 3 to 5 % of patients with CHH or KS were found to have a *CHD7* mutation (Vizeneux et al., 2013).

Previous studies on *CHD7* suggested that the analysis of this gene should be performed in KS patients having at least two CHARGE syndrome features. Supporting this guideline, Bergman and colleagues (2012), performed the *CHD7* analysis in a cohort of 36 Dutch KS patients (previously excluded to carry mutations in *FGFR1*, *PROK2*, *PROKR2* and *FGF8*), identifying 3 heterozygous *CHD7* mutations in patients having same features of CHARGE syndrome. Conversely, in the study of Kim et al. (2008b), mutations in this gene were found also in KS patients not carrying any CHARGE features (Table 3). Thus, considering these findings, new studies on *CHD7* and hypogonadic patients are needed.

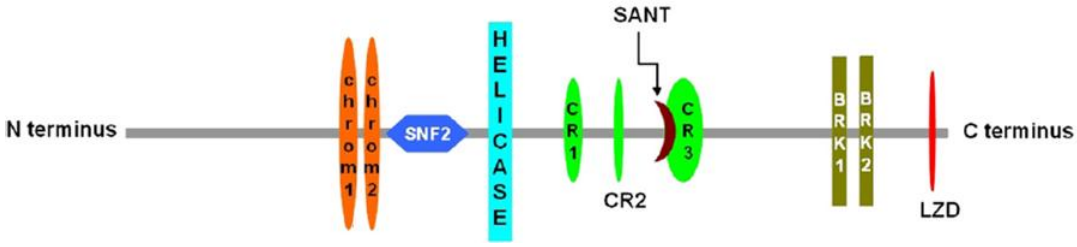

Figure 9. Structure of CHD7 protein. All functional domains are shown.
Chrom1 / 2: chromodomain 1 / 2; Helicase: helicase ATP-binding; CR1 / 2 / 3: function unknown, SANT: DNA binding domain; LZD: leucine zipper domain. (Kim et al., 2008b).

Table 3. CHD7 mutations identified by Kim et al (2008) in sporadic IHH/KS patients. Four patients, harbouring a CHD7 mutation, do not show addictional CHARGE syndrome features. IHH: Idiopathic Hypogonadotropc Hypogonadism.

Patient	Gender and Phenotype	Exon or Intron	Nucleotide Change	Amino Acid Change
1	Male, IHH; no other anomalies	Intron 8	IVS8+5G→A	Premature termination
2	Female, KS, cleft lip and palate, hearing loss	Intron 6	IVS6+5G→C	22 amino acid deletion
3	Male, IHH and cleft lip; cryptorchidism	Exon 8	c.2501C→T	Ser834Phe
4	Male, KS; no other anomalies	Exon 2	c.164A→G	His55Arg
5	Male, IHH, myopia; no other anomalies	Exon 38	c.8365G→A	Ala2789Thr
6	Male, IHH; cryptorchidism	Exon 38	c.8639C→T	Pro2880Leu
7	Male, KS; no other anomalies	Exon 38	c.8842A→G	Lys2948Glu

1.5.5 *NELF*

NMDA receptor synaptonuclear signaling and neuronal migration factor, also known as Nasal Embryonic LHRH Factor (NELF), maps on chromosome 9 (www.ncbi.nlm.nih.gov/gene/26012).

It is likely to be autosomal recessive because biallelic mutations, reducing protein expression in vitro, have only been described in the literature in one KS patient without any mutation in other genes, whereas heterozygous NELF mutations were only found in affected normosmic HH/KS patients with heterozygous mutations in another gene (Quaynor et al., 2011).

NELF is an attractive candidate gene for a role in GnRH neuron migration, mammalian puberty, and the pathophysiology of KS. Over ten years ago, the mouse *Nelf* was cloned from a differential cDNA library screen of migratory versus non migratory GnRH neurons (Kramer and Wray, 2000). Its expression was aligned along the plasma membrane of olfactory and GnRH neurons before they entered the hypothalamus and levels were down-regulated when GnRH neurons reached the forebrain. Moreover, reducing NELF protein expression, GnRH neurons were reduced in number and GnRH nerve fiber

decreased in complexity and length (Kramer and Wray, 2000). Two studies (Miura et al., 2004; Pitteloud et al., 2007) implicated NELF in KS. Subsequently, that data were confirmed by Xu et al. (2010), who correlated human NELF mutations with monogenic normosmic HH/KS. Xu and colleagues (2010) demonstrated that NELF protein was more highly expressed in migratory vs. postmigratory GnRH neuronal cells and NELF knockdown dramatically impaired GnRH neuronal cell migration in vitro. A functional nuclear localization signal and two putative zinc fingers, identifies NELF as a nuclear protein, possibly a transcription factor. (Xu et al., 2011, 2010).

Figure 10. All Nelf motifs identified by multiple alignments.
Shaded residues indicate approximately 75% similarity, $ indicates charged amino acid.

1.5.6 *GNRH1 and GNRHR*

GnRH plays a key role in the control of reproductive function. It is synthesized in a small number of hypothalamic neurons and released in a pulsatile manner into the hypophyseal portal circulation reaching the anterior pituitary, where, binding to specific receptors (GnRHRs), regulates the gonadal steroidogenesis in both sexes, stimulating synthesis and release of the two gonadotropins (LH and FSH) (Limonta and Manea, 2013).

Human *GNRH1*, on chromosome 8, composed by four exons separated by three introns, encodes a 92 amino acids preproprotein (pre-pro-GnRH), consisting of a signal sequence (23 amino acids) sequentially followed by two serine residues, the GnRH decapeptide (Table 3), a GKR sequence and a 56-amino acid peptide called GAP (GnRH associated peptide) (Chan et al., 2009;

Limonta and Manea, 2013). The mature decapeptide sequence is conserved among most mammals (Maione et al., 2013).

In two studies, “hpg” mice, carrying a deletion of Gnrh1, showed a complete absence of GnRH synthesis (Cattanach et al., 1977; Mason et al., 1986). Moreover, these mice were sexually immature, infertile, and exhibited low sex steroid and gonadotropin levels (Cattanach et al., 1977; Mason et al., 1986). These findings in the mouse suggested that mutations in human *GNRH1* could cause CHH. Variants of the human *GNRH1* gene are very rare. Naturally occurring mutations in human *GNRH2* (locus 20p13) were not described so far. The p.R31C mutation, is the sole missense mutation affecting the GnRH decapeptide sequence, encoded by GNRH1, described so far (Maione et al., 2013) (Table 4). This mutation, in which arginine is substituted by cysteine in position 8 of the mature decapeptide (Table 4), represents a hot spot (Chan et al., 2009; Quaynor et al., 2011).

The other variants described, rarer than p.R31C, were detected in the extra decapeptide positions such as p.R73X, p.T58S, p.V18M (Chan et al., 2009). All these variants were described in heterozygous state but curiously, Chan et al. (2009) reported also a homozygous mutation, p.G29GfsX12, in a male patient with severe congenital CHH. This single base-pair deletion causes a frameshift that was predicted to disrupt the GnRH decapeptide (Chan et al., 2009) (Table 5).

Table 4. Isoforms of the human GnRH.

GnRH-I is considered the only isoform implicated in CHH. Glp: pyroglutamic acid. (Limonta and Manea, 2013).

Amino acid sequences of natural GnRH isoforms.

GnRH (also designated GnRH-I)	Glp-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
GnRH-II	Glp-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2
GnRH-III	Glp-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2

Table 5. Recurrence of *GNRH* R31C in CHH families and in healthy controls (Maione et al., 2013).

Authors	Year	CHH	Healthy controls
Chan et al.	2009	4/310	0/192
Quaynor et al.	2011	2/48	0/188
Maione et al.	2013	3/410	0/545
Total.		9/768	0/925

Table 6. Rare *GNRH* variants as described by Chan et al. (2009).

Base pair change	Amino acid change	Ethnicity	Inheritance	Notable phenotypes	Other gene variants
[c.87delA] + [c.87delA]	[p.G29GfsX12] + [p.G29GfsX12]	Caucasian	Sporadic	Bilateral cryptorchidism, microphallus	None identified
[c.91C>T] + [=]	[p.R31C] + [=]	Caucasian	Familial	Reversal at age 42	*FGFR1*, *PROKR2*
[c.217C>T] + [=]	[p.R73X] + [=]	Caucasian	Familial	Right retractile testis, microphallus	None identified
[c.172A>T] + [=]	[p.T58S] + [=]	Asian	Sporadic	Microphallus, retinitis pigmentosa	None identified
[c.52G>A] + [=]	[p.V18M] + [=]	Caucasian	Familial	Adrenal insufficiency	*NR0B1/DAX1*

The GnRH receptor (*GNRHR*) gene, differently from that encoding for its ligand, accounts for many inactivating mutations, resulting in impairement of its ligand action (Beate et al., 2012).

GnRHR is a 328-amino acid protein encoded by a gene located on chromosome 4q21.2. This receptor belongs to the family of rhodopsin-like G protein coupled receptors (GPCR) and its activation results in the increased activity of phospholipase C and the mobilisation of intracellular calcium by means of the Gq/G11 group of G proteins (Bonomi et al., 2012). GnRHR contains seven transmembrane domains and an extracellular 35-amino acid amino-terminal domain with two putative glycosylation sites. Interestingly, this receptor does not have a carboxy-terminal cytoplasmic tail, thus, it internalizes relatively slowly and it does not rapidly desensitize (Limonta and Manea, 2013).

Inactivating mutations of *GNRHR* were the first recognized as monogenic causes of CHH condition (Beneduzzi et al., 2012). Null Gnrhr mice models display a similar phenotype to clinical human CHH (Bonomi et al., 2012). Over 22 human GnRHR inactivating mutations, with no hotspot, have now been described (Beate et al., 2012) and these different genotypes result in a wide phenotypic spectrum, ranging from fertile eunuch syndrome and partial hypogonadotropic hypogonadism to the most complete form of GnRH resistance, characterized by cryptorchidism, micropenis, undetectable gonadotropins and the absence of pubertal development (Bonomi et al., 2012). Although many defects in a large number of different genes were associated to CHH, *GNRHR* is still the most commonly affected gene in this pathogenic condition (Beneduzzi et al., 2012). Since the vast majority of patients harbouring *GNRHR* mutations are resistant to GnRH, the effective fertility treatment is achieved with gonadotropins (Beate et al., 2012).

1.5.7 *KISS1 and KISS1R (GPR54)*

Kisspeptin 1, encoded by *KISS1* gene (chromosome 1q32), was early identified in 2001 as a 54-amino acid peptide, also called kisspeptin 54, which corresponds to residues 68–121 of the preproprotein (Figure 11).

Features of the human kisspeptin aminoacidic sequence.

Signal peptide	1 – 19	19	Potential	
Chain	20 – 138	119	Metastasis-suppressor KiSS-1	
Peptide	68 – 121	54	Metastin	
Peptide	108 – 121	14	Kisspeptin-14	
Peptide	109 – 121	13	Kisspeptin-13	
Peptide	112 – 121	10	Kisspeptin-10	

Figure 11. Isoforms of the human kisspeptin 1.
The isoform, involved in CHH, is called kisspeptin 1 or kisspeptin 54, and it is composed by 54 amino acids. The other less studied shorter isoforms are supposed to be active because they maintain the region in positions 112-121, essential for receptor binding and subsequent receptor activation (www.uniprot.org).

Two years later, inactivating mutations in homozygous state in the G-protein-coupled KISS1 receptor (also known as GPR54, encoded by *KISS1R*, on chromosome 19p13.3), found in members of consanguineous families with a history of normosmic HH, revealed the reproductive roles of KISS1R and its ligand (Bianco and Kaiser, 2009). So far, mutations in the genes encoding the kisspeptin 1 and TAC3, as well as mutations in their receptors (KISS1R and TACR3, respectively), are associated with GnRH deficiency and a failure to initiate and/or progress through puberty (Lippincott et al., 2013). Inactivating mutations in *KISS1R* show an autosomal recessive pattern of transmission, as well as *KISS1*. Only few patients with *KISS1R* mutations have to date been reported (Figure 12) (Brioude et al., 2013).

So far, thanks to experiments in mice, researchers hypothesize that the system ligand/receptor TAC3/TACR3 is able to regulate the kisspeptin 1 release, which, through the interaction with the receptor KISS1R, stimulates the GnRH release and the normal reproductive function. Studies indicate that in

the case of abnormal TAC3/TACR3 pathway, KISS1 and GnRH are present in low doses in patients with mild CHH or reversal CHH but, in case of KISS1/KISS1R deficiency, patients present a more severe clinic associated to CHH (Lippincott et al., 2013).

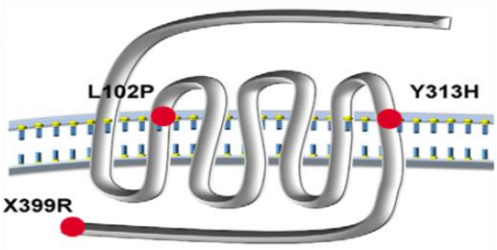

Figure 12. Schematic representation of human KISS1R and location of the mutations associated to hypogonadism described by Brioude et al. (2013).

1.5.8 *LEP and LEPR*

Adipose tissue, through expression and secretion of leptin, plays a dynamic role in whole-body energy homeostasis by acting as an endocrine organ (Hausman et al., 2012). Twenty years ago, a mouse model, named ob/ob, was discovered to have an inactivating mutation of the ob gene in both alleles, causing a complete deficiency of the ob gene product, to date known as leptin (Zhang et al., 1994). Interestingly, overweight in these mice, with metabolic, endocrine, and immune disturbances, regressed under exogenous leptin administration (Dardeno et al., 2010).

Leptin, encoded by human *LEP* gene, (on chromosome 7q31.3), is a 167-amino acid peptide with a four-helix bundle motif similar to that of a cytokine. It is produced, in a pulsatile manner, following a circadian rhythm, principally in adipose tissue but also in other different districts such as placenta, ovaries, and mammary epithelium (Dardeno et al., 2010). So far, it is known that leptin is present in human spermatozoa and seminiferous tubules together with its receptor (Landry et al., 2013). A few studies described

human families with congenital leptin deficiency with early onset of obesity, hyperphagia, hypogonadotropic hypogonadism and delayed puberty. Interestingly, inactivating mutation of the leptin receptor gene, (encoded by human *LEPR*, on chromosome 1p31), caused more less severe clinical features, indicating that probably, in case of receptor dysfunction leptin is able to interact with other molecules to exert its action (Dardeno et al., 2010; Landry et al., 2013). On the basis of recent findings, human leptin is supposed to be implicated in the secretion of GnRH through stimulation of several hypothalamic neurons, secreting neuropeptide-Y, proopiomelanocortin and kisspeptin-1 (Figure 12, panel A) (Dardeno et al., 2010; Landry et al., 2013). In the presence of leptin resistance, due to obesity, leptin is supposed to be unable to stimulate GnRH secretion, with consequent low levels of FSH and LH and hypogonadism (Figure 12, panel B) (Dardeno et al., 2010; Landry et al., 2013).

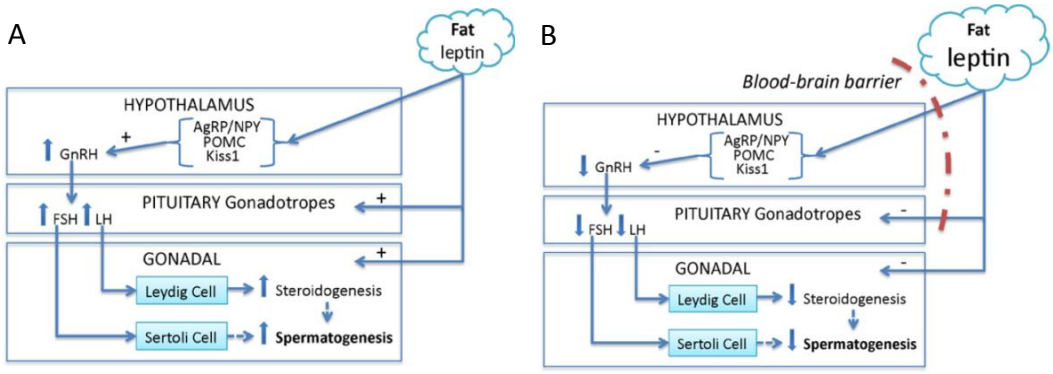

Figure 13. Schematic representation of supposed human leptin action. Panel A. Leptin action in human not obese healthy subjects. Panel B. Leptin resistance and consequent hypogonadism due to obesity. NPY: neuropeptide-Y, POMC: proopiomelanocortin, Kiss1: kisspeptin-1. (Landry et al., 2013).

1.5.9 *TAC3* and *TACR3*

Mammalian tachykinins comprise a proteins family including substance P (SP), neurokinin A (NKA), neurokinin B (NKB) and hemokinin-1 (HK-1). Human tachykinins, characterized by a common C-terminal amino-acid

sequence (Phe-X-Gly-Leu-Met-NH2) (Rance et al., 2010), are encoded by three different genes. *TAC1* encodes for SP and NKA, thanks to mRNA alternative splicing. The *TAC3* gene encodes NKB. The *TAC4* gene, through alternative splicing, generates four mRNA, all coding HK-1 (Pinto et al., 2010). Studies suggest that these peptides have a role as mediators of nonadrenergic and noncholinergic excitatory neurotransmission and recent data show that tachykinins are present in human spermatozoa and participate in the regulation of sperm motility (Rance et al., 2010). Tachykinins SP, NKA, and NKB interact with three receptors, belonging to a family of G-protein-coupled receptors (GPCRs), NK1R, NK2R, and NK3R, respectively (Navarro, 2013; Rance et al., 2010; Tusset et al., 2012). Although the specific interactions of the three tachykinins with each of these receptors have been described previously, their potency of affinity varies as follows: NK1R, SP > NKA > NKB; NK2R, NKA > NKB > SP; and NK3R, NKB > NKA > SP (Navarro, 2013).

The particular ligand/receptor system NKB/NK3R, (encoded by *TAC3*/*TACR3*), previously investigated only for pre-eclampsia, alcohol and cocaine dependence (Foroud et al., 2008; Laliberte et al., 2004; Page et al., 2006), gained an increasingly important role in human reproductive axis and in CHH onset in the last six years. So far, over 40 CHH patients with TAC3 and TACR3 mutations have been reported, with a worldwide distribution and a diverse racial mix. These patients display an absence of pubertal development with low circulating levels of serum LH and low gonadal steroids and high prevalence of microphallus, indicating that NKB/NK3R signaling is essential for the normal activation of the reproductive axis late in gestation (Gianetti et al., 2010; Guran et al., 2009; Topaloglu and Kotan, 2010; Topaloglu et al., 2009; Young et al., 2010). Recent evidences suggest

that NKB is able to modulate gonadotropin release through its action on Kiss1 neurons (Navarro, 2013) (Figure 13), but many aspects of the physiology of the NKB/NK3R system in the context of reproduction remain to be fully characterized.

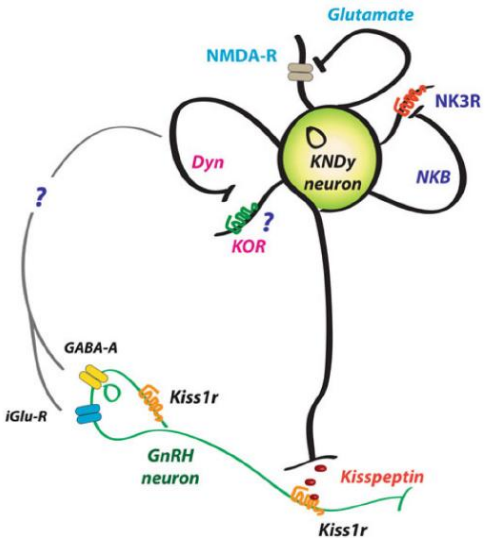

Figure 14. Schematic representation of a Kisspeptin/N KB/Dyn (KNDy) neuron depicting possible autoregulatory loops and interactions with GnRH neurons (Navarro, 2013).

The *TAC3* gene maps on chromosome 12q13-q21 and it is composed of 7 exons, 5 of which are translated to form the preprotachykinin B peptide. This prepropeptide undergoes enzymatic cleavages to form first proneurokinin B, and then mature NKB. The amino acid sequence of the final active peptide, widely conserved across vertebrates (Page et al., 2009), is encoded by exon 5 only (Page et al., 2000) (Figure 15). So far, only three mutations, a splicing variant (c.209-1G>C), a frame-shift variant (G20fsX39) and a missense variant (M90T), were detected in CHH patients (Table 7). All these mutations occurred in homozygous state, suggesting an autosomic recessive inheritance. The variant c.209-1G>C involves the intron 3 splice acceptor site (Fathi and Luo, 2013; Young et al., 2010). G20fsX39, due to a deletion of a single nucleotide G in position 60, causes a frame-shift and the consequent

introduction of a premature STOP codon in amino acidic position 39 (Gianetti et al., 2010) whereas M90T causes the substitution of the highly conserved Methyonine (in orthologs and homologs), falling inside the mature decapeptide, at the NH end (Topaloglu et al., 2009).

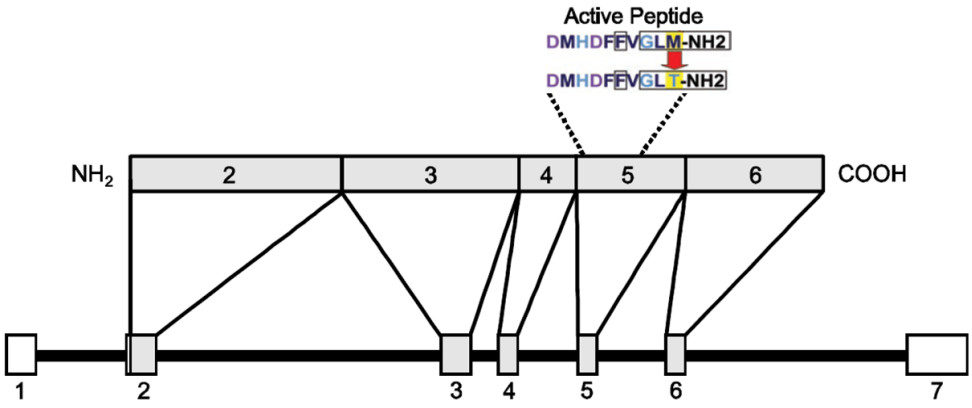

Figure 15. Schematic diagram of human TAC3 gene (bottom), preprotachykinin B (connected by lines above the gene) and the mature active decapeptide with the identified missense mutation M90T (on top). The TAC3 gene contains 7 exons denoted by boxes 1–7 with introns represented by lines. Exons 2–6 (shaded boxes) are translated from mRNA to form preprotachykinin B. Exon 5 encodes the active NKB peptide. Figure modified from Page et al (2001), Topaloglu et al (2009) and Rance et al. (2010).

The gene encoding the NKB receptor (*TACR3*), on chromosome 4q25, is composed by five exons. The mature protein has a cellular and an extracellular tail and seven transmembrane domains. The identified mutations, either synonymous, or non-synonymous or affecting physiologic splicing, are widely distributed along the gene, covering all main domains (Brioude et al., 2010; Fathi and Luo, 2013; Fukami et al., 2010; Gianetti et al., 2010; Guran et al., 2009; Topaloglu et al., 2009) (Figure 16). The TAC3 and TACR3 discovered so far mutations are listed in Table 7.

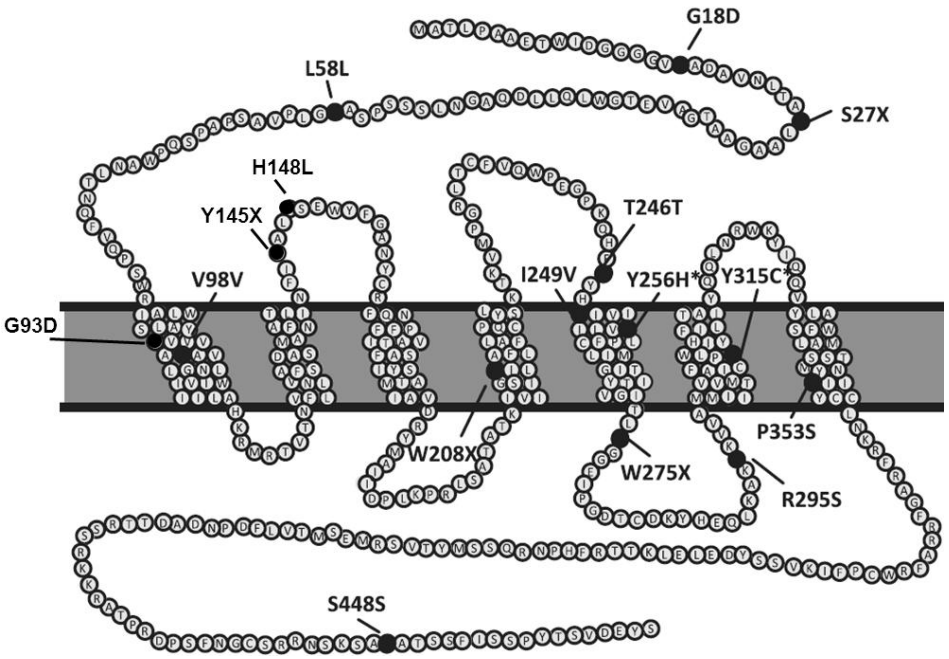

Figure 16. Schematic diagram of the NK3R protein with known synonymous and non synonymous variants. Figure modified from Page et al (2001), Topaloglu et al (2009) and Rance et al. (2010).

Table 7. Nucleotide variants identified in the two genes *TAC3* and *TACR3*.
(Last update: Genuary 2014).

GENE	VARIANTS		
	synonymous	non synonymous	splicing
TAC3		**G20fsX39**	**c.209-1G>C**
		M90T	
TACR3	**L58L**	**G18D**	**IVS1+1delG**
	V98V	**S27X**	**c.738-1G>A**
	T246T	**G93D**	
	S448S	**Y145X**	
		H148L	
		W208X	
		I249V	
		Y256H	
		W275X	
		R295S	
		Y315C	

1.5.10 *PCSK1*

Proprotein/neuroendocrine convertase deficiency, caused by rare mutations in *PCSK1* gene (Figure 17), has been associated with obesity, severe malabsorptive diarrhea, and certain endocrine abnormalities (Martín et al., 2013).

Neuroendocrine convertases are enzymes that process larger precursor peptides to release bioactive fragments, as in the case of the pro-opiomelanocortin, wich is processed by neuroendocrine convertase 1 (NEC 1), (encoded by *PCSK1* gene, on chromosome 5q15-q21), in the corticotroph to produce adrenocorticotropic hormone and lipotropin (Bianco and Kaiser, 2009). The first mutation was identified in 1995, in a patient with obesity and hypogonadotropic hypogonadism a compound heterozygous mutation in *PCSK1* (Gly483Arg and a premature STOP codon). In other two patients with a similar phenotype, an infant female and a 6-year-old boy, a compound heterozygous mutation (Glu250X and Ala213del) and a homozygous Ser307Leu substitution were identified in *PCSK1*, respectively. So far, *PCSK1* is thought to act on GnRH prohormone processing, even if the molecular mechanisms are still unclear (Bianco and Kaiser, 2009; Martín et al., 2013).

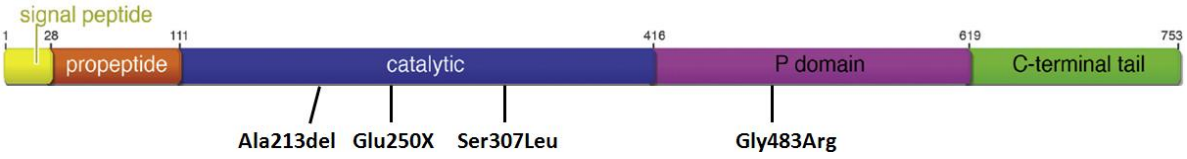

Figure 17. Schematic diagram of the PCSK1 product with the known domains and the mutations associated to hypogonadotropic hypogonadism. Image modified from Martin et al. (2013).

1.5.11 *WDR11*

The *WDR11* gene, on chromosome 10q26, encodes for a 1224 amino acid protein, highly conserved throughout vertebrate evolution. WDR11, originally identified as a potential tumor suppressor in human glioblastoma cells. It contains twelve WD domains, useful for protein-protein interactions. The gene was previously named as *BRWD2* (Bromodomain and WD repeat domain containing 2) but recently its name was changed with *WDR11* because it contains WD domains but no Bromo domains. The identification of human WDR11 mutations in normosmic HH/KS, absent in controls and supported by animal studies and in vitro analysis, indicate that WDR11 plays an important role in human puberty. So far, five missense mutations were identified (R395W, H690Q, F1150L, A435T and R448Q). Four of these, R395W, H690Q, F1150L and A435T (Figure 18) are completely conserved in all 11 available mammalian orthologs, suggesting that these substitutions in six independent sporadic patients could be very deleterious. All five detected variants were in heterozygous state, suggesting an autosomal-dominant inheritance. The absence of truncating nonsense and frameshift mutations could indicate a more severe phenotype or an embryonic lethality. Kim et al (2010) described that WDR11 protein colocalizes with EMX1 in vivo and in vitro, and three of the human mutations, previously described, failed to bind EMX1, suggesting an important role in human puberty also for EMX1 protein (Kim and Layman, 2011; Kim et al., 2010).

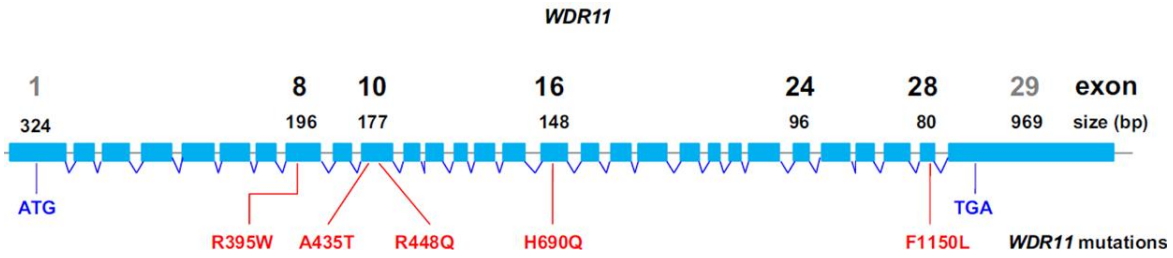

Figure 18. Schematic diagram of WDR11 gene.
Exon and intron structure of the 58 kb gene WDR11 (NM_018117.11). Locations of human missense mutations are identified in sporadic IHH and KS patients. Notable exons are shown to scale as blue rectangles and are numbered with exon size. The sizes of introns are not to scale. Mutations, in red, are indicated. (Kim et al., 2010).

1.5.12 *HS6ST1*

Recent in vivo and in vitro studies on *C. elegans* suggest that anosmin-1 requires specific modifications, through heparan solfates, to exert its function. This process seems to be required also for FGFR1 and FGF8 action.

Hs6st1 (Heparan sulfate 6-O-sulfotransferase 1), belonging to a class of molecules involved in neuronal development, is highly expressed in the brain (Tornberg et al., 2011). Recently, the gene *HS6ST1*, mapping on chromosome 2q21, was found to be mutated (Figure 19) in seven patients with hypogonadism, both normosmic and hypo/anosmic. The functional study of mutated Hs6st1 associates these variants with a reduction sulfotransferasic activity followed by GnRH deficiency. *HS6ST1* mutations were found in patients who had IHH with either normal olfaction (nHH) or variable degrees of olfactory dysfunction (KS). All identified mutations affect amino acid residues that are highly conserved in HS6ST1 (Figure 19) but segregates as a complex trait in families, not following Mendelian criteria. The study of Tornberg et al. (2011) suggests that the identified *HS6ST1* missense mutations could not be sufficient to cause disease, indicating a probable co-occurrence of other mutated genes. Patients with *HS6ST1* variants exhibited heterogeneous characteristics. Two patients had microphallus, one of whom

also had unilateral cryptorchidism. Three patients presented with absent puberty (severe GnRH deficiency), whereas three male patients had partial puberty. After several years of testosterone therapy, one patient demonstrated reversal of his hypogonadism, after suspension of therapy. Thus, CHH associated to HS6ST1 mutations display incomplete penetrance and variable expressivity of phenotype (Tornberg et al., 2011).

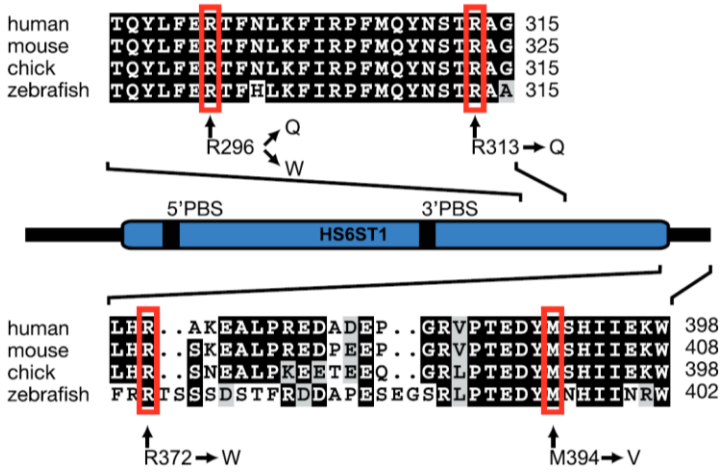

Fig. 19. Sequence of human HS6ST1 and positions of mutated amino acids in CHH patients. Schematic representation of the human HS6ST1 protein with the conserved sulfotransferase domain indicated in blue. 5′PBS and 3′PBS indicate the phosphoadenosylphosphosulfate (PAPS) cofactor binding sites. A multiple sequence alignment of two sections of the C terminus is shown with non synonymous changes indicated and amino acid positions denoted on the right. Amino acids shaded in black and gray indicate identical and similar residues, respectively. (Tornberg et al., 2011).

1.5.13 *SEMA3A*

It is well known that GnRH neurons are generated outside the brain, in the nasal placode, and migrate along olfactory/vomeronasal nerves reaching the hypothalamus by the time of birth. This migration occurs thanks to specific key players as semaphorins (Cariboni et al., 2011). Their important role was reinforced with the identification of semaphorin mutations in patients with developmental neuroendocrine deficiencies associated with infertility (Cariboni et al., 2011; Hanchate et al., 2012; Messina and Giacobini, 2013;

Young et al., 2012). More than twenty semaphorin coding genes have been identified, named and grouped into eight classes. All these molecules have a SEMA domain, important for dimerization and binding with their receptors, and a PSI domain (plexins, semaphorins, and integrins) (Figure 20). The semaphorins receptors are called plexins. Nine vertebrate plexins have been identified to date, grouped into four subfamilies. Some semaphorins require the presence of co-receptors named neuropilins (Nrp-1 and Nrp-2) (Messina and Giacobini, 2013). Some studies reported that a particular semaphorin, semaphorin 3A, encoded by human SEMA3A on chromosome 7p12.1, if mutated in humans and mice, could lead to abnormal migration of GnRH neurons to the hypothalamus, leading to hypogonadism and infertility (Cariboni et al., 2011; Hanchate et al., 2012; Young et al., 2012). So far, nine different *SEMA3A* mutations, leading to KS phenotype, were detected in patients of both sexes. All these variants, one deletion of 213 kb removing the last 11 of the 17 SEMA3A exons (Young et al., 2012), one frameshift deletion of 14 nucleotides (Figure 20) and seven missense mutations (p.R66W, p.N153S, p.I400V, p.V435I, p.T688A, p.R730Q, p.R733H) affecting evolutionarily conserved amino acids (Figure 20) (Hanchate et al., 2012), were identified in heterozygous state, suggesting an autosomal dominant transmission, similar to the inheritance mode reported in KS patients with *FGFR1* mutations (Dodé and Hardelin, 2009).

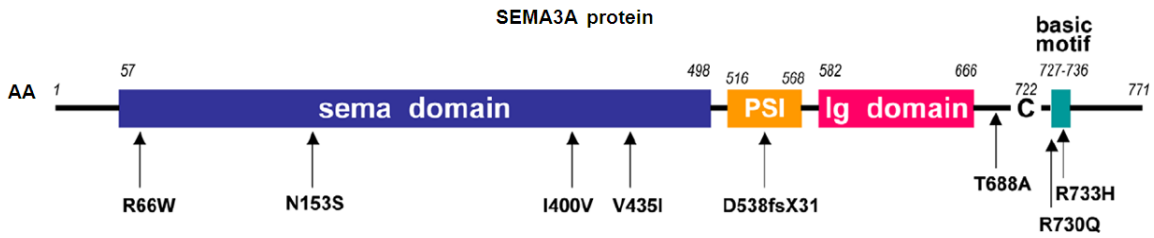

Figure 20. Diagram of Sema3A protein with 8 out of 9 mutations found in Kallmann Syndrome patients. Mutations are shown with the positions of the corresponding aminoacid residues in the protein domains. Sema: semaphorin; PSI: plexin/semaphorin/integrin; Ig: immunoglobulin-like; C: cysteine residue involved in Sema3A dimerization (interchain disulfide bond). (Hanchate et al., 2012).

1.5.14 *LHβ e FSHβ*

Gonadotropins are eterodimers composed by a α-subunit, (common for TSH, FSH, LH e hCG), and a specific β-subunit. So far, no mutations in the gene *CGA*, encoding the α-subunit, were identified, whereas, in some male and female patients, presenting with delayed puberty, several mutations were detected in the genes encoding the β-subunits of FSH and LH (Berger et al., 2005; Layman et al., 2002).

The *FSHβ* gene, located on chromosome 11p13, is composed of three exons but only exons 2 and 3 encode for the mature peptide. To date, four distinct FSHβ mutations (Figure 21) were described in four unrelated female patients with hypogonadism and three mutations were described in three CHH male patients. Affected women presented delayed puberty, lack of or poor breast development and primary amenorrhea. After treatment with exogenous FSH, follicular maturation, ovulation, and fertility were achieved in two women. All affected men presented with small testes and azoospermia, but only one man presented absence of pubertal development. Data in the literature suggest that the presence of undetectable serum FSH and high serum LH levels in CHH patients of both sexes could be strongly due to molecular defects in the *FSHβ* gene (Berger et al., 2005; Layman et al., 2002).

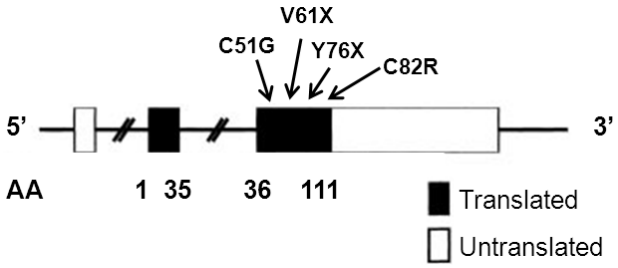

Figure 21. Schematic representation of human *FSHB* gene.
All reported mutations fall inside the exon 3 (coding amino acids 36 – 111). Picture adapted from Layman et al. (2002).

Homozygous mutations in the *LHβ* gene (Figure 22), (chromosome 19q13.32), abolishing the activity of LH have been reported to date in seven men and two women (Basciani et al., 2012; Lofrano-Porto et al., 2007; Valdes-Socin et al., 2004; Weiss et al., 1992). Despite different genotypic abnormalities, patients with *LHB* inactivating mutations had similar clinical phenotypes. In affected men, sexual differentiation is normal, but the absence of LH alters Leydig cells' proliferation and maturation, impairing spermatogenesis (Basciani et al., 2012). Women, harbouring an inactivating *LHβ* mutation, have normal pubertal development and menarche, followed by oligomenorrhea and secondary amenorrhea. Few data present in the literature, describing LHβ mutations, suggest that one copy of the LH beta is sufficient for normal LH secretion and function of the gonadotropic axis, indeed, only the patients harbouring homozygous mutations showed hypogonadism whereas their relatives, harbouring a heterozygous variant, did not show clinical manifestations (Basciani et al., 2012; Lofrano-Porto et al., 2007).

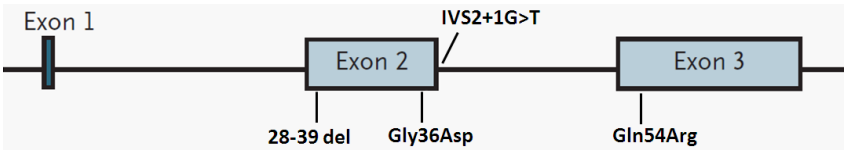

Figure 22. Schematic representation of the LHB gene.
All described mutations are reported. Picture adapted from Valdes-Socin et al. (2004).

1.5.15 *NDN*

Necdin belongs to the protein superfamily named MAGE and it is able to activate GnRH expression and GnRH neurons development in rodents. Human necdin, encoded by *NDN* gene (chromosome 15), has a potential role in the onset of hypogonadism in patients affected by Prader-Willi syndrome (Miller et al., 2009). Few years ago, Beneduzzi et al. (2011) identified a rare necdin variant in association with a mutation in *FGFR1*, in a patient with familial KS. Nevertheless, functional studies showed that the mutated necdin was able to activate the GnRH expression as the wild type protein (Beneduzzi et al., 2011). Further studies are needed to clarify the role of this protein in puberty and in human reproduction.

1.6 Digenic and oligogenic inheritance

For a long period CHH were considered a monogenic pathology with Mendelian inheritance. In 2006, researchers began thinking about hypogonadotropic hypogonadism as a digenic disorder (Dodé et al., 2006). Since that time, a number of other researchers described cases of digenic mutations in normosmic HH/KS (Quaynor et al., 2011). Indeed, defects in different genes could act synergistically to induce the CHH phenotype, or to modify the severity of the GNRH deficiency (Brioude et al., 2010; Quaynor et al., 2011). In 2010, Sykiotis et al. identified 10 CHH patients harbouring rare digenic protein-altering variants and 18 patients harbouring oligogenic known or predicted deleterious mutations (Figure 23). Recently, Quainor et al. (2011) contributed to these data describing 48 normosmic CHH patients, screened for 13 disease-related genes. In 12.5 % of these affected subjects digenic mutations were identified. Moreover, Quaynor et al. (2011) suggested that a proportion of isolated GnRH deficiency, could be attributable, in addition to

digenic/oligogenic component, to nongenetic components, as in the cases of occasional adult onset of the disease after normal puberty and reproductive function in subjects without mutations.

Nevertheless, findings from the literature indicate that monogenic mutations account for most cases of CHH (over 80 %) (Brioude et al., 2010; Quaynor et al., 2011; Sykiotis et al., 2010).

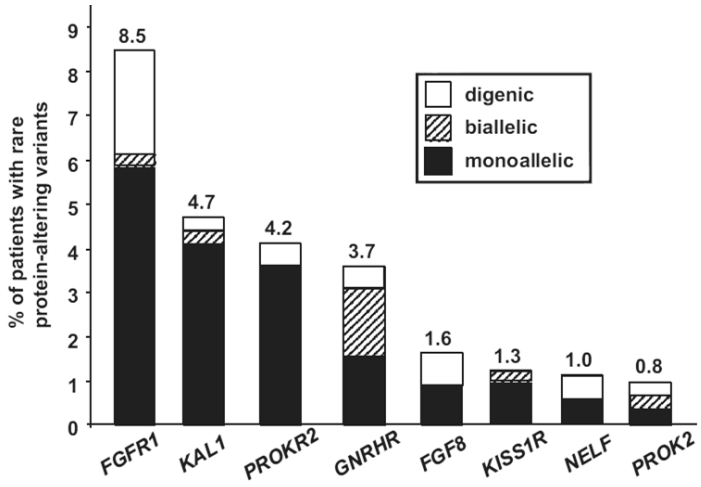

Figure 23. Percentages of patients with monoallelic, biallelic, and digenic rare protein-altering variants in eight CHH-related genes (Sykiotis et al., 2010)**.**

2. Aim of the study

The genetic causes of about 60% of patients affected by normosmic/anosmic hypogonadotropic hypogonadism remain unknown. Thus, the main purpose of this study was to identify novel and known genetic defects in CHH patients.

The identification of the genetic causes of this pathology in the major proportion of the affected individuals, presenting a wide phenotypic variety, may contribute to better define the correct genotype – phenotype correlations, helping clinicians in patient management, and it may contribute to the improvement of pharmacological treatments.

3. Materials and methods

This study, part of a multicentric project, under the patronage of the Italian Society of Endocrinology (SIA), has been approved by Modena's Ethic Committee. The project (GR2008-1137632) was performed in collaboration with the Endocrine Units of Milan, directed by Prof. L. Persani, Florence, directed by Prof. M. Maggi and Dr. C. Krausz, Naples, directed by Prof. A. Sinisi and Prof. A. Jolascon, Genova, directed by Prof. M. Maghnie and Dr. L. Perroni, Modena, directed by Prof. M. Simoni and Prof. C. Carani. The Endocrine Unit of Milan is the centre for the coordination of the project, which collects and distributes to the others Units the DNA samples, useful to perform the genetic analyses.

The genes *FGFR1*, *PROK2*, *PROKR2*, *GNRH1* and *GNRH2* were sequenced in Milan; *GNRHR* and *FGF8* in Florence; KAL1 gene in Genova; *PROK2* and *PROKR2* were sequenced also in Naples. *TAC3* and *TACR3* have been analysed in the laboratory of molecular endocrinology ENDOLAB, belonging to the Endocrine Unit of Modena (Department of Biomedical, Metabolic and Neural Sciences).

According to the agreements between the various Endocrine Units, only patients from Modena will be treated here extensively, reporting also their clinical characteristics.

3.1 Patients

400 CHH patients were enrolled in all Endocrine centres.

These patients were enrolled in the period comprising January 2009 – December 2013. Written informed consent was obtained from all participants, who donated a blood sample for this study.

A total number of 47 patients, (Mean age 37±13), 7 females and 40 males (Figure 24), were enrolled at the Endocrine Unit of Modena. 20/47 patients, 4 females and 16 males, were diagnosed as KS (Figure 24). The remaining 27 patients, 3 females and 24 males, were classified as normosmic HH (Figure 24), on the basis of the Smell test (described in the paragraph 3.2).

In addition to Modena's cohort, 25 CHH patients, enrolled at the other Italian Endocrine Units, coordinated by the Endocrine Unit of Milan, have been screened at ENDOLAB for both *TAC3* and *TACR3*.

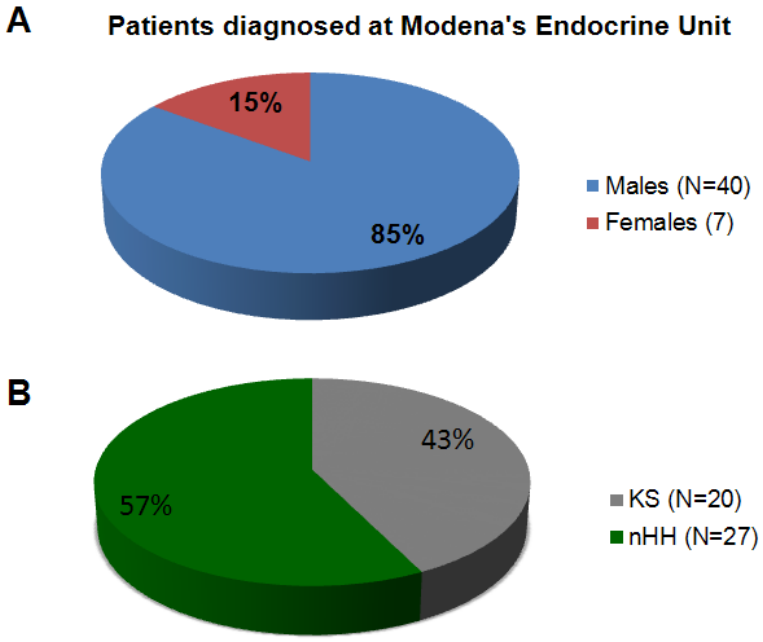

Figure 24. Characteristics of the Modena's cohort.
A. The ratio between males and females patients is shown. B. The ratio between Kallmann Syndrome and normosmic hypogonadic patients corresponds to 43% and 57%, respectively.

All diagnoses were performed by an experienced clinician evaluating the absence of puberty, low levels of sexual steroids and low or normal levels of gonadotropins.

Inclusion criteria were: age of enrolment > 18 years, normal or low levels of LH and FSH, (testosterone in males < 100 ng/dl), low level of estradiol and primary amenorrhea in females, normal other pituitary functions.

Exclusion criteria were: patients with other pituitary malfunctions, positive imaging for hypothalamic-pituitary alterations, secondary hypogonadism.

Data were collected about familial history of hypogonadism and other diseases, pubertal develop and infertility. Magnetic resonance imaging (MRI) of the forebrain has been carried out in all subjects to investigate hypoplasia or aplasia of the olfactory bulbs and tracts and to exclude hypothalamic or pituitary lesions.

All subjects were evaluated for gynecomastia, mirror movements, palate or nasal anomalies, coloboma, microphthalmia, renal agenesis, hearing impairment, manual synkinesis, and tooth agenesis. Moreover, Body Mass Index (BMI), height, arm span, and testicular volume (through Prader's orchidometer and ultrasounds), were measured.

3.2 Instrumental analyzes

MRI was performed to detect hypothalamic-pituitary anomalies; abdominal and transvaginal ultrasounds to exclude urinary and uterine abnormalities and to evaluate uterine volume; audiometry to define the presence of hearing problems; Dual X-ray Absorptiometry (DEXA) to assess osteopenia or osteoporosis; biochemical tests to assess hematocrit, prostate-specific antigen (PSA), glucidic metabolism, testosterone and estradiol. PRL, IGF1, TSH, FT4, ACTH, ferritin, cortisol and free urinary cortisol have been checked to assess the pituitary function and to exclude a secondary hypogonadism.

The sense of smell was evaluated through the Brief Smell Identification Test (B-SIT Sensonics, New Jersey, USA) wich is a little book containing twelve pages. Every page contains a particular odor, released after scratching the surface of the page. Patients must sniff immediately the scratched page and choose one out of four possible answers, written on the same page. After

comparing patients' answers with the answers of a healthy control group, the deficit of smell can be established.

3.3 Molecular analyzes of *TAC3* and *TACR3*

A total of six ml of peripheral blood was collected from each patient in two sterile tubes containing EDTA as anticoagulant. Genomic DNA was purified from peripheral blood with "Nucleon genomic DNA Extraction kit" (GE Healthcare), according to the manufacturer's instructions. The amount of DNA was measured with the UV spectrophotometer UV–1601 (Shimadzu). The concentration ranged from 50 to 250 ng/ul. Polymerase Chain Reaction (PCR) was performed to amplify all coding exons and intron-exon boundaries of *TAC3* and *TACR3* genes. Specific primers (forward and reverse) were designed at ENDOLAB (Tables 8 and 9).

Table 8. Primers used to perform PCR and sequencing of the *TAC3* gene.

Exon	Forward Primer	Oligonucleotide sequence	Reverse primer	Oligonucleotide sequence
2	2F	5'-CAAGCCAAGCTGCTGGTAATG-3'	2R	5'-GTCCGTCAGAGGGCATTT-3'
3 - 4	3F	5'-GATTCAGGATGGGCTCAGG-3'	4R	5'-CAAACAATATGCCAGCTCCC-3'
5	5F	5'-GGAACAGAGACCAGAAACCCA-3'	5R	5'-CACCAGAGGGAAAGACAGGA-3'
6	6F	5'-CCCCAGTCTCCCAACTCTGTC-3'	6R	5'-CCCATGCTACAGGTATTAAAGC-3'

Table 9. Primers used to perform PCR and sequencing of the *TAR3* gene.

Exon	Forward Primer	Oligonucleotide sequence	Reverse primer	Oligonucleotide sequence
1	1F	5'- AGCAGGGATTGCAGTATC-3'	1R	5'-CCCATTTGTAGCCCTCGAGT-3'
2	2F	5'-GCCATGATTACCATTCTACGC-3'	2R	5'-GATTTGTGTGTGGTCAATAAGTTG-3'
3	3F	5'-CAACTGGCAGCATTTGAAAC-3'	3R	5'-GCTGCTGTCCACATACTGTAATC -3'
4	4F	5'-CTGTCCGTATATTGCTTCACC-3'	4R	5'-CTGAGAGAGGCACAGGCTTT-3'
5	5F	5'-TGTGACATAAATTCTAAGAGTCTGGC-3'	5R	5'-GCTATGGTCAAATTGAGAAAGG-3'

All reactions (Table 10 and 11) were performed, under a PCR hood in an amplicon-free area, in a final volume of 50 µl, on a C1000 thermal cycler (Biorad) using the Expand™ High Fidelity as DNA polymerase (Roche).

Table 10. PCR reagents, to perform amplifications of *TAC3* and *TACR3*.

PCR reagents	
Genomic DNA	100 ng
Primer Forward [25pm/µl]	1 µl
Primer Reverse [25pm/µl]	1 µl
dNTPs [10mmol]	1 µl
Expand™ High Fidelity Polymerase 1U	0.7 µl
10X Buffer with $MgCl_2$	5 µl
Sterile H_2O	40.3 µl

Table 11. PCR protocol for TAC3 and TACR3.

	STEPS	TEMPERATURE AND TIMES
1	Initial Denaturation	94°C for 2 minutes
2	Denaturation	94°C for 15 seconds
3	Annealing	55° - 57°C for 30 seconds
4	Elongation:	72°C for 45 seconds (The steps 2 -4 are repeated 35 times)
5	Final Elongation	72°C for 7 minutes

PCR products were checked, by agarose gel electrophoresis and visualized by Syber Safe DNA gel staining (Invitrogen). Amplicons migration was compared with the migration of a known molecular DNA ladder (MVIII, Roche). Then, PCR products were purified with High Pure PCR Product Purification Kit (Roche).

The purified amplicons were sequenced through "cycle sequencing" method, based on the principle of Sanger, using a mixture of dNTPs, ddNTPs, $MgCl_2$ and a thermostable polymerase called Big Dye Terminator Ready Reaction Mix V1.1 (Life Technologies). All sequence reactions were performed, using the same PCR primers (Table 8 and 9), on a thermocycler C100 (BIORAD), in a final volume of 20 µl. In order to eliminate traces of unincorporated fluorochromes, enzymes, and other contaminants, the sequence reactions were purified through acetic sodium and ethanol precipitation, lyophilized and resuspended in 6 ul of sterile water. Following the electrophoretic run with a 4-capillary ABI PRISM® 3130 sequencer (Life Technologies), all results (raw data) were analyzed by Sequencing Analysis 5.3 software to obtain nucleotide sequences visualyzed as electropherograms. Finally, nucleotide sequences of *TAC3* and *TACR3* were investigated for novel and known nucleotide variants, including single nucleotide polymorphisms (SNPs), through multiple alignments between the nucleotide sequences reported on NCBI database and

the nucleotide sequences obtained, using SeqScape 2.6 software (Life Technologies).

3.4 Statistical analyzes

All the statistical analyzes were carried out using the GraphPad Prism 5 software (www.graphpad.com). A P value <0.05 was considered to be statistically significant.

4. Results

4.1. Patients enrolled at Endocrine Unit of Modena

The main developmental abnormalities of the patients recruited in Modena, subdivised in nHH and KS, were collected and the two groups were compared (Table 12).

Table 12. Developmental abnormalities in the Modena's cohort.
**** Highly statistically significant difference between normosmic (nHH) and ipo/anosmic (KS) patients. A P value <0.05 was considered to be statistically significant.

Developmental abnormalities	Total Patients N = 47	nHH N = 27	KS N = 20	P (Fisher's exact test, two-tailed, CI 95%)
Pubertal delay	37/47 (78%)	19/27 (70%)	18/20 (90%)	0.1544
Midline defects	22/46 (48%)	14/26 (54%)	8/20 (40%)	0.3880
Dental agenesis	9/47 (19%)	6/27 (22%)	3/20 (15%)	0.7130
Pectus excavatum	5/47 (11%)	2/27 (7%)	3/20 (15%)	0.6377
Bimanual synkinesis	2/46 (4%)	1/26 (4%)	1/20 (5%)	1
Bilateral cryptorchidism	4/40 (10%)	1/24 (4%)	3/16 (19%)	0.2832
Monolateral cryptorchidism	4/40 (10%)	1/24 (4%)	3/16 (19%)	0.2832
Kidney abnormalities	12/46 (26%)	7/26 (27%)	5/20 (25%)	1
Abnormalities of olfactory structures	14/34 (44%)	2/19 (11%)	12/15 (80%)	**<0.0001 ******

So far, the genetic investigation regarding the CHH subjects, enrolled at the Endocrine Unit of Modena, is completed in 29 patients for *TAC3*, *TACR3* and *PROK2* genes, in 30 patients for *FGFR1*, *PROKR2*, *GNRH1* and *GNRH2*, in 6 for *KAL1*, and in 20 patients for *GNRHR* (Table 12a,b).

4.1.1. *TAC3*

In our cohort, only one *TAC3* mutation was identified.

- The intronic variant IVS+87 G>T (Figure 25), in heterozygous state, was detected in *TAC3* in one male patient with KS. The same variant was also found in two proband's sisters (Figure 26). One sister presented only delay of puberty while the other sister, presenting only anosmia, harboured also the *TACR3* polymorphism c.103C>T (rs3733632), in heterozygous state (Figure 26).

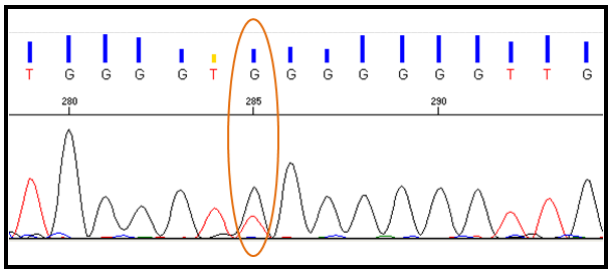

Figure 25. Part of the nucleotide sequence (electropherogram) of intron 2 belonging to *TAC3* gene. Orange circled double peak, corresponding to the nucleotides G and A (IVS2+87 G>T), denotes a heterozygous mutation.

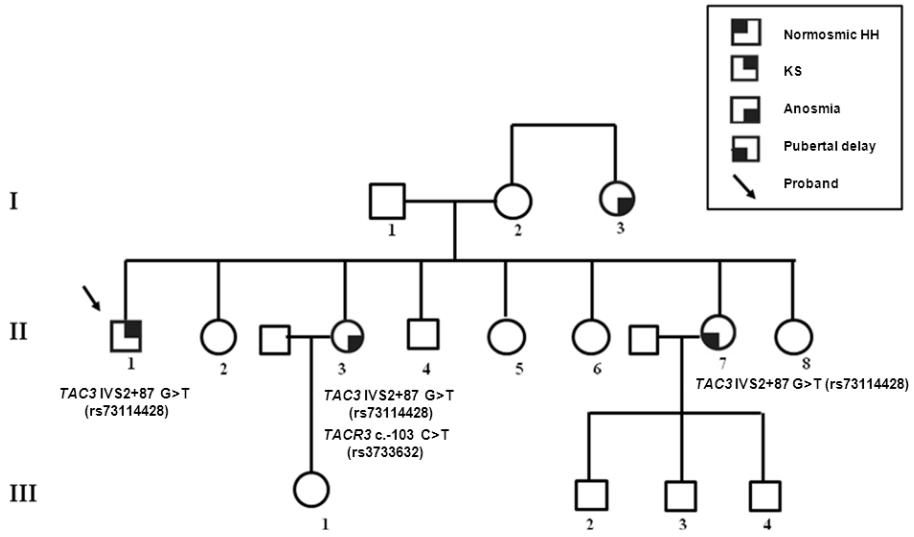

Figure 26. Genetic pedigree of a male KS proband, harbouring a SNP in *TAC3*.

The identified *TAC3* variant, at the moment of genetic analysis, was present neither inside the International SNP database (dbSNP) (www.ncbi.nlm-.nih.gov/snp/), nor in PubMed (www.ncbi-.nlm.nih.gov/pubmed). Therefore, we proceeded to verify the possible impact of IVS+87 G>T on splicing. Splicing is a modification of the nascent pre-messenger RNA transcript, in which introns are removed and exons are joined to form the mature mRNA. Several intron sites are important in this process, such as intronic splicing enhancers (ISEs), splicing silencers (ISSs) and the branch point (BP) (Calandra et al., 2011). Performing in silico analyzes, using the two prediction software Automated Splice Site Analyses (https://splice.uwo.ca/) and ESE Finder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi) (Calandra et al., 2011), we found no associations between the variant and an hypotetical altered splicing. Recently, the variant IVS+87 G>T was inserted in the dbSNP, as rs73114428 (http://www.ncbi.nlm.nih.gov/snp/?-term=rs73114428). This SNP, in Caucasian populations, has an allele

frequency of 91.7 and 8.3 % for the allele G and T, respectively (www.ncbi.nlm.nih.gov-/projects/SNP-/snp_ref.cgi?rs=73114428).

4.1.2. *TACR3*

- SNP rs2765 (IVS5+73 C>T) was detected, in heterozygous state, in three unrelated male patients, two KS and one nHH. This polymorphism falls inside intron 5, in the portion corresponding to the Untranslated Region (UTR) at 3'end of the mature mRNA. This SNP was reported earlier as a nonpathologic variant (www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=2765).

  One of these patients harboured also the *TACR3* variant c.103C>T (rs3733632), in heterozygous state. In another KS patient, with this variant, the mutation p.V273M in *FGFR1* gene, considered causative, was addictionally detected (mutation treated in the paragraph 4.1.4).
- The SNP rs3733632 (c.-103 C>T), in homozygous state, was found in three unrelated male patients with normosmic hypogonadism. This variant falls upstream the first exon, in the region corresponding to the UTR at 5' end of the mRNA. This SNP is described in literature as a nonpathologic variant (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=3733632).
- The variant p.T389T (c.1166 C>T), in heterozygous state, was detected in one male patient with normosmic HH (Figure 27). At the time of this finding, this variant was absent in dbSNP and in PubMed. The variant, considered new, belongs to the mutations classified as synonymous. These mutations imply a nucleotide change not followed by an amino acid change, due to the "degenerated" genetic code (Strachan and Read, 1999). Indeed, a nucleotide triplet (codon), for instance CGT that

encodes the amino acid Arginine, when mutated, as in the case CGT>CGA or CGT>CGG, continues to encode the same amino acid, as CGA or CGG that encodes always for Arginine. Recently, the variant p.T389T has been inserted in db SNP as a nonpathogenic mutation.

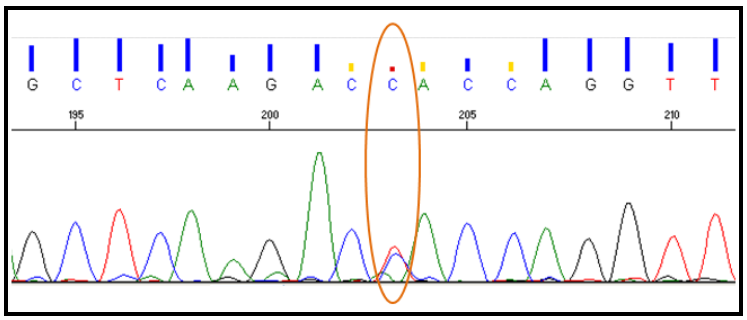

Figure 27. Part of the exon 5 nucleotide sequence (electropherogram) of *TACR3* gene. The orange circled double peak, corresponding to the nucleotides C and T (c.1166 C>T; p.T389T), indicates a heterozygous mutation.

4.1.3. PROKR2

- The missense mutation c.190 A>G; p.M64V, in heterozygous state, was identified in one KS male patient. The heterozygous nucleotide change in the first exon, at position 190, is considered sufficient to cause the pathology (Sykiotis et al., 2010).
- The new missense variant c.295 G>A; p.D99N, in heterozygous state, was identified in two unrelated male patients with nHH. Studies about its biological effects were not performed. To date, we screened only one proband's family for *PROKR2*. As a result, both the father and the daughter of the proband showed the same mutation but they were asymptomatic. This finding suggests that p.D99N in a single allele is not sufficient to determine the pathology. This proband harboured also the SNP p.T389T (rs201473070) in *TACR3*. Unfortunately, his family has not yet been screened for this gene.

- The missense variant c.254 G>A; p.R85H, in heterozygous state, was detected in two unrelated patients, a KS female and a nHH male. The female proband harboured also the SNP rs4986941 in *GNRHR*, in homozygous state. p.R85H is a known causative mutation, involving the first exon of the receptor. Functional studies demonstrated that R85H causes a highly significant reduction of the receptor action, compared to the wild type protein (Caronia et al., 2011; Dodé et al., 2006).
- The frameshift variant c.58delC; p.H20MfsX43, was identified in heterozygous state, in a male patient with a delayed puberty and reversal normosmic hypogonadism. This mutation was already described and causes a frameshift with the consequent introduction of a premature STOP codon in amino acid position 43 (Dodé et al., 2006).

4.1.4. FGFR1

- The missense mutation c.817 G>A; p.V273M, in heterozygous state, was identified in two siblings with KS, one male and one female. This nucleotide variant falls in exon 7 and causes a structural change in the protein domain, named D3, because the amino acid Methyonine replaces Valine in position 273. This change has an elevated steric hindrance, altering the three-dimensional protein structure (Pitteloud et al., 2006; Rajith and George Priya Doss, 2013). Curiously, their father, bearing the causative variant p.V273M, is asymptomatic and does not show any phenotypic features of CHH (Figure 28). The investigation regarding the other CHH related genes revealed the GNRHR SNP rs4986942, in homozygous state, and the TACR3 SNP rs2765, in heterozygous state, harboured by sister and brother, respectively.

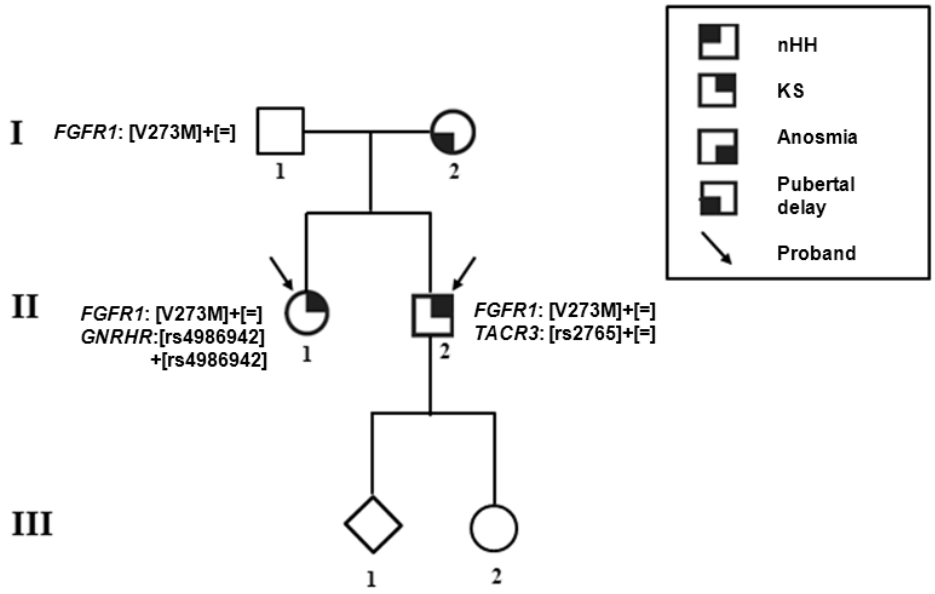

Figure 28. Genetic pedigree of two KS siblings.
The female proband, harbours the variant V273M in *FGFR1* and one SNP in *GNRHR*. The brother harbours V273M and rs2765 in *FGFR1* and *TACR3*, respectively. The probands' father (I1) harbours the variant V273M in *FGFR1* but he is asymptomatic.

- The non sense mutation c.880 G>T; p.E294X was detected, in heterozygous state, in a male patient affected by normosmic HH,. This nucleotide variant, falling inside exon 7, causes the formation of a truncated protein with a premature STOP codon at amino acid position 294. To date, functional studies about this mutation are not available in the literature. The proband also harboured the SNP rs3733632 of *TACR3*, in homozygous state.

4.1.5. KAL1

- A entire deletion of the exon 3 was identified in a male patient with Kallmann Syndrome. This mutation, already described in the literature (Dodé and Hardelin, 2009), is sufficient to cause the pathology.

4.1.6. GNRHR

In the Modena's cohort, only SNPs were found in GNRH Receptor gene.

- The polymorphism rs4986941 (c.415A>G) (www.ncbi.nlm.nih.gov-/projects/SNP/-snp_ref.cgi?rs=4986941) was identified, in homozygous state, in one female patient with KS, harbouring the causative mutation in *PROKR2* p.R85H, described above.
- The polymorphism rs4986942 (c.453C>T) (http://www.ncbi.nlm.nih-.gov/projects/SNP/snp_ref.cgi?rs=4986942) was detected, in homozygous state, in three unrelated patients, two nHH males and one KS female, harbouring the mutation p.V273M in *FGFR1*, discussed in paragraph 4.1.4.

Table 13a. Genetic results of the Modena's CHH cohort.

ID	GENDER	PHENOTYPE	*TAC3 29/47 (61.7%)*	*TACR3 29/47 (61.7%)*	*FGFR1 30/47 (63.8%)*	*PROK2 29/47 (61.7%)*	*PROKR2 30/47 (63.8%)*	*GNRH1 30/47 (63.8%)*	*GNRH2 30/47 (63.8%)*	*KAL1 8/47 (17%)*	*GNRHR 20/47 (42.6%)*
1	Male	nHH	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type		[c.453C>T (rs4986942)]+ [c.453C>T (rs4986942)]
2	Male	nHH	Wild Type	Wild Type	Wild Type	Wild Type	[c.254 C>A, p.R85H]+[=]	Wild Type	Wild Type		
3	Male	KS	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type
5	Male	KS	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type		
6	Male	KS	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type		Wild Type
8	Male	nHH	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type		Wild Type
9	Male	nHH	Wild Type	[c.-103C>T (rs3733632)] +[c.-103C>T (rs3733632)]; [IVS5+73 C>T (rs2765)]+[=]	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type		Wild Type
12	Male	KS								[del exon 3]	
13	Female	nHH	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type		
15	Male	nHH	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type		[c.453C>T (rs4986942)]+ [c.453C>T (rs4986942)]
17	Female	KS	Wild Type	Wild Type	Wild Type	Wild Type	[c.254 C>A, p.R85H]+[=]	Wild Type	Wild Type	Wild Type	[c.415A>G (rs4986941)]+ [c.415A>G (rs4986941]
18	Male	KS	Wild Type	Wild Type	Wild Type	Wild Type	[c.190 A>G; p.M64V]+[=]	Wild Type	Wild Type		
20	Male	nHH	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type		Wild Type
21	Male	KS	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	
23	Female	KS	Wild Type	Wild Type	[c.817 G>A (p.V273M)]+ [=]	Wild Type	Wild Type	Wild Type	Wild Type		[c.453C>T (rs4986942)]+ [c.453C>T (rs4986942)]
24	Male	KS	Wild Type	[IVS5+73 C>T (rs2765)]+[=]	[c.817 G>A (p.V273M)]+ [=]	Wild Type	Wild Type	Wild Type	Wild Type		Wild Type

Table 13b. Genetic results of the Modena's CHH cohort.
Patients 4, 7, 8 – 11, 14, 16, 19, 22, 28, 32, 35, 37 – 41, have not yet been screened for any gene.

ID	GENDER	PHENOTYPE	*TAC3 29/47 (61.7%)*	*TACR3 29/47 (61.7%)*	*FGFR1 30/47 (63.8%)*	*PROK2 29/47 (61.7%)*	*PROKR2 30/47 (63.8%)*	*GNRH1 30/47 (63.8%)*	*GNRH2 30/47 (63.8%)*	*KAL1 8/47 (17%)*	*GNRHR 20/47 (42.6%)*
25	Male	KS	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type		Wild Type
26	Male	nHH	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type		Wild Type
27	Male	nHH	Wild Type	[c.1166 C>T; p.T389T (rs201473070)]+[=]	Wild Type	Wild Type	[c.295 G>A; p.D99N]+[=]	Wild Type	Wild Type		
29	Male	nHH	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	
30	Male	nHH	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type		
31	Male	KS	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	
33	Male	nHH	Wild Type	Wild Type	Wild Type	Wild Type	[c.295 G>A; p.D99N]+[=]	Wild Type	Wild Type		Wild Type
34	Male	KS	[IVS2+87 G>T (rs73114428)]+[=]	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type
36	Male	KS	Wild Type	[IVS5+73 C>T (rs2765)]+[=]	Wild Type		Wild Type	Wild Type	Wild Type		Wild Type
42	Male	KS	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type
43	Male	nHH	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type		Wild Type
44	Male	nHH	Wild Type	Wild Type	Wild Type	Wild Type	[c.58delC; p.H20MfsX23]+[=]	Wild Type	Wild Type		
45	Male	nHH	Wild Type	[c.-103C>T (rs3733632)]+ [c.-103C>T (rs3733632)]	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type		Wild Type
46	Male	nHH	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type	Wild Type		Wild Type
47	Male	nHH	Wild Type	[c.-103C>T (rs3733632)]+ [c.-103C>T (rs3733632)]	[c.880 G>T (p.E294X)]+[=]	Wild Type	Wild Type	Wild Type	Wild Type		Wild Type

4.2. Patients enrolled at Endocrine Units collaborating with Modena

As already described in the paragraph 3.1, in addition to the Modena's cohort, 25 CHH patients, enrolled at the other Italian Endocrine Units of the network, were screened at ENDOLAB for both *TAC3* and *TACR3* genes.

4.2.1. *TAC3*

- The intronic,nonpathologic SNP rs73114428 (IVS+87G>T), in heterozygous state, was detected in *TAC3* in one patient only.
- In one subject, the new mutation c.293-7A>C (NM_013251.3), in heterozygous state, was identified. This variant, to date, is neither present in the literature nor in the dbSNP. This variant falls in the intron 5, 17 nucleotides downstream the Branch point (Smith et al., 2006) and 7 nucleotides upstream the 3' acceptor splice site (Figure 29).

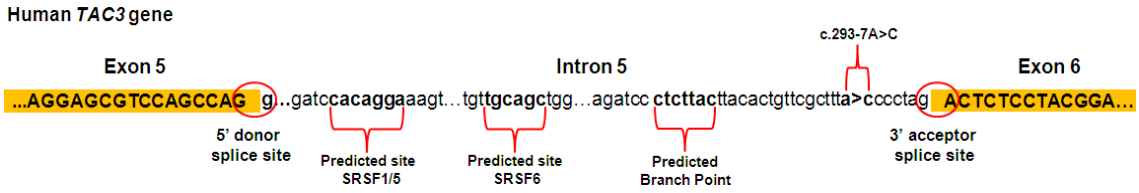

Figure 29. Graphical view of a portion of *TAC3* gene.
Both donor and acceptor splice sites, SRSF sites, the Branch point and the variant c.293-7A>C, in the intron 5, are indicated.

Considering the particular position of this variant, we decided to use several prediction software, such as Automated Splice Site Analysis (Nalla and Rogan, 2005), Ese finder 3.0 (Smith et al., 2006) and Human Splicing Finder (Desmet et al., 2009), to investigate possible relations between mutation and altered splicing. The results obtained indicate that c.293-7A>C is not espected to interfere with the physiologic splicing process involving the exons 5 and 6 and the intron 5 of *TAC3*.

4.2.2. TACR3

- In 14/25 affected patients, only the SNP rs2765 (IVS5+73 C>T), in heterozygous state, have been detected. This polymorphism falls inside intron 5, corresponding to the 3'UTR of the mRNA (www.ncbi.nlm.nih.-gov/projects/SNP/snp_ref.cgi?-rs=2765).

5. Discussion and Conclusion

Central Hypogonadotropic Hypogonadism is a complex disease including different phenotypic alterations regarding the HPG axis, olfactive structures, kidney structures, puberty onset and many other phenotypic anomalies.

So far, only few studies performed a comparison between normosmic and ipo/anosmic patients for main phenotypic abnormalities.

In our cohort pubertal delay was observed in 70% of nHH and in 90% of KS patients but this difference was not statistically significant (Table 12).

The prevalence of midline defects in our patients was high, 48%, without statistical differences between normosmic and ipo/anosmic groups (Table 12). On the contrary, in the literature midline defects were described in 6.6% of affected patients, with higher frequency in KS subjects (Bonomi et al., 2012).

We identified dental agenesis, 22% for nHH and 15% for KS subjects, without significant differences among the two groups (Table 12), while in the literature a prevalence of this anomaly has been reported in KS patients only (Bailleul-Forestier et al., 2010).

In our group pectus excavatum and bimanual synkinesis were found in 11% and 4% respectively, with a prevalence in KS patients of 15% and 5%, respectively (Table 12). In the study of Bonomi et al. (2012), bimanual synkinesis is rare and described only in KS patients.

Bilateral and monolateral cryptorchidism was prevalent in our KS subjects, 19%, without statistically significant differences among the two groups (Table 12). The prevalence of cryptorchidism in KS subjects has been also described by Bonomi et al. (2012), but with different frequencies, 35% in KS and 10% in normosmic patients.

The prevalence of kidney anomalies was very similar in our groups, 27% and 25% for nHH and KS patients, respectively (Table 12). Our data are similar to the percentages reported in the literature (Sato et al., 2004). We found three unrelated KS patients with renal agenesis. Interestingly, one out of the three patients harbours a large deletion of the entire exon 3 of the *KAL1* gene (Table 13a, b). Anosmin-1, encoded by *KAL1*, is known to have a role during the embryonic development of the renal ducts (Hardelin and Dodé, 2008). Thus, mutations in *KAL1* could determine renal agenesis and dysplasia.

The best known phenotype feature present in KS patients is aplasia of the olfactory bulbs (Koenigkam-Santos et al., 2011; Quinton et al., 1996) (Table 12). The frequency of this feature in our two groups, normosmic and hypo/anosmic patients, is highly significant and different ($P < 0.0001$, table 12). Nevertheless some normosmic patient, with anomalies of the olfactory structures, and viceversa, have been described in the literature (Quinton et al., 1996). Indeed, in this study were identified 2 out of 19 normosmic patients with agenesis and bulb hypoplasia and 3 out of 15 anosmic patients without abnormalities of olfactory bulbs by MRI (Table 12). These findings could be due to a wrong smell test, defective batch of smell test or to a preserved olfactory function through a compensation mechanism implemented by the contralateral bulb.

In the present study, we report the first Italian data about the mutational status of *TAC3* and *TACR3* in 29 out of 47 patients, 13 KS and 17 nHH (Table 13a, b). No *TAC3* causative mutations were identified. Only one SNP, in heterozygous state, was found in a KS patient (Table 13b). In addition, we found seven SNPs in *TACR3* in six unrelated patients, four normosmic and three ipo/anosmic. No mutations in *TACR3* were detected (Table 13a, b). Despite this relatively small cohort, our results suggest that causative mutations in the system *TAC3*/*TACR3* are rare in subjects affected by CHH, not confirming the frequency reported by Gianetti et al. (2010).

Regarding the additional group of 25 CHH patients, enrolled at Endocrine Units collaborating with Modena, we found a *TACR3* SNP in fourteen patients, a *TAC3* SNP in one patient, and the new *TAC3* mutation c.293-7A>C (NM_013251.3), in heterozygous state, in another patient. Despite the position of the nucleotide change, which suggests probable alteration of the normal splicing mechanism, subsequent analyses with predictive algorithms indicated that the nucleotide variant should not interfere with the splicing process involving exons 5-6 and the intron 5 of *TAC3*.

In our cohort we did not detect any causative mutation in *PROK2*, as well as in *GNRH1*, *GNRH2*, and *GNRHR*. Only two SNPs in *GNRHR* were detected in a total of four patients.

Mutations in *FGFR1* have been identified both in normosmic and anosmic patients (Table 13a, b), as described in literature (Topaloglu et al., 2009). The same mutation of *FGFR1*, reported in this study for two siblings (Table13a, b) (Figure 28), was described by Pitteloud et al. (2006), in a male patient with a family history of KS. In our subjects, a KS family history is confirmed (mother with slightly delayed puberty and KS sibling). The wide phenotypic variability expressed also between individuals, belonging to the same family,

in case of identical mutations in *FGFR1* agrees with a variable penetrance of such mutations (Silveira et al., 2010) and with a possible oligogenetic etiology. Our female proband, with a normal BMI (22.4), presents an insulin resistance, while her brother did not show insulin resistance but a slight overweight (BMI of 25). Curiously, their father, bearing the same probands variant, is asymptomatic and does not show any phenotypic features of CHH (Figure 28). A concomitant mutation or polymorphism in the probands could be the cause of this wide phenotypic variability.

Regarding the *PROKR2* investigation in our cohort, we report four different mutations in six unrelated patients, four nHH and two KS (Table 13a, b). The mutations p.M64V and p.H20MfsX43 cause the pathology, as described by Sykiotis et al. (2010) and Dodè et al. (2006). p.R85H, identified in one female KS seems to be causative, as suggested by Caronia et al. (2011).

Two unrelated male nHH patients, with the same new mutation (p.D99N) (Table 13a, b) present different phenotypes. Indeed, only one of these patients presents an overweight and a renal hypoplasia. The expression of *PROKR2* is present also in the endothelial cells of kidney, suggesting a possible pathogenic role of *PROKR2* in renal anomalies (Martin et al., 2011).

Only one out of eight patients screened for *KAL1* showed a mutation in this gene. The identified variant is a deletion, already described by Dodé and Hardelin (2009), eliminating the entire exon 3 and causing the pathology. This mutated KS patient is a 21 years old male with delay of puberty, pectus excavatum, bilateral cryptorchidism, renal agenesis and bilateral hypoplasia of the olfactory structures.

In conclusion, considering the different phenotypic characteristics of our cohort and after comparing the two groups, normosmic and hypo/anosmic patients, for nine different developmental abnormalities, the present study supports the hypothesis that nHH and KS are two different phenotypic expressions of the same heterogeneous disease.

In this study, we found some genetic alterations and confirmed the previously suggested wide phenotypic variability of CHH and the variable expressivity of the analysed genes.

Moreover, CHH is confirmed to be an oligogenic disease.

In 17 screened subjects (57%), we did not detect any causative variants, considering the genes analysed so far.

Although the polymorphic variants, described in this work, are not considered pathologic, they could influence the phenotype, especially if in combination with other mutations in other genes. In the near future, we will try to complete all genetic studies and phenotypic characterization in our wide cohort, composed by 400 CHH patients, enrolled in all Endocrine Centres of our network.

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7. List of abbreviations

ACTH: adrenocortocotropic hormone
AMH: anti-mullerian hormone
ARC: arcuate nucleus
BMI: Body Mass Index
BP: Branch point
BRWD2: Bromodomain and WD repeat domain containing 2
CHD7: chromodomain helicase DNA binding protein 7
CPHD: combined pituitary hormone deficiency
dbSNP: International SNP database
DEXA: Dual X-ray Absorptiometry
DHEA: deidroepiandrosterone
DHEAS: deidropiandrosterone solfato
FGF8: fibroblast growth factor 8
FGFR1: fibroblast growth factor receptor 1
FnIII: fibronectin-like type III
FSH: follicle-stimulating hormone
FSHB: follicle stimulating hormone beta polypeptide
GABA: gamma-aminobutyric acid
GH: growth hormone
GnRH: gonadotrophin-releasing hormone
GNRHR: gonadotrophin-releasing hormone receptor
GH: growth hormone
GPCR: G protein coupled receptor
hCG: human chorionic gonadotropin
HESX1: homeobox expressed in ES cells 1
HGF: hepatocyte growth factor
HH: Ipogonadismo ipogonadotropo
HK-1: hemokinin-1
HPG: Hypothalamic-pituitary-gonadal
HS6ST1: heparan sulfate 6-O-sulfotransferase 1
HSPG: heparin sulphate proteoglycans
IGF-1: insulin growth factor-1
Ig-like: immonuglobulin-like
ISE: intronic splicing enhancer

ISS: splicing silencers
KAL1: Kallmann syndrome 1
KISS1: kisspeptin 1
KISS1R: kisspeptin 1 receptor
KS: Kallmann Syndrome
LEP: leptin
LEPR: leptin receptor
LH: luteinizing hormone
LHB: luteinizing hormone beta polypeptide
LHX3: LIM/homeobox protein
MAF: Minor allele frequency
MRI: Magnetic resonance imaging
NDN: necdin
NEC 1: neuroendocrine convertase 1
NELF: nasal embryonic LHRH factor
nHH: normosmic Hypogonadotropic Hypogonadism
nICH: normosmic Idiopathic Central Hypogonadism
NKA: neurokinin A
NKB: neurokinin B
NROB1: nuclear receptor subfamily 0 group B member 1
Nrp-1: neuropilin 1
Nrp-2: neuropilin 2
PCR: Polymerase Chain Reaction
PROK2: prokineticin 2
PROKR2: prokineticin receptor 2
PSA: prostate-specific antigen
SNP: single nucleotide polymorphism
SF1: steroidogenic factor-1
SP: substance P
SVZ: subventricular zone
TAC3: tachykinin-3
TACR3: tachykinin-3 receptor
TM: transmembrane domain
TSH: thyroid-stimulating hormone
UTR: Untranslated Region
VNNs : olfactory-vomeronasal nerves
WAP: whey acid protein
WDR11: WD repeat domain

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