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MOLECULAR GENETICS OF KALLMANN SYNDROME AND CONSTITUTIONAL DELAY OF GROWTH AND PUBERTY IN FINLAND

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ACADEMIC DISSERTATION

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To my Family

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals I-V:

I Laitinen E-M*, Vaaralahti K*, Tommiska J, Eklund E, Tervaniemi M, Valanne L, Raivio T. Incidence, phenotypic features and molecular genetics of Kallmann syndrome in Finland. *Orphanet Journal of Rare Diseases*. 2011; 6: 41. *Joint first authorship.

II Tommiska J, Toppari J, Vaaralahti K, Känsäkoski J, Laitinen E-M, Noisa P, Kinnala A, Niinikoski H, Raivio T. *PROKR2* mutations in autosomal recessive Kallmann syndrome. *Fertility and Sterility*. 2013; 99: 815-818.

III Vaaralahti K, Tommiska J, Tillmann V, Liivak N, Känsäkoski J, Laitinen E-M, Raivio T. *De novo SOX10* nonsense mutation in a patient with Kallmann syndrome and hearing loss. *In press / Pediatric Research*.

IV Vaaralahti K, Raivio T, Koivu R, Valanne L, Laitinen E-M, Tommiska J. Genetic overlap between Holoprosencephaly and Kallmann syndrome. *Molecular Syndromology*. 2012; 3: 1-5.

V Vaaralahti K, Wehkalampi K, Tommiska J, Laitinen E-M, Dunkel L, Raivio T. The role of gene defects underlying isolated hypogonadotropic hypogonadism in patients with constitutional delay of growth and puberty. *Fertility and Sterility*. 2011; 95: 2756-2758.

Publication I has appeared also in the thesis by Eeva-Maria Laitinen (2012).

ABBREVIATIONS AND DEFINITIONS

AD	Autosomal dominant
AR	Autosomal recessive
ARC	Arcuate nucleus
AVPV/PeN	Anteroventral periventricular nucleus / rostral periventricular nuclei
BF	Basal forebrain
BMP4	Bone morphogenetic protein 4
CDGP	Constitutional delay of growth and puberty
cDNA	Complementary DNA
CHARGE	Acronym derived from the cardinal features of the syndrome (coloboma, heart defects, choanal atresia, retarded growth and development, genital abnormalities, and ear anomalies)
CHD7	Chromodomain helicase DNA binding protein 7
CO	Cryptorchidism
CP	Cribriform plate
DP	Delayed puberty
DNA	Deoxyribonucleic acid
DS	Direct sequencing
DUSP6	Dual specificity phosphatase 6
DYN	Dynorphin A
EAP1	Enhanced at puberty 1 (approved name: interferon regulatory factor 2 binding protein-like)
ES cells	Embryonic stem cells
EV	Empty vector
F	Female
FGF	Fibroblast growth factor 2/8/17/18
FGFR1	Fibroblast growth factor receptor 1
FOXH1	Forkhead box H1
FLRT3	Fibronectin leucine rich transmembrane protein 3
FSH	Follicle-stimulating hormone
GH	Growth hormone
GHSR	Growth hormone segretagogue receptor
GLI2/3	GLI family zinc finger 2/3
GnRH	Gonadotropin-releasing hormone
GNRH1	Gonadotropin-releasing hormone 1
GNRHR	Gonadotropin-releasing hormone receptor
GPR54	G protein-coupled receptor 54 (approved name: KISS1 receptor)
HA	Human influenza hemagglutinin
HESX1	HESX homeobox 1
HGNC	HUGO gene nomenclature commitee
HH	Hypogonadotropic hypogonadism
HPE	Holoprosencephaly
HPG axis	Hypothalamic-pituitary-gonadal axis
HRP	Horseradish peroxidase
HSPG	Heparan sulfate proteoglycan
HS6ST1	Heparan sulfate 6-O-sulfotransferase 1
ICD	International classification of disease
IG	Immunoglobulin-like

IGFALS	Insulin-like growth factor binding protein, acid-labile subunit
IL17RD	Interleukin 17 receptor D
JM	Juxtamembrane
KAL1	Kallmann syndrome 1 sequence
KISS1	KiSS-1 metastasis-suppressor
KISS1R	KISS1 receptor
KP	Kisspeptin
KS	Kallmann syndrome
LEP	Leptin
LEPR	Leptin receptor
LH	Luteinizing hormone
LHRH	Luteinizing hormone-releasing hormone (obsolete)
LIN28B	Lin-28 homolog B (C. elegans)
M	Male
MAF	Minor allele frequency
MAPK	Mitogen-activated protein kinase
MLPA	Multiplex ligation-dependent probe amplification assay
MP	Micropenis
MRI	Magnetic resonance imaging
NA	Not assessed
NC	Neural crest
NDN	Necdin, melanoma antigen (MAGE) family member
NELF	Nasal embryonic LHRH factor (approved name: NMDA receptor synaptonuclear signaling and neuronal migration factor
NGS	Next-generation sequencing
nHH	Normosmic hypogonadotropic hypogonadism
NKB	Neurokinin B
NP	Nasal placode
OB	Olfactory bulb
OE	Olfactory epithelium
ON	Olfactory neurons
OP	Olfactory placode
OSN	Olfactory sensory neuron
OT	Olfactory tract
OTX2	Orthodenticle homeobox 2
PBS	Phosphate buffered saline
PCWH	Acronym derived from the cardinal features of the syndrome (peripheral demyelinating neuropathy, central dysmyelination, Waardenburg syndrome, and Hirschsprung disease)
PHS	Pallister-Hall syndrome
POA	Pre-optic area
PROK2	Prokineticin 2
PROKR2	Prokineticin receptor 2
RSV	Rare sequence variant
SCC	Semicircular canal
SD	Standard deviation
SEMA3A	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A
SHH	Sonic hedgehog
SIX3	SIX homeobox 3

SNP	Single nucleotide polymorphism
SOD	Septo-optic dysplasia
SOX2/10	SRY (sex determining region Y)-box 2/10
SP	Signal peptide
SPRY2/4	Sprouty homolog 2/4 (Drosophila)
SRY	Sex-determining region of the Y-chromosome
TAC3	Tachykinin 3
TACR3	Tachykinin receptor 3
TDGF1	Teratocarsinoma-derived growth factor 1
TGIF1	TGFB-induced factor homeobox 1
TK	Tyrosine kinase
TM	Transmembrane
TTF1	Transcription termination factor, RNA polymerase I
VNN	Olfactory, vomeronasal and terminal nerve fibers
VNO	Vomeronasal organ
WDR11	WD repeat domain 11
WS	Waardenburg syndrome
WT	Wild-type
XR	X-linked recessive

In the text, human genes are in italics and in caps. Mouse genes are in italics. From genes only abbreviations are used.

ABSTRACT

Puberty is governed by ~2000 hypothalamic gonadotropin-releasing hormone (GnRH) neurons. GnRH neurons originate from the neural crest and from the olfactory placode. From the olfactory placode GnRH neurons migrate to the hypothalamus along the axons of developing olfactory nerves. Defects in GnRH neuron development, migration or in GnRH secretion or action cause congenital hypogonadotropic hypogonadism (HH), which is a rare developmental disorder characterised by delayed or absent puberty. If HH appears with defects in sense of smell, the condition is termed Kallmann syndrome (KS). Clinical and genetic features of KS and congenital HH with normal sense of smell (normosmic HH) are heterogeneous. Only for ~35% of congenital HH patients a molecular genetic diagnosis can be given.

The most common cause of delayed puberty is constitutional delay of growth and puberty (CDGP). CDGP is a variant of the normal spectrum of pubertal timing and is characterized by first pubertal signs appearing at an age that is 2.0 standard deviations above the mean age for pubertal onset. The genetic background of CDGP is unknown.

The aim of this thesis study was to characterize the molecular genetic features of KS patients in Finland. We also investigated whether mutations in known holoprosencephaly (HPE) or septo-optic dysplasia (SOD) genes could underlie some cases of KS. In addition, we investigated the role of congenital HH genes in CDGP.

Thirty-four subjects with KS (6 females, 28 males) were screened for mutation(s) in genes involved in development and/or migration of GnRH neurons and in which mutations are known to cause KS: *KAL1*, *FGFR1*, *FGF8*, *PROK2*, *PROKR2*, *CHD7*, *WDR11*, and *SOX10*. The effects of FGFR1 missense mutations G48S, R209H, and E670A on receptor function were analysed *in vitro*. Patients remaining without identified molecular genetic cause in established KS genes were also screened for mutation in *SOX2*, *SHH*, *SIX3*, *TGIF1*, *TDGF1*, *FOXH1*, and *GLI2*, in which mutations are known to cause HPE or SOD (*SOX2*). Furthermore, *GNRHR*, *FGFR1*, *TAC3*, and *TACR3* were screened in 146 subjects with CDGP (*TAC3* and *TACR3* in females only).

Out of 34 KS patients, 15 got a molecular genetic diagnosis. Nine patients (5 females, 4 males) had an *FGFR1* mutation, three males had a *KAL1* mutation, one male of Iraqi origin

carried a homozygous *PROKR2* mutation, one male with CHARGE (coloboma, heart defects, choanal atresia, retarded growth and development, genital abnormalities, and ear anomalies) syndrome associated features had a *CHD7* mutation, and one male with KS and deafness carried a *de novo SOX10* mutation. FGFR1 missense mutants G48S and E670A displayed impaired mitogen-activated protein kinase signalling *in vitro*. One male KS patient carried heterozygous missense variants in *GLI2* and in *SIX3*. One male subject with CDGP carried a previously undescribed heterozygous deletion in *GNRHR*, which segregated with delayed puberty in his family.

In conclusion, KS is a male predominant condition. 44% of KS patients received a molecular genetic diagnosis. A clear difference was seen in the distribution of molecular genetic diagnoses in this study and in those reported previously, as the leading molecular genetic cause of KS, mutation in *FGFR1*, accounted for 26% of the cases, and mutations in *PROK2* and *PROKR2* were almost completely absent. Also, a significantly higher proportion of women (83%) carried an *FGFR1* mutation compared with men (14%). Considerable genotypic and phenotypic overlap is seen between KS, Waardenburg syndrome and CHARGE syndrome. Therefore hearing impairment and/or ear anomalies in KS patient should be considered as an indication for both *CHD7* and *SOX10* molecular analyses and, in case of identified mutation the possibility of more severely affected future children should be taken under consideration. Mutations in known HPE genes are not a common cause for KS in Finland. Finally, mutations in *FGFR1*, *GNRHR*, *TAC3* or *TACR3* are not a common cause of CDGP.

1 INTRODUCTION

Intact hypothalamic-pituitary-gonadal axis is essential for normal pubertal development: hypothalamic gonadotropin-releasing hormone (GnRH) neurons produce GnRH, GnRH stimulates the production and secretion of gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary, which in turn, stimulate gonadal function and secretion of sex steroids.

In a series of adolescents with delayed puberty from a large academic center, more than 50 reasons for pubertal delay were recorded (Sedlmeyer & Palmert 2002). The most common cause for pubertal delay is constitutional delay of growth and puberty (CDGP), which is a variant of the normal spectrum of pubertal timing. CDGP tends to aggregate in families, and, although many genes may be involved, the inheritance patterns suggest that there are also single genes with major effects (Sedlmeyer et al. 2002, Wehkalampi 2008a).

Delayed or absent puberty can also be caused by hypogonadotropic hypogonadism (HH), which is a condition characterized by diminished functional activity of the gonads due to an impaired secretion of FSH and LH. HH can be acquired or syndromic, or it can be isolated. Acquired/syndromic HH is relatively common and results from different causes, including brain tumours, head trauma, and certain systemic diseases and syndromes (Palmert & Dunkel 2012). In contrast, isolated HH, also called as congenital HH, is a rare developmental disorder of sexual maturation, which results from GnRH deficiency. Reasons for GnRH deficiency are a lack or reduced number of hypothalamic GnRH-producing neurons, disturbed secretion of GnRH from the hypothalamus, or inadequate action of GnRH in the anterior pituitary (Seminara et al. 1998, Young 2012). When congenital HH occurs with defects in sense of smell, the condition is termed Kallmann syndrome (KS), named after an American geneticist, Franz Josef Kallmann (Kallmann et al. 1944). Genetics of congenital HH is complex and heterogenous. Although several genes have been implicated in the etiology congenital HH, still about 65% of the cases remain without identified genetic cause (Mitchell et al. 2011).

The aim of this study is to characterize the molecular genetic features of delayed and absent puberty, and it focuses on patients with KS and CDGP.

2 REVIEW OF THE LITERATURE

2.1 Function of the hypothalamic-pituitary-gonadal (HPG) axis

The proper function of HPG axis is essential for normal gonadal development, sex steroid production and fertility. In short, hypothalamic GnRH neurons secrete GnRH that binds to its receptors on the gonadotropic cells of the anterior pituitary. Activation of these receptors leads to expression and secretion of LH and FSH, which in turn stimulate the testes and ovaries to produce sex steroids estrogen and testosterone. The HPG axis is active already during fetal life and in early childhood, but then becomes quiescent until onset of puberty (Winter et al. 1975, Wu et al. 1996)

2.1.1 HPG axis in infancy

Hypothalamic GnRH secretion begins by the end of the first trimester. In both sexes, LH and FSH are important for proper development of fetal gonadal tissue. At birth, levels of LH and FSH are low due to inhibitory effect of placental steroids (Winter et al. 1975, Debieve et al. 2000) but they rise again when the HPG axis is reactivated within two weeks after birth. This "minipuberty" is considered to be important for normal reproductive development especially in boys, since during this period, there is an increase in the number of Sertoli cells, growth of penis and testes, and increased testosterone secretion. In girls, increase in FSH levels indicates maturation of the ovaries. During minipuberty, gonadotropin levels are at their highest level between 1 to 3 months, after which they gradually decline to low levels by six months of age. (Winter et al. 1975, Kuiri-Hänninen et al. 2011a,b)

2.1.2 Puberty

Puberty is a transitional state between childhood and adulthood when fertility is achieved. GnRH pulses followed by LH pulses are evident during sleep from mid-childhood onwards (Wu et al. 1996). Approximately two years before the first physical signs of puberty, there is a marked increase in the amplitude of these pulses, and finally they occur also during the daytime. LH and FSH promote gonadal maturation and production of sex steroids, which are essential for the development of secondary sex characteristics and for the pubertal growth spurt.

Puberty proceeds in specific order, and it is usually described by Tanner stages 1 to 5. In girls, the appearance of breast tissue (telarche) is usually the first sign of puberty (Tanner stage B2). In boys, the first sign is the enlargement of testes to a volume of 3ml or more (gonadarche, Tanner stage G2). In normal situation, gonadarche and thelarche indicate the activation of the HPG axis and central onset of puberty. Menarche, the onset of menstruation occurs in girls approximately 2 years after reaching B2 (Marshall & Tanner 1969, Marshall & Tanner 1970). Pubertal hair growth (pubarche/adrenarche) indicates the activation of the hypothalamic-pituitary-adrenal axis which occurs independently from HPG axis activation (Sklar et al. 1980). The gradually increasing secretion of gonadal sex steroids also drives the pubertal growth spurt together with rising growth hormone (GH) levels (Tanner & Whitehouse 1976).

An active pulsatile GnRH-stimulated secretion of LH and FSH is important for the maintenance of secondary sexual characteristics and for sexual function throughout adult life. In men, LH promotes testosterone production from Leydig cells and FSH is needed for spermatogenesis supported by Sertoli cells. In women, GnRH secretion is required for a normal menstrual cycle, where FSH and LH secretion stimulates folliculogenesis and steroidogenesis in ovaries during follicular phase, and LH surge leads to ovulation (Navarro & Tena-Sempere 2011).

A global secular trend towards earlier puberty observed in last century reflects improved nutrition and health (Wyshak & Frisch 1982, Parent et al. 2003). Novel environmental influences, such as endocrine-disrupting chemicals and the increasing problem of childhood obesity, have been suggested to explain the more recent observations of earlier pubertal onset in developed countries (Herman-Giddens 2001, Aksglaede et al. 2009, Sorensen et al. 2010). Overall, the timing of puberty varies greatly in the general population. Based on twin studies and similarities in the ages at pubertal onset between family members, it appears that the onset is largely regulated by genetic factors, and influenced by environmental and individual factors (Figure 1) (Sedlemeyer et al. 2002, Parent et al. 2003, Silventoinen et al. 2008).

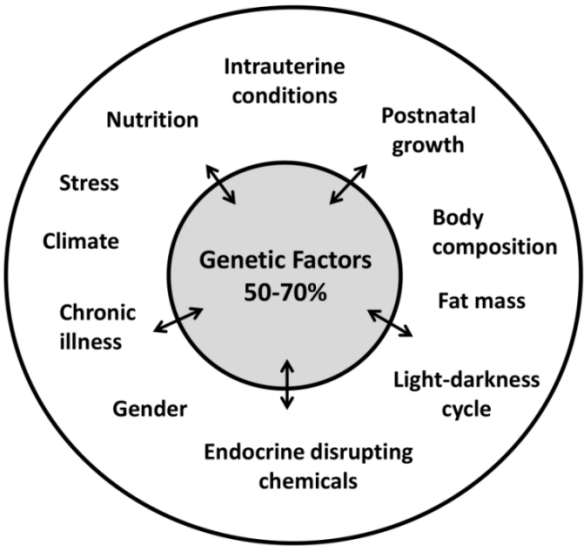

Figure 1. Timing of puberty is largely regulated by genetic factors, and influenced by environmental and individual factors. Modified from Parent et al. 2003.

Although regulation of pubertal timing likely derives from modest additive effects of multiple genes (Parent et al. 2003), important information may result from identification of single high impact genes, in which mutations cause abnormalities in the onset of pubertal development. A number of genes important for the onset of puberty have been discovered by investigating individuals with congenital HH (chapter 2.3).

2.1.3 Regulation of HPG axis

The reactivation of HPG axis at puberty is under the control of regulatory networks that gather and interpret hormonal, nutritional, metabolic, and environmental signals. The balance and the interplay of these signals determine the onset of puberty. But what is the exact mechanism/factor that ultimately activates GnRH secretion is not known (Sisk & Foster 2004). One identified upstream regulator of GnRH neurons is kisspeptin. Kisspeptin is secreted from Kiss1 neurons located in the arcuate nucleus (ARC) and anteroventral periventricular nucleus/rostral periventricular nuclei (AVPV/PeN) of the hypothalamus. GnRH neurons express KISS1 receptor, and kisspeptin signalling via this receptor stimulates GnRH secretion (de Roux et al. 2003, Seminara et al. 2003, Navarro & Tena-Sempere 2011). In response, LH and FSH are released from the anterior pituitary, which in turn, stimulate

gonadal function and secretion of sex steroids and Inhibin B. Sex steroids and Inhibin B regulate HPG axis activation through a negative feedback loop at the level of hypothalamus and pituitary (Cariboni et al. 2007) (Figure 3).

Kisspeptin neurons in the ARC are also called as KNDy-neurons as in addition to kisspeptin they co-express neurokinin B (NKB) and dynorphin A (DYN) (Lehman et al 2010). These neurons may have an important role in mediating negative feedback of sex steroids to the GnRH neurons as they express estrogen receptor α and androgen receptor. KNDy-neurons are thought to autoregulate themselves through positive (NKB) and negative (DYN) feedback loops. Also Kiss1 neurons located in the AVPV/PeN express estrogen receptor α and may be involved in the positive feedback regulation by estradiol, and could be responsible for the GnRH - LH surge that leads to ovulation (Figure 2) (Roa et al. 2008, Oakley et al. 2009, Navarro & Tena-Sempere 2011).

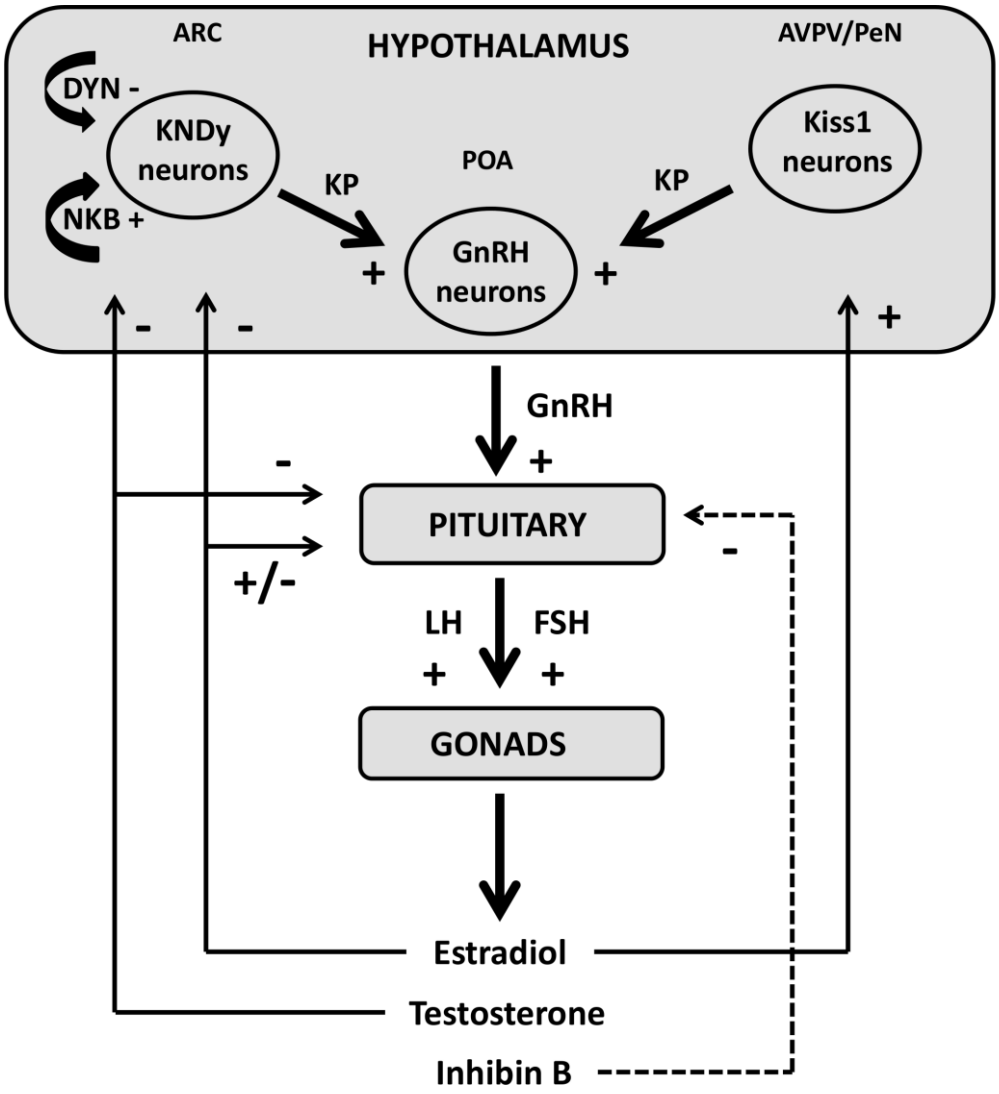

Figure 2. Hypothalamic-pituitary-gonadal axis and its regulation. KNDy neurons and the Kiss1 neurons located in the arcuate nucleus (ARC) and anteroventral periventricular nucleus/rostral periventricular nuclei (AVPV/PeN) of the hypothalamus secrete kisspeptin (KP), which stimulates the Gonadotropin-releasing hormone (GnRH) neurons to produce GnRH. GnRH stimulates the production and secretion of gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary, which in turn, stimulate gonadal function and secretion of sex steroids and Inhibin B. Sex steroids and Inhibin B regulate the secretion of GnRH and gonadotropins through negative feedback loops. KNDy-neurons co-express kisspeptin, neurokinin B (NKB) and dynorphin A (DYN) and are thought to autoregulate themselves through positive (NKB) and negative (DYN) feedback loops. Before ovulatory LH surge in women, estradiol has a short positive feedback effect on pituitary and on the Kiss1 neurons located in the AVPV/PeN. POA, pre-optic area. (Navarro & Tena-Sempere 2010).

2.2 Embryonic development of GnRH neurons

2.2.1 Dual origin of GnRH neurons

GnRH neurons are viewed as one of the most upstream regulatory components of the HPG axis (Wray et al. 1989). The embryonic origin of GnRH neurons has been a matter of debate for several decades. Studies with different animal models have provided evidence of both placodal and neural crest (NC) origin for these cells (Wray et al. 1989, Forni et al. 2011, Sabado et al. 2011, Berghard et al. 2012, Metz & Wray 2010, Whitlock et al. 2003).

Cranial placodes are spelialized regions of the ectoderm, which give rise to the various sensory ganglia and contribute to the pituitary gland and the sensory organs of the vertebra head (Schlosser 2006). The olfactory placode (OP) gives rise to the olfactory epithelium (OE) and epithelium of vomeronasal organs (Whitlock 2004). These epithelia contain chemoreceptive sensory cells that project their axons towards the developing olfactory bulb (OB), in which they synapse with dendrites of mitral cells to form the olfactory tract (Cariboni & Maggi 2006). Historically, GnRH neurons have been thought to originate exclusively from the OP. Immunohistochemistry and *in situ* hybridization experiments with mice in two landmark studies showed that GnRH expressing cells could be found only in the OP on embryonic day 11.5 (Schwanzel-Fukuda et al. 1989, Wray et al. 1989). Ablation studies have both provided support and contradicted the OP origin of GnRH neurons; amphibians that undergo OP removal end up lacking GnRH neurons (Murakami et al. 1992, Northcutt et al. 1994), whereas OP removal from chick and rat embryos does not result in total loss of GnRH neurons (Daikoku-Ishido et al. 1990, Daikoku & Koide 1998), suggesting that they do not arise solely from OP.

The NC has been implicated as a possible contributor to the formation of OP (Whitlock & Westerfield 2000, Forni et al. 2011, Katoh et al. 2011). The pluripotent NC cells arise at the junction between the neural tube and the epidermis, from where they migrate throughout the organism and give rise to many different cell types including neurons and glia of the peripheral nervous system, bone and cartilage of the skull, and melanocytes (Le Douarin 1999, Selleck et al. 1993). In addition, NC cells have been shown to migrate towards the presumptive OP and are likely to contribute to the developing OP and its derivates (Schilling & Kimmel 1994). Studies with zebrafish have shown that when pre-migratory NC cells were

labelled, subset of these cells later on expressed GnRH peptide (Whitlock et al 2003). This data was recently replicated in mice. Lineage tracing experiment performed in two mouse models showed that NC cells indeed intermix in the OP and give rise to all olfactory ensheating cells which support the growth and targeting of olfactory axons, and to a subpopulations of GnRH neurons (about 30%), olfactory, and vomeronasal cells (Forni 2011, Katoh 2011, Forni & Wray 2012). Thus, GnRH neurons are likely to originate from both of the NC and OP.

2.2.2 Migration to the hypothalamus

GnRH neurons migrate from the OP to the hypothalamus in association with olfactory, vomeronasal and terminal nerve fibers (Schwanzel-Fukuda et al. 1989, Wray et al. 1989) (Figure 3). Once GnRH cells gain motility, they exit from the OP and migrate through the nasal compartment. As they move from the nasal compartment through the cribriform plate into brain tissue, they travel across the nasal–forebrain junction. At this point, GnRH cells appear to pause. The pause may ensure that the cells mature and/or that the correct migratory route is established (Wray et al. 2010). When GnRH neurons have crossed the nasal-forebrain junction, they follow axons that express peripherin toward the basal forebrain. Once they have reached their final destination in the developing hypothalamus, they detach from the vomeronasal axons and extend their own axons to the median eminence (Cariboni et al. 2007, Wray et al. 2010). In humans, the first GnRH neurons appear in the hypothalamus by 14 weeks of gestation, and the migration is completed by 19 weeks (Schwanzel-Fukuda et al. 1989, Quinton et al. 1997).

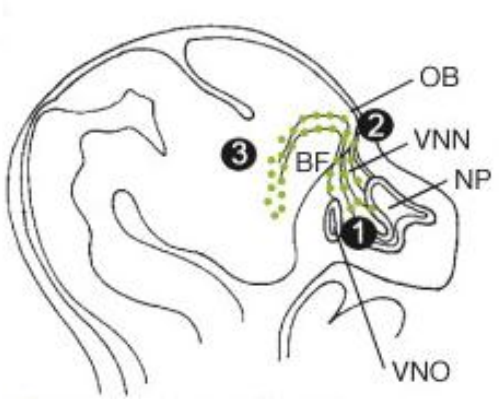

Figure 3. Migratory route of Gonadotropin-releasing hormone neurons (green dots) begins from the olfactory/nasal placode (NP) in the region of vomeronasal organ (VNO). They migrate along the olfactory, vomeronasal and terminal nerve fibers (VNN), enter the brain in the proximity of olfactory bulbs (OB), and continue through the basal forebrain (BF) to the hypothalamus. Migratory route takes them through three chemically distinct environments: 1) the nasal compartment, 2) the nasal-forebrain junction, and 3) the basal forebrain. Adapted from Cariboni et al. (2007).

Migratory journey from the OP to the hypothalamus takes GnRH neurons through a changing molecular environment with numerous factors controlling different aspects of their movement (Wray et al. 2010). Many of these molecules work in multiple areas and may even produce a different response depending on the relative dose to which GnRH cells are exposed (Wray et al. 2010). These factors include for example anosmin-1, fibroblast growth factor receptor 1 (FGFR1), semaphorins, necdin and nasal embryonic luteinizing hormone-releasing hormone factor (NELF) (see details in chapter 2.4), but many of them still remain elusive.

Despite of their key role in reproduction, the adult hypothalamus has very limited number of GnRH neurons, less than 2000 (Quinton et al. 2003). Studies on rodents suggest that original number of GnRH neurons is much bigger and that during migration process there is also an inherent selection process which pares down the number (Wu et al. 1997).

2.3 Congenital hypogonadotropic hypogonadism (HH)

Congenital HH is a disorder characterized by absent or incomplete sexual maturation by the age of 18 years, in conjunction with low levels of circulating gonadotropins and sex steroids

and no other abnormalities of the HPG axis. It is caused by defects in GnRH neuron development or in GnRH secretion/action (Seminara et al. 1998, Young 2012).

2.3.1 Clinical features and variable reproductive phenotype

Congenital HH is classically defined as 1) absent or incomplete puberty by age 18 years; 2) low serum testosterone level in men or estradiol level in women in association with low or normal levels of serum gonadotropins; 3) otherwise normal pituitary function; 4) normal serum ferritin concentrations; and 5) normal magnetic resonance imaging (MRI) of the hypothalamic-pituitary region (Pitteloud et al. 2007b, Raivio et al. 2009). Congenital HH is suspected usually on the basis of clinical signs of hypogonadism in infancy, such as micropenis and/or cryptorchidism, or failure to undergo pubertal development (Seminara et al. 1998, Young 2012). In a large series of adolescents with delayed pubertal development assessed in a tertiary center, congenital HH was an underlying cause in ~3% of cases, and the condition appears to be 4-5 times more frequent in men (Seminara et al. 1998, Sedlmeyer & Palmert 2002).

The reproductive phenotype of the patients with congenital HH can vary from severe to milder forms (Figure 4) (Dode et al. 2003, Pitteloud et al. 2006a, Raivio et al. 2009). In severe cases, failure of HPG axis activation during fetal life and in early childhood may manifest as bi- or unilateral cryptorchidism in association with micropenis (Lee et al. 1980). Approximately two-thirds of HH male patients are cryptorchid and one-third has a micropenis at birth (Quinton et al. 2001, Pitteloud et al. 2002a). Approximately 70% of male patients with congenital HH have no pubertal development (Pitteloud et al. 2002a), whereas up to 50% of the female congenital HH patients have spontaneous thelarche and 10% have experienced 1-2 menses (Shaw et al. 2011). In mild forms of congenital HH, some irregular and / or infrequent LH pulsatility may be detected that may be sufficient to activate the HPG axis to achieve partial pubertal development (Seminara et al. 1998, Pitteloud et al. 2002a, Shaw et al. 2011). Differentiating congenital HH from the more common case of delayed puberty, CDPG, is complicated and requires evaluation in a pediatric endocrinology unit (see chapter 2.5) (Palmert & Dunkel 2012).

Previously, congenital HH was thought to require lifelong hormone therapy, but in recent years it has been shown that up to 10% of congenital HH patients may undergo reversal of

HH later in life (Raivio et al. 2007, Laitinen et al. 2012). These reversal variants undergo spontaneous pubertal development commonly in early adulthood, and some may even attain normal fertility. No single phenotypic or genotypic feature predicts the reversal of HH at the time of diagnosis, but spontaneous testicular growth during androgen therapy is highly indicative (Laitinen et al. 2012). Reasons leading to recovery are elusive, but testosterone exposure has been suggested to predispose to recovery of the HPG axis (Raivio et al. 2007, Morelli et al. 2009).

After normal pubertal development, central activity of the HPG axis may decline, leading to adult-onset HH. These patients frequently have sexual dysfunction, decreased libido and infertility, and low levels of circulating gonadotropins and sex steroids (Nachtigall et al. 1997). On rare occasions, partial HPG axis activation in men produces sufficient amount of LH and FSH to result in testicular maturation and growth. However, the activation is inadequate to induce virilization. These patients, also termed fertile eunuchs, lack secondary sexual characteristics and have a eunuchoidal body habitus (Williams *et al.* 1975)

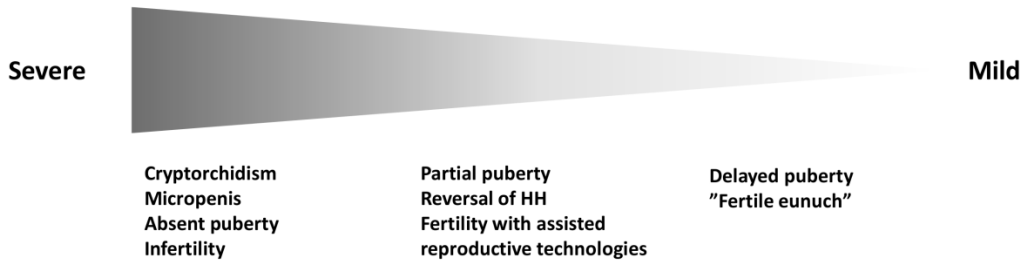

Figure 4. Reproductive phenotype is highly variable in patients with congenital hypogonadotropic hypogonadism (HH). Modified Brioude et al. 2010 and Laitinen 2012.

2.3.2 Associated nonreproductive features

Patients with congenital HH occasionally display additional nonreproductive phenotypic features listed in Table 1. These features may be suggestive of certain genetic etiologies (Costa-Barbosa et al. 2013, see also chapter 2.4).

Table 1. Associated features in congenital hypogogonadotropic hypogonadism

Associated feature	Reference(s)
Midline defects	
-Cleft lip/palate	Dode et al. 2003
-High-arched palate	Dode et al. 2003, Falardeau et al. 2008
-Dental agenesis	Dode et al. 2003, Falardeau et al. 2008
Unilateral renal agenesis	Wegenke et al. 1975, Georgopoulos et al. 2007
Hearing impairment	Dode et al. 2003
Limb and digit anomalies	Dode et al. 2003
External ear anomalies	Zenaty et al. 2006
Semicircular canal dysplasia	Jongmans et al. 2009
Coloboma of the eye	Kim et al. 2008, Jongmans et al. 2009
Mirror movements	Kallmann et al. 1944

2.3.3 Olfaction defects and Kallmann syndrome (KS)

Congenital HH has been traditionally divided into two entities according to olfactory phenotype; congenital HH and normal sense of smell is termed normosmic HH (nHH) whereas congenital HH with defective sense of smell is termed Kallmann syndrome (Kallman et al 1944, Schwanzel-Fukuda et al. 1989). About 60% of patients with congenital HH present with anosmia (Bianco & Kaiser 2009, Lewkowitz-Shpuntoff et al. 2012).

KS is suggested to result from abnormal guidance of olfactory axons, which further leads to disturbed migration of GnRH neurons from the OP to the hypothalamus. The evidence for this association comes from postmortem findings of a 19-week-old male fetus carrying a chromosomal deletion at Xp22.3 including *KAL1* and affected by KS. This fetus had no OBs, and there was a complete absence of GnRH neurons in the brain, whereas clusters of these cells were found in the nasal region and on the dorsal surface of cribriform plate, together with entangled fibers of the olfactory and terminal nerves that did not contact the forebrain (Schwanzel-Fukuda et al. 1989) (Figure 5). In contrast, nHH results from defects in GnRH secretion or action, although the more current view is that anosmia/normosmia and HH form a phenotypic continuum and can also result from the same genetic defect (Cadman et al. 2007).

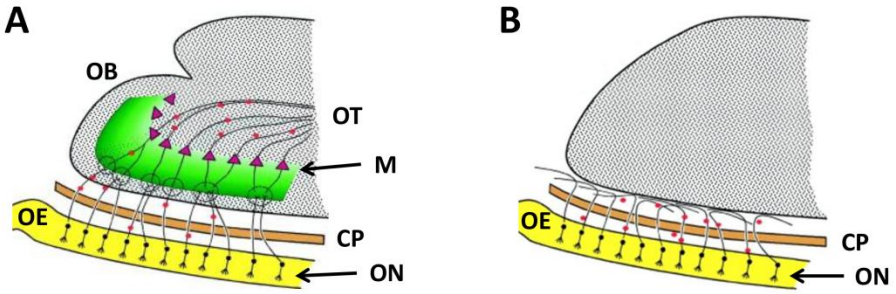

Figure 5. Model for Kallmann syndrome (KS) pathogenesis. In normal individuals (A), the olfactory neurons (ON) in the olfactory epithelium (OE) send their axons through the cribriform plate (CP) to reach the olfactory bulb (OB), where they make synapses with dendrites of mitral cells (M, purple triangles) whose axons will form the olfactory tract (OT). Gonadotropin-releasing hormone neurons (red dots) migrate along the olfactory nerves until they reach the forebrain. In an X-linked KS-affected human fetus (B), the olfactory axons were not targeted to the OB, ending their migration between the CP and the forebrain. In addition, OB morphogenesis was incomplete (Schwanzel-Fukuda et al. 1989, figure modified from Rugarli 1999).

Like the reproductive phenotype, the olfaction phenotype can also vary from absent sense of smell (anosmia) to decreased sense of smell (hyposmia) in KS patients and their affected family members. In addition, anosmia may be present as an isolated symptom without HH in KS-families (Dode et al. 2003, Pitteloud et al. 2006b). Although the contact between olfactory axons and the developing OB is essential for OB morphogenesis (Cadman et al. 2007), not all KS patients with olfactory defects have dysmorphic or absent OBs (Quinton et al. 1996, Sato et al. 2004).

2.3.4 Overlapping syndromes

Variable reproductive phenotype and wide range of additional features seen in congenital HH complicates the diagnosis of the condition. Although congenital HH together with defective sense of smell is highly indicative of KS, HH with or without anosmia can also be part of rare syndromes.

Congenital hypopituitarism is a condition with a wide variation in severity and with many underlying causes. It may manifest as an isolated deficiency of a single pituitary hormone (such as gonadotropin deficiency, leading to HH), or several pituitary hormone axes may be

defective, resulting in combined pituitary hormone deficiency (Kelberman & Dattani 2009). Mutations in genes that are involved in early development and patterning of the forebrain and pituitary (such as *HESX1, PITX2, SOX2, SOX3, OTX2, LHX3* and *LHX4*) or are involved in initial stages of pituitary cell differentiation (*PROP1* and *POU1F1*) may cause hypopituitarism (Kelberman & Dattani 2009, Pfaffle & Klammt 2011).

HH may also be present in the **Bardet Biedl** and **Prader-Willi syndromes** (Aminzadeh et al. 2010), or in the rare **Johnson neuroectodermal syndrome** (Johnson et al. 1983) or in the **Dandy-Walker brain malformation** (Aluclu et al. 2007). Mutations in *LEP* and *LEPR*, encoding leptin and its receptor, cause defective leptin signaling, which leads to **obesity and HH** due to abnormal appetite control and insuffient GnRH secretion (Farooqi et al. 2007). Mutations in *DAX1* cause abnormal development of adrenal cortex, hypothalamus, pituitary, and gonads leading to **combined adrenal hypoplasia congenita and HH** (Muscatelli et al. 1994).

In this thesis, CHARGE syndrome, Waardenburg syndrome, holoprosencephaly and septo-optic dysplasia are studied with more detail, as they all have clear phenotypic and genotypic overlap with congenital HH.

2.3.4.1 CHARGE syndrome

CHARGE syndrome is a clinically variable, multiple congenital anomaly condition occurring in 1 / 10 000 live births. CHARGE is characterized by ocular coloboma, heart defects, atresia of the choanae, retarded growth and development, genital abnormalities, and ear anomalies including deafness and vestibular disorders. Patient with CHARGE may also be anosmic and have congenital HH. In addition, OB hypoplasia is highly penetrant in CHARGE individuals (Pago et al. 1981, Pinto et al. 2005, Sanlaville & Verloes 2007). Patients with KS may also display some CHARGE features (Kim et al. 2008, Jongmans et al. 2009, Bergman et al. 2012). Heterozygous mutations in *CHD7*, which encodes a chromatin remodeling enzyme account for over 70% of CHARGE syndrome cases. As patients with KS may also have heterozygous mutations in *CHD7* (see chapter 2.4.1), KS can be considered as a milder allelic variant of CHARGE syndrome (Kim et al. 2008, Jongmans et al. 2009, Bergman et al. 2012).

2.3.4.2 Waardenburg syndrome

Waardenburg syndrome (WS) is characterized by the association of pigmentation abnormalities and sensorineural hearing loss, that result from an abnormal proliferation, survival, migration, or differentiation of NC-derived melanocytes (Waardenburg 1951, Read & Newton 1997). Pigmentation defects include depigmented patches of the skin and hair, vivid blue eyes or heterochromia irides. WS has been classified into 4 main types according to other features such as dystopia canthorum (lateral displacement of the inner corners of the eyes, present in WS1, not present in WS2), musculoskeletal abnormalities of the limbs (WS3), and Hirschsprung disease (WS4), found in subsets of patients (Read & Newton 1997, Pingault et al. 2010). These main types are yet divided into subtypes according to the underlying genetic cause. Mutations in transcription factor SOX10, which is involved in the development and differentiation of the NC, cause WS type 4C and WS type 2E (Pingault et al. 1998, Bondurand et al 2007) and also their neurological variant PCWH (peripheral demyelinating neuropathy, central dysmyelination, Waardenburg syndrome, and Hirschsprung disease) (Inoue et al. 2004). Recently, a high frequency of OB agenesis was found among patients with WS, and *SOX10* mutations were identified in KS patients with deafness (see 2.4.1) (Pingault et al. 2013). Studies with Sox10-null mutant mice suggested that KS in the patients resulted from defects in olfactory ensheating cell differentiation (Pingault et al. 2013, Barraud et al. 2013).

2.3.4.3 Holoprosencephaly and septo-optic dysplasia

Holoprosencephaly (HPE) is the most common malformation of the brain and face in humans, and it represents a contiguous clinical spectrum of disorders ranging from simple microform features such as closely spaced eyes to the extreme of single cyclopic eye (Dubourg et al. 2007). HPE results from varying degrees of incomplete cleavage of the prosencephalon into the cerebral hemispheres and ventricles that occurs between the 18th and the 28th day of gestation (Demyer & Zeman 1963, Cohen et al. 2006, Dubourg et al. 2007). Midline defects are frequently observed in HPE, including cyclopia, proboscis (elongated nose), median or bilateral cleft lip/palate in severe forms, ocular hypotelorism or solitary median maxillary central incisor in minor forms (Roessler et al. 2008, Dubourg et al. 2007). The less severe end of phenotypic spectrum includes absent olfactoty tracts and bulbs. Patients with HPE often have also endocrine disorders like diabetes insipidus, HH, adrenal hypoplasia, thyroid

hypoplasia, and GH deficiency because the midline malformation affects the development of the hypothalamus and the pituitary gland (Dubourg et al. 2007). HPE occurs approximately in 1 / 10 000 - 15 000 livebirths, but in 1 / 250 during early embryogenesis, since most affected fetuses are miscarried. Besides environmental causes, such as maternal diabetes, an abnormality of chromosome number is overall the most frequently identified etiology in HPE (up to 50% of cases). Out of these, trisomy 13 is the most common, but trisomy 18, triploidy, and small deletions and duplications have been reported (Goetzinger et al. 2008). HPE may also be a part of a recognizable syndrome, suc as Smith-Lemli-Opitz syndrome (Kelley et al 1996). In addition, autosomal dominant or *de novo* mutations in many genes (including *SHH, SIX3, TGIF1, TDGF1, FOXH1* and *GLI2*) have been shown to cause HPE, but a molecular genetic diagnosis is attained in only approximately 30% of the patients (Dubourg 2007). KS and HPE have been suggested as allelic syndromes, as mutations in *FGF8* have been identified in both (Arauz et al. 2010, McCabe et al. 2011).

Septo-optic dysplasia (SOD) is a highly heterogeneous condition with variable phenotypes including midline and forebrain abnormalities, and optic nerve and pituitary hypoplasia (Dattani et al. 1998). Most instances of SOD are sporadic, and several etiologies, including alcohol abuse of the mother during pregnancy, have been suggested to account for the pathogenesis of the condition. However, an increasing number of familial cases have been described, with mutations identified in transcription factors that are essential for normal forebrain development (Webb & Dattani 2010, McCabe et al. 2011). One of such factor is SOX2, which has a key role both in eye and pituitary development (Kelberman et al. 2006). *SOX2* mutations have also been identified in patients with congenital HH (Stark et al. 2011) (see chapter 2.4.4). In addition, mutations in known KS genes *FGFR1* and *FGF8* have been found in patients with SOD (Webb & Dattani 2010, McCabe et al. 2011, Raivio et al 2012).

In addition, it has also been shown that Sox2 and Chd7 physically interact and regulate a set of common target genes that are mutated in human syndromes (Engelen et al. 2011). These target genes include for example *Gli3*, in which mutations cause several diseases, such as Pallister-Hall syndrome (PHS). PHS is a rare disorder characterized by benign tumors or lesion of the hypothalamus, polydactyly, laryngeal anomalies, and various visceral and genital anomalies (Kang et al. 1997). Gli3 also mediates SHH signaling and has been suggested to act as a modifier in Sox10-dependent melanocyte defects (Matera et al. 2008)

2.4 Molecular genetics of congenital HH

Genetics of congenital HH is complex and heterogenous. Although several genes have been implicated in the etiology of congenital HH, still about 65% of the cases remain without identified genetic cause (Mitchell et al. 2011). Mutations in genes that are important for GnRH neuron development and migration cause congenital HH that is usually, but not always, accompanied with defects in sense of smell (KS). Mutations in genes that regulate GnRH secretion from hypothalamic neurons or its action at the pituitary level cause nHH (Figure 6). Summarizing table of genetics of congenital HH is presented at the end of this chapter (Table 2).

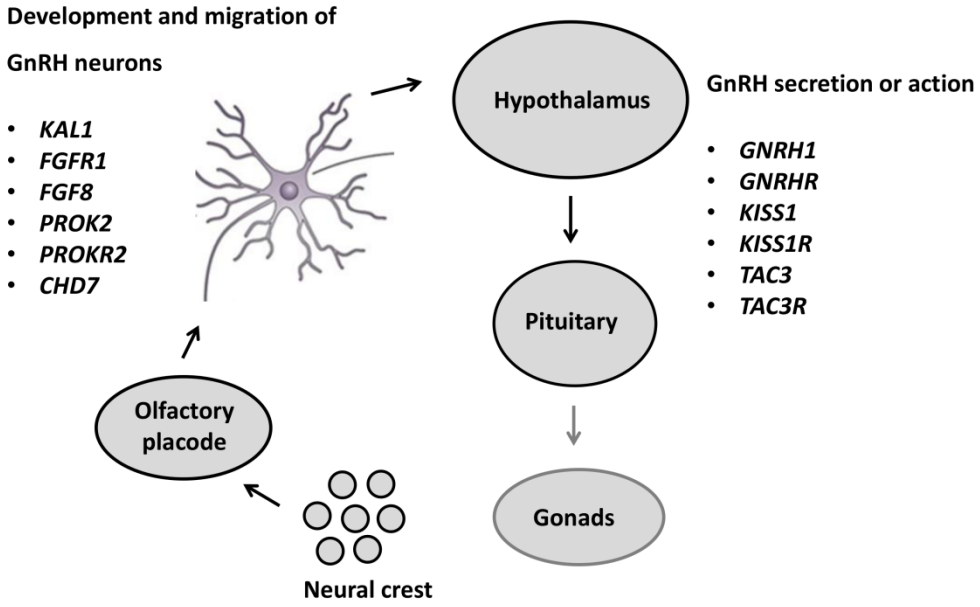

Figure 6. The genetic basis of congenital hypogonadotropic hypogonadism (HH). Mutations in genes that are important for Gonadotropin-releasing hormone (GnRH) neuron development and migration cause congenital HH that is usually, but not always, accompanied with defects in sense of smell (Kallmann syndrome). Mutations in genes that regulate GnRH secretion or action cause normosmic congenital HH (Bianco & Kaiser 2009).

2.4.1 Genes underlying KS

KAL1 **(HGNC ID: 6211)** located on chromosome Xp22.3, encodes anosmin-1. Anosmin-1 is an extracellularly expressed protein, which needs heparan sulphate proteoglycans (HSPGs) in

order to bind to adjacent cell surfaces (Franco et al. 1991, Legouis et al. 1991, Soussi-Yanicostas et al. 2002). Anosmin-1 is expressed during development in the presumptive OB region from embryonic week 5 onwards, and is thought to be important for initial OB differentiation and innervation of early olfactory axons. Anosmin-1 is also expressed in developing kidneys (Hardelin et al. 1999). The central role of anosmin-1 in GnRH neuron migration has been demonstrated by histopathological observations in two fetuses (one with *KAL1* deletion and one with *KAL1* nonsense mutation), both of which showed accumulation of GnRH neurons in the nasal region, and lack of them in the hypothalamic regions (chapter 2.3.3, Figure 5) (Schwanzel-Fukuda et al. 1989, Teixeira et al. 2010). Abnormal development of OB and interrupted connection of olfactory axons and terminal nerve fibres within the OB were also documented, suggesting that anosmin-1 influences the later migration route when GnRH neurons penetrate into the forebrain. This was supported by later studies in a zebrafish, showing that anosmin-1 directs terminal targeting of olfactory sensory neuron (OSN) axons within OB, and mediates OB mitral cell axons towards the olfactory cortex (Soussi-Yanicostas et al 2002, Yanicostas et al. 2009, Hu & Bouloux 2011). In addition, anosmin-1 has shown to be essential for cranial NC formation in chick embryos, where it modulates fibroblast growth factor (FGF), bone morphogenenic protein (BMP), and Wingless/INT-related signalling (Endo et al. 2012).

KAL1 was the first gene implicated in KS when two independent groups in 1991 identified point mutations or intragenic deletions in patients with KS (Franco et al. 1991, Legouis et al. 1991). Since then, nearly sixty mutations have been identified, most of which are deletion, frameshift or nonsense mutations (Kim et al. 2008, Sykiotis et al. 2010). To date, *KAL1* is the only gene implicated in X-linked recessive KS. KS patients with mutations in *KAL1* have usually severe HH and they display less variable phenotypes than patients with other identified molecular genetic causes (Sato et al. 2004, Salenave et al. 2008). Patients with *KAL1* mutations are typically anosmic due to abnormal OB development and OSN axon guidance defect (Schwanzel-Fukuda et al. 1989). Synkinesia (mirror movements) and unilateral renal agenesis are often encountered in patients with *KAL1* mutations (Quinton et al. 2001, Albuisson et al. 2005). Mutations in *KAL1* account for approximately 5-10% of all KS cases, and approximately 3-6% of all congenital HH cases (Albuisson et al. 2005, Pedersen-White et al. 2008, Bianco & Kaiser 2009).

FGF8 **(HGNC ID: 3686) and** ***FGFR1*** **(HGNC ID: 3688)**, located on chromosomes 10q24.32 and 8p11.23-p11.22, respectively, encode FGF8 and its receptor, FGFR1. The FGF signaling system is essential for the regulation of cell growth, tissue differentiation, and organogenesis during development (Cotton et al. 2008). Mouse studies have shown that both Fgf8 and Fgfr1 have important roles in GnRH neuron development, especially in promoting the formation and maintaining the survival of these neurons in the OP. In homozygous *Fgf8* hypomorphic mice GnRH neurons never emerge from the OP and are absent in every subsequent developmental stage (Chung et al. 2008), and heterozygous *Fgf8* and *Fgfr1* hypomorphs have reduced number of GnRH neurons in the pre-optic area and median eminence (Meyers et al. 1998, Falardeau et al. 2008, Chung et al. 2008). Targeted expression of dominant negative *Fgfr1* in mouse GnRH neurons resulted in 30% reduction in the neuron population and also in the projection of their axons to the median eminence, and these mice displayed delayed puberty and decreased fertility (Tsai et al. 2005, Gill & Tsai 2006). Fgf8 – Fgfr1 signaling is also required for OB morphogenesis, as both *Fgf8* and *Fgfr1* hypomorphs lack normal OBs (Hebert et al. 2003). FGFR1 signaling activation requires binding of FGFs and co-receptor HSPGs. Binding is regulated by the N-glykosylation pattern of the receptor (Duchesne et al. 2006), and it induces receptor dimerization, followed by the response of intracellular signal transduction cascade via three typical downstream pathways: classic mitogen-activated protein kinase (MAPK) p42/44, phosphatidylinositide 3-kinase and phospholipase C. Of note, anosmin-1 may facilitate FGFR1 signaling and development of GnRH neurons by interacting with the FGFR1/FGF complex via HSPGs, or by binding directly to FGFR1 with high affinity (Hu & Bouloux 2011, Hu et al. 2013).

Mutations in *FGFR1* and *FGF8* cause autosomal dominant congenital HH. The first mutations in *FGFR1* were described in KS patients in 2003 (Dode et al. 2003). To date, heterozygous *FGFR1* mutations underlie approximately 10% of KS cases and 6-8% of all congenital HH cases (Dode & Hardelin 2009, Bianco & Kaiser 2009). Phenotypic variability is typical for patients with an *FGFR1* mutation, even within families carrying the same mutation. The reproductive phenotype may vary from an unaffected mutation carrier via delayed puberty via reversal of HH to severe HH, and the olfaction may vary from normal sense of smell via hyposmia to anosmia (Dode et al. 2003, Pitteloud et al. 2005, Pitteloud et al. 2006a, Trarbach et al. 2006, Xu et al. 2007, Salenave et al. 2008, Raivio et al. 2009). Cleft lip/palate, dental agenesis, external ear anomalies, and limb anomalies are seen in *FGFR1* mutation carriers, consistent with several functions and wide expression pattern of the

receptor (Dode et al. 2003, Albuisson et al. 2005, Kim et al. 2005, Zenaty et al. 2006, Dode et al. 2007).

Mutations in *FGF8* are rare; they account for less than 5% of KS cases and less than 2% of all congenital HH cases (Bianco & Kaiser 2009). The first heterozygous mutations in *FGF8* were reported in 2008 in congenital HH patients with variable olfaction (Falardeau et al. 2008). Reproductive phenotype of patients with an *FGF8* mutation is variable, and associated features include cleft lip/palate and hearing impairment (Falardeau et al. 2008, Trarbach et al. 2010).

PROK2 **(HGNC ID: 18455) and** ***PROKR2*** **(HGNC ID: 15836)** are located at chromosome 3p13 and 20p12.3, respectively, and encode prokineticin-2 and its G-protein coupled receptor prokineticin receptor-2. Prokineticin signalling influences several physiological events in the central nervous system and peripheral tissues, including intestinal contraction, spermatogenesis, neuronal survival, and circadian rhythm (Ngan & Tam 2008). Prok2 is expressed in the developing OB where it serves as a chemoattractant for the migrating OB neuron progenitors, and both Prok2 and Prokr2 are critical for the establishment of a normal OB architecture (Ng et al. 2005, Matsumoto et al. 2006, Pitteloud et al. 2007b). *Prok2-/-* and *Prokr2-/-* mice show similarity to KS phenotype: *Prok2-/-* mice have sexual immaturity/infertility, severely decreased number of GnRH neurons as the migration is arrested after crossing the cribiform plate, and 50% of mice display asymmetric OBs (Ng et al. 2005, Pitteloud et al. 2007b). *Prokr2-/-* mice show severe atrophy of the reproductive system, absence of GnRH neurons in the hypothalamus, and OB malformation (Matsumoto et al. 2006).

First homozygous and compound heterozygous mutations in *PROKR2* were discovered from KS patients in 2006 (Dode et al. 2006). A year later, a homozygous *PROK2* deletion was found in siblings with KS/nHH (Pitteloud et al. 2007b). Since then, biallelic mutations in *PROK2* and *PROKR2* have been reported in congenital HH patients with severe HH and various olfactory phenotypes (Abreu et al. 2008, Cole et al. 2008, Leroy et al. 2008, Sarfati et al. 2010), accounting together approximately 5-10% of KS cases and 3-6% of all congenital HH cases (Bianco & Kaiser 2009). Heterozygous *PROK2* or *PROKR2* mutations have also been suggested to contribute to congenital HH phenotype either alone or in combination with mutations in other genes (Dode et al. 2006, Cole et al. 2008, Canto et al. 2009, Sarfati et al.

2010). However, the presence of heterozygous *PROK2* and *PROKR2* mutations in healthy carriers (Dode et al. 2006, Pitteloud et al, 2007b, Abreu et al. 2008, Sinisi et al. 2008, Leroy et al. 2008), lack of dominant-negative effect of these mutations in *in vitro* functional studies (Monnier et al. 2009) and normal phenotype of heterozygous knock-out mice (Matsumoto et all. 2006, Pitteloud et al. 2007b), support the recessive nature of *PROK2* and *PROKR2* mutations. Also, convincing evidence of digenetic inheritance in congenital HH is lacking (see chapter 2.4.5) and there are no reports of patients carrying heterozygous mutations in both *PROK2* and *PROKR2*.

CHD7 **(HGNC ID: 20626)** located on chromosome 8q12 encodes a choromodomain helicase deoxyribonucleic (DNA)-binding protein-7. CHD7 functions in the nucleus where it regulates access to chromatin by hydrolyzing adenosine triphosphate and altering nucleosome structure (Marfella & Imbalzano 2007), and it may also affect DNA binding, cell cycle regulation and apoptosis (Zentner et al. 2010). Chd7 is expressed in the developing nervous system and its derivates. Particularly high expression levels are seen in mice OP during GnRH neuron development, and at later stage in the OE, cochlea, anterior pituitary, and spinal cord (Kim et al. 2008). In these tissues, Chd7 promotes neurogenesis by regulating expression of *Fgfr1, Bmp4* and *Otx2* (Layman et al. 2011). Mice with heterozygous loss of *Chd7* have genital and heart anomalies, hypoplastic semicircular canals, olfactory defects (Bossman et al. 2005, Hurd et al. 2007), delayed puberty and reduced number of GnRH neurons in the hypothalamus (Layman et al. 2011). In addition, studies with *Xenopus* and human NC-like cells showed that Chd7 is essential for the formation of NC, regulation of NC gene expression, and cell migration (Bajpai et al. 2010).

A mutation in *CHD7* is found in 70% of patients with the CHARGE syndrome (chapter 2.3.4.1) (Vissers et al. 2004, Zentner et al. 2010). First *CHD7* mutations in patients with congenital HH were found in 2008 when Kim et al. hypothesized that congenital HH might be a mild allelic variant of CHARGE syndrome, as patients with CHARGE may also have HH and olfaction defects (Pinto et al. 2005, Ogata et al. 2006). Kim et al. found a *CHD7* mutation in 6% of congenital HH patients with variable olfaction defects (Kim et al. 2008) and a year later Jongmans et al. described 3 KS patients with CHARGE associated features and a *CHD7* mutation (Jongmans et al. 2009). To date, heterozygous mutations in *CHD7* account less than 5% of all congenital HH cases, and most mutations are *de novo* (Kim & Layman 2010).

2.4.2 Genes underlying normosmic congenital HH

***GNRH1* (HGNC ID: 4419) and *GNRHR* (HGNC ID: 4421)** are located on chromosomes 8p21.2 and 4q13.2, respectively, and encode GnRH and its receptor. GnRH is a decapeptide that is synthetized from 92-amino acid preprohormone. Hypothalamic GnRH neurons secrete GnRH in pulsatile fashion to the portal circulation, which carries it to the gonadotropic cells of anterior pituitary that express GnRH receptor. Binding of GnRH to this G-coupled receptor leads to activation of phospholipase C, production of second messenger inositol trisphosphate and rise of intracellular calcium, which leads to subsequent release of both FSH and LH (de Roux et al. 2006).

Homozygous or compound heterozygous mutations in *GNRHR* cause autosomal recessive normosmic HH (de Roux et al. 1997, Layman et al. 1998). First cases were described in 1997, and to date over 20 different mutations have been described (de Roux et al. 1997, Tello et al. 2012). Most *GNRHR* mutations are point mutations and have been shown to cause impaired cell surface expression of the receptor, ligand binding or intracellular signaling in *in vitro* functional studies (Tello et al. 2012). Reproductive phenotype of nHH patients with biallelic *GNRHR* mutations varies from severe HH via partial puberty to delayed puberty, whereas heterozygous carriers are unaffected (de Roux et al. 1997, Layman et al. 1998, Kottler et al. 2000, Beranova et al. 2001, Lin et al. 2006). In addition, *GNRHR* mutations have been reported among patients with reversal of HH (Pitteloud et al. 2001, Dewailly et al. 2002, Raivio et al. 2007, Lin et al. 2006, Laitinen et al. 2011), and it has been suggested that HH patients with a R262Q mutation in *GNRHR* in a compound heterozygous or homozygous state are especially prone to reversal of HH (Laitinen et al. 2011). *GNRHR* mutations are estimated to cause 16% - 40% of nHH (Beranova et al. 2001, Bhagavath et al. 2005).

Mutations in *GNRH1* are extremely rare cause of nHH. The first, and so far only *GNRH1* mutations were described in 2009, when two independent groups found homozygous *GNRH1* mutations in one sib-pair and in a male patient with nHH (Bouligand et al. 2009, Chan et al. 2009). Thereafter, no patients with *GNRH1* mutations have been reported.

***KISS1* (HGNC ID: 6341) and *KISS1R* (HGNC ID: 4510)** are located on 1q32.1 and 19p13.3, respectively. *KISS1* encodes a 145 amino acid long pre-protein, which is cleaved to shorter (54, 14, 13 and 10 amino acids) kisspeptins (Kotani et al. 2001). The 54 amino acid

kisspeptin and its G-protein coupled receptor KISS1R (previously known as GPR54) are expressed in the hypothalamus and the role of kisspeptin signaling in regulation of GnRH expression was described in chapter 2.1.3. Both *Kiss1r-/-* and *Kiss1-/-* mice display HH phenotype, although the phenotype of latter is more variable (Seminara et al. 2003, Lapatto et al. 2007).

Mutations in *KISS1* and in *KISS1R* cause autosomal recessive nHH (Seminara et al.2003, de Roux et al. 2003, Topaloglu et al. 2012). First homozygous loss-of-function mutations in *KISS1R* in nHH were described in 2003 by two independent groups. In both cases, mutations were found in large consanguineous families (de Roux et al. 2003, Seminara et al. 2003) Patients with homozygous or compound heterozygous mutations in *KISS1R* have severe HH with little phenotypic variability (de Roux et al. 2003, Seminara et al. 2003, Lanfranco et al. 2005, Teles et al. 2010, Nimri et al. 2011). Mutations that impair kisspeptin signaling are a rare cause of nHH: *KISS1R* mutations account for less than 5% of nHH cases (Bianco & Kaiser 2009), and thus far there has been only one report of *KISS1* mutations causing nHH in a consanguineous family (Topaloglu et al. 2012).

***TAC3* (HGNC ID: 11521) and *TACR3* (HGNC ID: 11528).** *TAC3* is located on chromosome 12q13.3 and it encodes preprotachykinin B which is modified into NKB. NKB signals most efficiently through tachykinin receptor-3 (TACR3), a G-protein coupled receptor encoded by *TACR3* located in 4q24 (Patacchini & Maggi 1995). NKB and TACR3 are expressed in the KNDy-neurons in the hypothalamus where they are suggested to participate in the autoregulation of KNDy-neurons (chapter 2.1.3) (Navarro & Tena-Sempere 2012).

First homozygous mutations in *TAC3* and *TACR3* causing nHH were found in four Turkish consanguineous families in 2009 (Topaloglu et al. 2009). Since then, more homozygous or compound heterozygous mutations have been reported (Guran et al. 2009, Young et al. 2010, Gianetti et al. 2010, Francou et al. 2011) and they account for approximately 5% of nHH cases (Francou et al. 2011). Patients with *TAC3* or *TACR3* mutations have severe HH (Topaloglu et al. 2009, Young et al. 2010, Francou et al. 2011), although few patients have reportedly undergone a spontaneous recovery of the HPG axis (Gianetti et al. 2010). Unaffected heterozygous carriers of *TAC3* or *TACR3* mutations support autosomal recessive mode of inheritance (Topaloglu et al. 2009, Guran et al. 2010, Young et al. 2010, Francou et al. 2011), although heterozygous mutations have been reported in patients with nHH.

2.4.3 Recent advances in molecular genetics of KS

In spring 2013, Pingault et al. described a high prevalence of *SOX10* (HGNC ID: 11190) mutations in patients with KS and deafness (Pingault et al. 2013). Mutations in *SOX10* are known to cause WS (specifically types WS4C, its neurological variant PCWH, and WS2E) which is a rare developmental disorder characterized by pigmentation abnormalities and deafness (chapter 2.3.4.2) (Pingault et al. 1998, Inoue et al. 2004, Bondurand et al. 2007). Involvement of *SOX10* mutations also in KS was suspected when MRI revealed a high frequency of OB agenesis among patients with WS. Screening of *SOX10* from altogether 103 KS patients revealed 7 novel loss-of-function mutations. Five mutations were found among 17 KS patients with one or more WS associated features, and two in 86 KS patients without any WS features. Notably, six of these seven mutation carriers had a hearing impairment. As in WS, the mutations were found in the heterozygous state together with a dominant mode of inheritance (Pingault et al. 2013).

SOX10 is a transcription factor involved in the development and differentiation of the NC by regulating several transcriptional targets (Wegner 2005). It is important for the development of melanocytes, enteric ganglia neurons, Schwann cells and oligodendrocytes and for the formation of the structures of inner ear (Britch et al. 2001, Breuskin et al 2009). Studies with *Sox10-/-* mice revealed that Sox10 is also important for the formation of olfactory ensheating cells that arise from NC and support the growth and targeting of olfactory axons (Pingault et al. 2013, Forni et al. 2011). *Sox10-/-* mice lack these ensheating cells, have misrouted olfactory nerve fibers, impaired migration of GnRH neuron, and disorganization of the olfactory nerve layer of the OB, consistent with the KS phenotype (Pingault et al. 2013).

2.4.4 Other genes associated with congenital HH

In addition to genes listed above, there are a number of other genes in which mutations/variants have been reported in patients with congenital HH. Mutations in these genes are either extremely rare, and/or their role in the pathophysiology of congenital HH is not yet confirmed.

WDR11 **(HGNC ID: 13831)** encodes WD repeat domain-11 protein, which is involved in olfactory neuron development**.** Kim et al. (2010) identified *WDR11* by positional cloning of a

translocation breakpoint in a KS patient, and later discovered heterozygous missense mutations in this gene in 3% of patients with KS or nIHH. Mutations were absent from controls and seemed to be pathogenic in *in vitro* studies (Kim et al. 2010). Thereafter, no mutations in *WDR11* have been reported, suggesting that *WDR11* mutations are an extremely rare cause of congenital HH.

SEMA3A **(HGNC ID: 10723)** encodes semaphoring 3A. Semaphorins are a class of secreted and membrane proteins that act as axonal growth cone molecules (Zhou et al. 2008). Sema3a is essential for patterning vomeronasal axons and in *Sema3a* mutant mice migration of GnRH neurons is arrested and OSN axons remain outside the central nervous system – a phenotype that resembles X-linked KS (Cariboni et al. 2011). Heterozygous mutations in *SEMA3A* have been recently reported in patients with KS (Young et al. 2012, Hanchate et al. 2012, Känsäkoski et al. 2014). As some of these mutations have also been reported in single nucleotide polymorphism (SNP) databases (such as exome variant server database), some have been found in healthy controls, and some have been found in patients with mutations in other HH-associated genes, it has been suggested that heterozygous mutations in *SEMA3A* are not sufficient to cause the disease phenotype alone, but may contribute to it (Hanchate et al. 2012, Känsäkoski et al. 2014). Further studies are needed to clarify the role of semaphorins in the etiology of KS.

NELF **(HGNC ID: 29843, approved name: NMDA receptor synaptonuclear signaling and neuronal migration factor)** encodes embryonic LHRH factor. This factor serves as common guidance molecule for olfactory axon projections and, either directly or indirectly, in the migration of GnRH neurons (Kramer & Wray 2000). Miura et al. (2004) identified a heterozygous missense variant in *NELF* in an nHH patient without previous family history. Xu et al. (2011) described an nHH patient with intronic *NELF* variants in a compound heterozygous state. In most cases *NELF* variants are encountered in congenital HH patients with mutation(s) in other HH-associated genes (Miura et al. 2004, Pitteloud et al. 2007a, Xu et al. 2011, Tornberg et al. 2011), and it has been suggested that *NELF* variants contribute to the disease phenotype by acting as modifiers (Sykiotis et al. 2010). The significance of *NELF* variants in congenital HH warrants further studies.

HS6ST1 **(HGNC ID: 5201)** encodes Heparan sulfate 6-O-sulfotranferase-1 which is an enzyme that modifies sugar residues of heparan sulfates. This enzyme is important for

example for the normal function of anosmin-1 and the FGFR1-FGF complex (Soussi-Yanicostas et al. 1998). Genetic experiments in *C. elegans* revealed that heparan-6O-sulfotransferase cell-specifically regulates neural branching *in vivo* in concert with kal-1 (Bülow wt al. 2002). Tornberg et al. (2011) identified rare variants in *HS6ST1* in 2% of congenital HH patients. Most of these patients carried also mutations in *FGFR1* and *NELF*. Tornberg et al. suggested that variants in *HS6ST1* may contribute to the disease phenotype although alone they are not sufficient to cause the phenotype. Role of *H6ST1* variants in congenital HH remains unconfirmed.

NDN **(HGNC ID: 7675)** encodes necdin, which plays a critical role in neuronal differentiation. Loss of necdin function has been reported to cause HH in Prader-Willi syndrome (Miller et al. 2009), and in mice necdin has been shown to regulate GnRH neuron development and migration, and *Gnrh1* gene expression (Muscatelli et al. 2000, Barker & Salehi 2002, Miller et al. 2009). Sequencing of *NDN* in 160 patients with congenital HH did not reveal conclusive mutations; only one rare variant was identified in a family with KS and *FGFR1* mutation (Beneduzzi et al. 2011)

FGF8 **synexpression group** consists of genes that are similarly expressed and regulated during development as FGF8, and which also modulate signaling efficiency of FGF8 through FGFR1 as enhancers or inhibitors (Fürthauer et al. 2002, Niehrs & Meinhardt 2002). Miraoui et al. (2013) selected five members of this group as candidate genes for congenital HH: *IL17RD* (HGNC ID: 17616), *DUSP6* (HGNC ID: 3072), *SPRY2* (HGNC ID: 11270), *SPRY4* (HGNC ID: 15533), and *FLRT3* (HGNC ID: 3762). These genes were sequenced from 386 patients with congenital HH and from 155 controls. *FGF17* (HGNC ID: 3673) and *FGF18* (HGNC ID: 3674) were also screened due to their high homology to *FGF8*. Altogether 27 different mutations in five genes (*FGF17, IL17RD, DUSP6, SPRY4, FLRT3*) were found in 30 unrelated patients but not in controls. Most mutations were heterozygous and some were accompanied by an *FGFR1* mutation. A bioinformatics method, interactome-based affiliation scoring, predicted *FGF17* and *IL17RD* as the two top candidates for congenital HH in the entire proteome, so mutations in these genes were further characterized *in vitro*. Authors concluded that mutations in genes encoding components of the FGF-pathway act primarily as contributors to the phenotype (Miraoui et al. 2013). Future studies are needed to elucidate the exact role of these genes in the etiology of congenital HH.

***SOX2* (HGNC ID: 11195)** is a member of the sex-determining region of the Y-chromosome related high-mobility group box (SOX) family of transcription factors. SOX2 regulates embryonic neurogenesis and is especially critical for eye development (Stevanovic et al. 1994, Fantes et al. 2003). Heterozygous mutations in *SOX2* are the most common single-gene cause of anophtalmia/microphtalmia (Fantes et al. 2003, Ragge et al. 2005), and cause also SOD (chapter 2.4.3.3), a heterogeneous condition with midline and/or forebrain abnormalities (Dattani et al. 1998, Webb & Dattani 2010). *SOX2* mutations have been reported in patients with HH, but in these cases HH occurred together with severe ocular anomalies (Kelberman et al. 2006, Kelberman et al. 2008). However, Stark et al. (2011) reported a family where children had anophthalmia/microphthalmia but their mother had congenital HH without ocular phenotype, and they all carried a frameshift mutation in *SOX2*. This has been thus far the only report of *SOX2* mutation in a patient with congenital HH without any associated features.

***HESX1* (HGNC ID: 4877)** encodes an embryonic transcription repressor important for organ commitment, cell differentiation, and proliferation. Hesx1 is essential for normal forebrain development, and homozygous mutations in *HESX1* cause SOD (Dattani et al. 1998). Heterozygous mutations have been reported in patients with isolated GH deficiency and in patients with combined pituitary hormone deficiency (McNay et al. 2007, Coya et al. 2007). Newbern et al. (2013) identified three heterozygous missense variants among 83 KS patients without any associated features or familial background of congenital HH, and suggested that KS could be a milder manifestation of heterozygous *HESX1* variants. Further studies are needed to elucidate the role of *HESX1* mutations in KS.

2.4.5 Inheritance of congenital HH

Approximately ~40% of patients with congenital HH have a positive family history of congenital HH or associated features; the remaining 60% appear as isolated cases (Seminara et al. 1998, Oliveira et al. 2001). KS may be inherited as an X-linked recessive trait, KS as well as nHH can also follow the autosomal dominant or autosomal recessive mode of inheritance (Table 2) (Waldstreicher et al. 1996, Oliveira et al. 2001). Variable expressivity and incomplete penetrance of the mutations is seen especially in patients and their relatives with *FGFR1* or *GNRHR* mutations (Dode et al. 2003, Pitteloud et al. 2005, Pitteloud et al.

2006a, Trarbach et al. 2006, Xu et al. 2007, Salenave et al. 2008, Raivio et al. 2009, de Roux et al. 1997, Layman et al. 1998, Kottler et al. 2000, Beranova et al. 2001, Lin et al. 2006).

Traditionally, congenital HH has been regarded as a monogenic disorder in which mutation(s) in a single gene leads to the phenotype. However, several reports suggesting the presence of di- or oligogenic inheritance in congenital HH have been published (Dode et al. 2006, Pitteloud et al. 2007a, Canto et al. 2008, Sykiotis et al. 2010, Sarfati et al. 2010, Quaynor et al. 2011, Miraoui et al. 2013). In most of these reports, patients harbor heterozygous mutations in genes that underlie recessive congenital HH (most commonly *PROKR2*, *GNRHR*, *TACR3*, *KISS1R*), or that still have an uncertain role in the disease pathogenesis (such as *NELF*, *HS6ST1*, *IL17RD*, *FLRT3*), in combination with an autosomal dominant mutation in *FGFR1* (Pitteloud et al. 2007a, Quaynor et al. 2011 Miraoui et al. 2013) or a hemizygous mutation in *KAL1* (Dode et al. 2006, Canto et al. 2008, Quaynor et al. 2011). As mutations in *FGFR1* or *KAL1* are sufficient to cause the disease phenotype alone, di- or oligogenic inheritance in these cases is questionable.

Sarfati et el. (2010) reported that male patients with biallelic mutations in *PROK2* or *PROKR2* have less variable and more severe reproductive phenotypes than patients with monoallelic mutations in these genes, and that nonreproductive/nonolfactory associated features appeared to be restricted to patients with monoallelic mutations. Authors suggested that this phenomenon is due to di- or oligogenic inheritance in patients with monoallelic mutations (Sarfati et al. 2010). However, it is also possible that these patients have causal mutations in genes not yet implicated in the etiology of congenital HH, and monoallelic mutation in *PROK2* or *PROKR2* does not contribute to the phenotype.

It appears that patients with congenital HH more often harbor rare protein-altering variants in genes associated with congenital HH than the healthy controls. Sykiotis et al. (2010) reported that 10 out of 397 (2.5%) patients had rare protein-altering variants in two or more different genes, whereas none of the controls (n=179) did. In study of Miraoui et al. (2013) 24 out of 350 (19%) patients carried at least two rare-protein altering variants in different genes whereas none of the controls (n=155) did. These differences between patients and controls were considered as possible evidence of oligogenicity. It should be noted that in both studies the number of patients far exceeded the number of controls.

Table 2. Genes in which mutations cause or are suggested to contribute to congenital hypogonadotropic hypogonadism.

Gene	Locus	HGNC ID	Product	Phenotype	Mode of inheritance	Reference(s)
KAL1	Xp22.3	6211	Anosmin-1	KS	XR	Franco et al. (1991); Legouis et al. (1991)
FGFR1	8p11.23	3688	Fibroblast growth factor receptor 1	KS, nHH	AD	Dodé et al. (2003)
FGF8	10q24.32	3686	Fibroblast growth factor 8	KS, nHH, HPE	AD	Falardeau et al. (2007)
PROK2	3p13	18455	Prokineticin 2	KS, nHH	AR	Dodé et al. (2006), Pitteloud et al. (2007)
PROKR2	20p12.3	15836	Prokineticin receptor 2	KS, nHH	AR	Dodé et al. (2006), Pitteloud et al. (2007)
CHD7	8q12	20626	Chromodomain helicase DNA binding protein 7	KS, nHH, CHARGE	AD	Kim et al. (2008); Jongmans et al. (2009)
GNRH1	8p21.2	4419	Gonadotropin releasing hormone 1	nHH	AR	Bouligand et al. (2009); Chan et al. (2009)
GNRHR	4q13.2	4421	Gonadotropin releasing hormone receptor	nHH	AR	de Roux et al. (1997); Layman et al. (1998)
KISS1	1q32.1	6341	Kisspeptin	nHH	AR	Topaloglu et al. (2012)
KISS1R	19p13.3	4510	Kisspeptin receptor	nHH	AR	de Roux et al. (2003); Seminara et al. (2003)
TAC3	12q13.3	11521	Neurokinin B	nHH	AR	Topaloglu et al. (2009)
TACR3	4q24	11528	Neurokinin B receptor	nHH	AR	Topaloglu et al. (2009)
SOX10	22q13.1	11190	SRY (sex determining region Y)-box 10	KS, WS	AD	Pingault et al. (2013)
WDR11	10q25.12	13831	WD repeat domain-11	KS, nHH	AD?	Kim et al. (2010)
SEMA3A	7q21.11	10723	Semaphorin 3A	contributes to the KS phenotype?	AD?	Young et al. (2012), Hanchate et al. (2012)
NELF	9q34.3	29843	Nasal embryonic LHRH factor	contributes to the HH phenotype?	NA	Miura et al. (2004), Xu et al. (2011)
HS6ST1	2q14.3	5201	Heparan sulfate 6-O-sulfotranferase 1	contributes to the HH phenotype?	NA	Tornberg et al. (2011)
NDN	15q11.2	7675	Necdin	NA	NA	Beneduzzi et al. (2011)
IL17RD	3p14.3	17616	Interleukin 17 receptor D	contributes to the HH phenotype?	NA	Miraoui et al. (2013)
DUSP6	12q21.33	3072	Dual specificity phosphatase 6	contributes to the HH phenotype?	NA	Miraoui et al. (2013)
SPRY2	13q31.1	11270	Sprouty* homolog 2 (Drosophila)	contributes to the HH phenotype?	NA	Miraoui et al. (2013)
SPRY4	5q31.3	15533	Sproyty* homolog 4, (Drosophila)	contributes to the HH phenotype?	NA	Miraoui et al. (2013)
FLRT3	20p12.1	3762	Fibronectin leucine rich transmembrane protein 3	contributes to the HH phenotype?	NA	Miraoui et al. (2013)
FGF17	8p21.3	3673	Fibroblast growth factor 17	contributes to the HH phenotype?	NA	Miraoui et al. (2013)
FGF18	5q35.1	3674	Fibroblast growth factor 18	contributes to the HH phenotype?	NA	Miraoui et al. (2013)
SOX2	3q26.33	11195	SRY (sex determining region Y)-box 2	HH?, eye anomalies, SOD	AD	Stark et al. (2011)
HESX1	3p14.3	4877	HESX homeobox 1	KS? SOD	NA	Newbern et al. (2013)

HGNC, HUGO gene nomenclature committee; XR, X-linked recessive; AD, autosomal dominant; AR, autosomal recessive; HH, congenital hypogonadotropic hypogonadism; KS, Kallmann syndrome; nHH, normosmic HH; CHARGE syndrome (coloboma, heart defects, choanal atresia, retarded growth and development, genital abnormalities, and ear anomalies); HPE, holoprosencephaly; WS, Waardenburg syndrome; SOD, septo-optic dysplasia; SRY, Sex-determining region of the Y-chromosome; LHRH, luteinising hormone-releasing hormone; NA, not assessed; TK, tyrosine-kinase; ES cells, embryonic stem cells

*Sproyty is an antagonist of FGF-signaling in Drosophila

2.5 Constitutional delay of growth and puberty (CDGP)

Clinically, onset of puberty can be assessed only indirectly by recording different physical changes related to pubertal maturation, which appear at different ages in healthy individuals, with the timing following a normal distribution (Tanner 1962). CDGP is the most common cause of delayed puberty. CDGP is a variant of the normal spectrum of pubertal timing and is characterized by first pubertal signs appearing at an age that is 2.0 standard deviations (SDs) above the mean age for pubertal onset in the absence of any illnesses causing pubertal delay (Sedlemeyer & Palmert 2002). According to British reference data (photographed breast, genital, and pubic hair stages of 420 institutionalized Caucasian British children from the 1960s (Marshall & Tanner 1969, 1970)), the mean age at achieving Tanner stage B2 is 10.8 years in girls and CDGP is diagnosed if no breast development occurs by 13.0 years. The B2 mean age has been reported to be 10.8 years also in Finland and in other parts of Europe (Ojajärvi 1982, Mul et al. 2001, Juul et al. 2006). Based on British reference data the mean age for Tanner stage G2 is 11.6 years in boys, and CDGP is diagnosed if no testicular enlargement occurs before 13.5 years (Marshall & Tanner 1969, 1970). The mean age at G2 in other Western European countries is 11.5 years (Mul et al. 2001, Juul et al. 2006) and 12.2 in Finland (Ojajärvi 1982).

2.5.1 Differentiating CDGP from congenital HH is challenging

CDGP is a diagnosis of exclusion. It is especially difficult to differentiate adolescents with CDGP from those with congenital HH, especially if early signs of gonadotorpin deficiency such as microphallus and/or cryptorchidism are absent (Root 2010). During initial evaluations adolescents with both etiologies are often prepubertal and have low levels of gonadotropins (Harrington & Palmert 2012). LH and FSH levels are low in CDGP because the HPG axis has not yet matured to secrete pubertal levels of GnRH, but in congenital HH levels are low because of a lack of GnRH secretion or action (Bianco & Kaiser 2009, Shaw et al. 2011). Family history of pubertal delay in siblings or parents is typical for CDGP (Sedlemeyer & Palmert 2002, Wehkalampi et al. 2008a), but individuals with delayed puberty are also seen among pedigrees with congenital HH, especially in those where the underlying genetic cause is *FGFR1* or *GNRHR* mutation(s) (Pitteloud et al. 2006b, Raivio et al. 2009, Lin et al. 2007). Adolescents with CDGP may have also delayed adrenarche and pubarche, whereas adolescents with congenital HH are more likely to have delayed gonadal development alone,

but this distinction is often blurred (Sedlemeyer & Palmert 2002, Harrington & Palmert 2012). To date, there is no diagnostic test that reliably distinguishes CDGP from HH, so in many cases only follow-up will reveal whether the condition is permanent HH or transient CDGP (Harrington & Palmert 2012, Palmert & Dunkel 2012).

2.5.2 Unknown genetic background of CDGP

Based on twin studies and similarities in the ages at pubertal onset between family members and within racial groups, it is evident that much of the variation of pubertal timing is due to genetic factors (Sedlemeyer et al. 2002). CDGP also tends to aggregate in families, and, although many genes may be involved, the inheritance patterns suggest that there are also single genes with major effects (Sedlmeyer et al. 2002, Wehkalampi 2008a). As CDGP is a variant of normal pubertal timing, identification of genes underlying CDGP would also provide information about genes important for pubertal onset in general, and vice versa.

Leptin is an adipose tissue derived satiety hormone that signals the information from energy reserves to the hypothalamic GnRH region (Campfield et al. 1995). Leptin is an important regulator of several endocrine functions in humans, and mutations in leptin (*LEP*, HGNC ID: 6553) or in its receptor (*LEPR*, HGNC ID: 6554) lead to early-onset obesity, but also to delayed puberty development, reduced GH and thyrotropin levels (Strobel et al. 1998, Clement et al. 1998). In the late 1990s, leptin was proposed to be the initial trigger for pubertal onset based on the observation that leptin induced pubertal pattern of LH release in a girl with congenital leptin deficiency (Farooqi et al. 1999) and reversed reproductive failure in leptin deficient mice (Chebab et al. 1996). The latter observation was contradicted by later studies showing that although leptin advanced puberty in food restricted rodents with delayed puberty, advancement was not observed relative to untreated animals (Cheung et al. 1997). Banerjee et al. (2006) genotyped *LEP* and *LEPR* polymorphisms from 81 CDGP subjects but no association with late pubertal development was found.

The acid-labile subunit is a GH-dependent peptide that is involved in carrying insulin-like growth factor-1 around in the circulation. Mutation in the acid-labile subunit gene *IGFALS* (HGNC ID: 5468) have been reported in patients with delayed puberty but with only a modest degree of growth failure (Domene et al. 2004, Domene et al. 2007). However, no mutations were found when *IGFALS* was screened from 90 subjects with CDGP (Banerjee 2008).

Based on animal studies, thyroid transcription factor 1 (Ttf1) and transcription factor enhanced at puberty 1 (Eap1) control female sexual development (Mastronardi et al. 2006, Heger et al. 2007). Gene expression profiling of the nonhuman primate hypothalamus revealed that expression of both *Ttf1* and *Eap1* increases at puberty, and mice with deleted *Ttf1*/inhibited *Eap1* exhibit delayed puberty and reduced reproductive capacity (Mastronardi et al. 2006, Heger et al. 2007). Very recently, *TTF1* (HGNC ID: 12397) and *EAP1* (HGNC ID: 14282) were sequenced from 133 patients with pubertal disorders, but no mutations were found (Cukier et al. 2013).

Ghrelin is primarily secreted by gastric cells, and through interaction with its receptor GH segretagogue receptor (GHSR), it stimulates GH secretion and has a potent appetite stimulating effect (Sun et al. 2004). GHSR is mainly expressed in the hypothalamus and pituitary. Mutations in *GHSR* (HGNC ID: 4267) have been implicated in the etiology of idiopathic short stature (Pantel et al. 2003) and in 2009, Pantel et al. reported an isolated GH deficiency patient with delayed puberty and compound heterozygous *GHSR* mutations (Pantel et al. 2009). Pugliese-Pires et al. (2011) sequenced *GHSR* in 96 patients with idiopathic short stature, of which 31 also had CDGP. They found five different heterozygous mutations, all in patients with CDGP, suggesting a possible link between *GHSR* and CDGP. However, future studies with larger cohorts of CDGP patients, including family members, are needed to verify this connection.

Besides candidate gene-based mutation screening strategies, linkage analyses and association analyses have also been employed in order to identify gene(s) important for pubertal onset.

Genes underlying congenital HH (chapter 2.4) are obvious candidates for CDGP. In 2005, Sedlmeyer et al. investigated the association of *GNRH1* and *GNRHR* sequence variants or haplotype structures in subjects with CDGP as well as in a large multiethnic cohort representing the full spectrum of normal pubertal timing. No association was found in either group. Gajdos et al. (2008) explored the association between common variants in *KAL1, FGFR1, PROK2, PROKR2, GNRH1, GNRHR, KISS1, KISS1R, LEP,* and *LEPR* and early or late menarche in 1801 girls, but no association was detected in this study, either. Genome-wide linkage study of 52 Finnish families with CDGP suggested that the pericentromeric region of chromosome 2 harbors a gene predisposing to pubertal delay (Wehkalampi et al. 2008b), but no significant signal was detected from this region in large genome-wide

association studies published in 2009, when four different research groups found that variation in or near the *LIN28B* (HGNC ID: 32207) was associated with pubertal timing in humans (Perry et al. 2009, Ong et al. 2009, He et al. 2009, Sulem et al. 2009). A year later, Widen et al. (2010) identified two distinct variants near and in *LIN28B* that were associated with human postnatal growth, especially with pubertal growth. However, subsequent sequencing of *LIN28B* from 145 subjects with CDGP revealed no sequence variation, so mutations in coding region of *LIN28B* are not causing CDGP (Tommiska et al. 2010). In 2011, meta-analysis combining data from 32 genome-wide association studies in over 87 000 women identified 30 novel loci for the timing of menarche, and provided evidence for a further 10 possible loci (Elks et al. 2011). In 2013, genome-wide association analyses in 18 737 European samples that utilized longitudinally collected height measurements identified significant associations at 10 loci, including *LIN28B*. Five loci associated with pubertal timing (Cousminer et al. 2013). However, despite the numerous loci identified in these studies, the actual causal variants and implicated genes remain unidentified, both in CDGP and in pubertal timing in general.

3 AIMS OF THE STUDY

* To characterize the molecular genetic features of KS patients in Finland (I, II, III)

* To investigate the genetic overlap between HPE and KS (IV)

* To further investigate the role of genes underlying congenital HH in CDGP (V)

4 SUBJECTS AND METHODS

4.1 Subjects

4.1.1 Patients with KS (I – IV)

Patients diagnosed with congenital HH were enrolled from all five university hospitals in Finland. Discharge registers of Helsinki, Turku, Tampere, Kuopio and Oulu university hospitals were queried by International classification of disease (ICD) edition 8 (if applicable), 9 and 10 codes for HH (ICD-8: 253.1; ICD-9: 253.4; ICD-10: E23.04). Patients previously diagnosed with congenital HH on the basis of 1) absent or incomplete puberty by the age of 18 years, 2) low sex hormone levels in association with normal or subnormal gonadotropin levels, 3) otherwise normal anterior pituitary function, 4) no organic cause for their condition, and, in case of KS, 5) defective sense of smell detected by formal testing, anamnesis, or absent or rudimentary OB in MRI, were requested to participate. In addition to adult patients, 12–18 year-old patients with unequivocal signs of severe HH (boys with a history of cryptorchidism and/or micropenis), absent puberty, and anosmia/hyposmia were enrolled. Relatives of patients were contacted with the permission of the proband.

Patients willing to participate were asked for a detailed medical history including history of cryptorchidism, micropenis, prior pubertal development, prior treatment, associated phenotypes, and the sense of smell. Patients underwent a complete physical examination. Olfaction was assessed by a 40-item smell identification test (University of Pennsylvania smell identification test, Sensonic Inc), and defective sense of smell was defined by score <5th percentile of age. Renal structures were assessed by abdominal ultrasound scan. Olfactory bulbs, sulci, and inner ears were visualized with MRI. Blood was drawn for serum biochemical testing (testosterone, estradiol, LH, FSH) and for DNA extraction for genetic analyses. The family members willing to participate were interviewed for prior pubertal development, fertility, associated phenotypes, and sense of smell.

Out of 102 patients who were identified from hospital registers and contacted by a letter, 38 (31%) agreed to participate. In addition, 17 patients were recruited during normal follow-up visits and 3 patients contacted our research group via webpage, so altogether 58 HH patients

were phenotyped. Recruitment yielded 45 patients meeting the criteria of congenitall HH (nHH or KS), and 34 KS patients were included in this thesis study.

The incidence of KS in Finland (I) was assessed separately by register searches in the five university hospitals (ICD codes 9 and 10 for HH, years 1996 to 2007). Patients of Finnish origin, born between 1976 and 1987, and diagnosed with KS between 1996 and 2007 were included. To estimate the incidence of KS, the observed number of KS patients was compared with the number of live-born children in Finland between 1976 and 1987 (http://pxweb2.stat.fi/database/StatFin/vrm/synt/synt_en.asp; Statistics Finland register database).

In study III, one KS patient was from Estonia. He was diagnosed at the age of 17 years, based on anosmia from history, lack of puberty, low circulating basal testosterone in association with low gonadotropin levels. At the age of 19 years, he participated in a study investigating KS in Estonia.

4.1.2 Subjects with CDGP (V)

The enrolment, clinical details and workup of 146 subjects with CDGP analysed in study V have been previously described in detail (Wehkalampi et al. 2008a,b). In short, all subjects (116 males and 30 females) fulfilled the diagnostic criteria for CDGP; Tanner genital stage II (testis volume of more than 3 ml) beyond the age 13.5 years in boys and Tanner breast stage II beyond the age 13.0 years in girls (Marshall et al. 1969, Marshall et al. 1970). The mean age for acceleration of pubertal height growth and the mean age for peak height velocity were more than 2 SDs later than the average for both sexes (Karlberg et al. 2003, Tanner et al. 1976). Medical history, clinical examination, and routine laboratory tests excluded chronic illnesses accounting for the delayed puberty. HH was excluded by GnRH testing and by clinical follow-up ensuring spontaneous pubertal development. Of the subjects 133 had familial background of CDGP. In 13 subjects familial occurrence was not known.

4.1.3 Controls

Controls used in genetic studies were from the same geographical region as the cases. Controls used in study I, IV and V were healthy anonymous blood donors obtained from the Finnish Red Cross Blood Service. Estonian controls were used in study III.

4.2 Mutation analyses (I -V)

Genomic DNA from blood leukocytes of the patients was extracted using NucleoSpin Blood XL kit (Macherey-Nagel). Genes implicated in congenital HH (I-V), HPE (IV) SOD (IV), and PHS (IV) (Table 3) were screened for mutations by direct sequencing. The coding exons and exon-intron boundaries of each gene were amplified with polymerase chain reaction using primers that were designed with Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/, Untergrasser et al. 2012, Koressaar et al. 2007) or previously published (most exons of *FGFR1*, Dode et al. 2003; most exons of *CHD7*, Lalani et al. 2005; all exons of *PROK2*, *PROKR2* Dode et al. 2006; and *WDR11*, Kim et al. 2010). The correct size of polymerase chain reaction products were verified with agarose gel electrophoresis before purifying the products with ExoSAP-IT (Amersham Biosciences) or Illustra ExoProStar (GE Healthcare) treatment. Samples were bi-directly sequenced using the ABI BigDyeTerminator Cycle Sequencing Kit (v3.1) and ABI Prism 3730xl DNA Analyzer automated sequencer (Applied Biosystems). The sequences were aligned and read with Sequencher® 4.9 software (Gene Codes Corporation).

In addition to direct sequencing, *FGFR1* and *CHD7* were screened by multiplex ligation-dependent probe amplification assay (MLPA, Salsa MLPA Kits P133 Kallmann-2 and P201-B1 CHARGE, MRC-Holland) according to the manufacturer's protocol (I). MLPA is able to detect large deletions, insertions, or duplications that could be missed with direct sequencing. MLPA was performed only for *CHD7* and *FGFR1* as in other KS genes such aberrations are very rare. Overview of mutation analyses is presented in table 3.

Table 3. Mutation analyses.

Gene	HGNC ID	RefSeq	Coding exons	Method	Subjects	Sudy
KAL1	6211	NM_000216.2	1-14	DS	KS	I - IV
FGFR1	3688	NM_023110.2	2-18 (8A, 8B) *	DS, MLPA	KS, CDGP	I - V
FGF8	3686	NM_033163.3	1-6	DS	KS	I - IV
PROK2	18455	NM_001126128.1	1-4	DS	KS	I - IV
PROKR2	15836	NM_144773.2	1-2	DS	KS	I - IV
CHD7	20626	NM_017780.2	2-38	DS, MLPA	KS	I, III, IV
WDR11	13831	NM_018117.11	1-29	DS	KS	I, III, IV
NELF	29843	NM_001130969.1	1-16	DS	KS	I
SOX10	11190	NM_006941.3	2-4	DS	KS	III
SOX2	11195	NM_003106.3	1	DS	KS	IV
SHH	10848	NM_000193.2	1-3	DS	KS	IV
SIX3	10889	NM_005413.3	1-2	DS	KS	IV
TGIF1	11776	NM_170695.3	1-3	DS	KS	IV
TDGF1	11701	NM_003212.3	1-6	DS	KS	IV
FOXH1	3814	NM_003923.2	1-3	DS	KS	IV
GLI2	4318	NM_005270.4	2-14	DS	KS	IV
GLI3	4319	NM_000168.5	2-15	DS	KS	IV
GNRHR	4421	NM_000406.2	1-3	DS	CDGP	V
TAC3	11521	NM_013251.3	2-6	DS	CDGP (females)	V
TACR3	11528	NM_001059.2	1-5	DS	CDGP (females)	V

HGNC, HUGO gene nomenclature commitee; RefSeq, the reference sequence database; DS, direct sequencing; MLPA multiplex ligation-dependent probe amplification assay; KS, Kallmann syndrome; CDGP, subjects with constitutional delay of growth and puberty. * In *FGFR1*, both exons 8A and 8B, generating isoforms FGFR1-IIIb and FGFR1-IIIc by alternative splicing were screened (Miura et al. 2010).

Nonsense mutations (changes of one nucleotide in the DNA sequence that introduce a premature stop codon in the transcript), frameshift mutations (caused by deletions, duplications or insertions of one or more nucleotides in the protein-coding sequence, and result in disrupted reading-frame) and splice-site mutations (nucleotide changes that occur in the highly conserved splice donor sites, branch sites or splice acceptor sites and cause incorrect splicing of the following exon) were categorized as pathogenic mutations. *FGFR1* and *PROKR2* missense mutations (nucleotide changes in the DNA sequence that cause amino acid to change), that were absent from the SNP databases were further characterized by functional *in vitro* studies.

4.3 Functional studies (I, II)

4.3.1 Mutagenesis (I, II)

To evaluate the functional consequences of FGFR1 (G48S, R209H, E670A) and PROKR2 (G234D) missense mutations *in vitro,* mutant construcs were created. Expression plasmid pcDNA3.1+ containing N-terminally myc-tagged FGFR1c wild type (WT) complementary DNA (cDNA) (I) was received from Dr. Nelly Pitteloud (Centre Hospitalier Universitaire Vaudois, Switzerland), and expression plasmid pcDNA3.1+ containing N-terminally HA (human influenza hemagglutinin) -tagged PROKR2 WT cDNA (II) was received from Dr. Yisrael Sidis (University of Lausanne, Switzerland). Mutations were created using QuikChange II XL site-directed mutagenesis kit (Stratagene) according to manufacturer's instructions. The nucleotide sequences of the mutants were confirmed by sequencing.

4.3.2 Cell lines, culture conditions and transfections (I, II)

COS-1 cells, are a fibroblast-like cell line derived from African green monkey kidney tissue, were used for FGFR1 expression and maturation studies (I) and for PROKR2 immunocytochemistry analysis (II). L6 cells, rat thigh myoblasts, were used for FGFR1 MAPK signalling studies (I). Both cells were maintained in Dulbecco's modified eagle medium (Sigma) supplemented with penicillin (25 U/ml), streptomycin (25 U/ml), 10% fetal bovine serum (v/v) and L-glutamine. For functional studies these cells were seeded on 24-well plates, 3 x 10^4 COS-1 cells/well or 1 x 10^4 L6 cells/well. Both cell lines were from American type culture collection.

Transfections were performed using Fugene HD transfection reagent (Roche) according to manufacturer's recommendation. For each 24-well plate well total of 300ng of DNA was transfected containing 50ng of Myc-FGFR1 or HA-PROKR2 expression vector and 250ng of empty pcDNA3.1+ vector (EV) or 300ng of EV alone (negative control). Transfection optimatization for both COS-1 and L6 cells was performed using expression plasmid containing green fluorescent protein. COS-1 cells were transfected at sub-confluent stage and L6 cells at 10% confluence stage.

4.3.3 FGFR1 receptor expression and maturation studies (I)

One day after COS-1 cells were transfected with Myc-tagged wild-type (WT) or mutated FGFR1 cDNA, cells were washed twice with PBS (phosphate buffered saline) and lysed with 100 µl of radioimmunoprecipitation assay buffer (Sigma) containing 1X Halt protease inhibitor cocktail (Pierce) and lysates of 3 replicate wells were pooled to one sample. Protein concentration of each sample was measured with Protein Quantification kit-Rapid (Sigma) and 5µg of protein was subjected to PNGasef or EndoHf endoglycosidase digestion according to manufacturer's recommendations (New England Biolabs). PNGase digestion removes all N-linked carbohydrate chains from the receptor whereas EndoHf treatment removes only high-mannose N-linked sugars, which are typical for immature forms of the receptor. Samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Gold Precast 4-20% Tris-Glycine Gels, Lonza) and transferred onto nitrocellulose membrane (GE Healthcare). Immunoblotting was performed with anti-Myc primary antibody (1:1000, clone 4A6, Millipore) and a goat anti-mouse horseradish peroxidase (HRP) -conjugated secondary antibody (1:3000, Bio-Rad). Immunoreactivity was visualized using Amersham western blotting detection reagents (GE Healthcare). To control for equal loading, blots were stripped using Restore western blot stripping buffer (Pierce) and reprobed using an anti-β-actin primary antibody (1:1000, sc-47778, Santa Cruz Biotechnology), and the secondary antibody (see above). Receptor expression levels were visualized from the PNGase treated samples and receptor maturation patterns from the EndoHf treated samples. Experiment was performed three times.

4.3.4 FGFR1 cell surface expression (I)

One day after COS-1 cells were transfected with Myc-tagged WT or mutated FGFR1 cDNA, cell were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 minutes. Cells were then blocked with 1% bovine serum albumine in PBS for 1 hour. FGFR1 cell-surface expression levels were determined using an anti-Myc primary antibody (1:1000, clone 4A6, Millipore) and a goat anti-mouse HRP-conjugated secondary antibody (1:3000, Bio-Rad) and assayed using 3,3`,5,5`tetramethylbenzidine (Sigma). HRP catalyzes the conversion of 3,3`,5,5`tetramethylbenzidine into coloured product. Reaction was stopped by adding 0.5 M H_2SO_4 and intensity of coloured product was measured with spectrophotometer (450nm). Experiment was performed in triplicates and repeated three times.

4.3.5 FGFR1 MAPK-signalling studies (I)

After 5 hours of transfecting L6 myoblasts with Myc-tagged WT or mutated FGFR1 cDNA, culture media was replaced with starvation media containing only 2% fetal bovine serum. 20 hours later, the cells were stimulated with FGF2 (Cell Signaling Technology) 50ng/ml for 0/2/10/30 min. At each time point, the stimulation was stopped by washing the cells with ice-cold PBS. Cells were then lysed with 50µl of radioimmunoprecipitation assay buffer buffer (Sigma) containing 1X Halt phosphatase inhibitor cocktail (Pierce), and lysates of 3 replicate wells at each time point were pooled. Samples containing 8µg of protein (Protein Quantification kit-Rapid, Sigma) were resolved as described above and subjected to western blot using a Phospho-p44/42 MAPK (Thr202/204) primary antibody (1:1000, Cell Signaling Technology), and a HRP-conjugated secondary antibody (1:3000, Bio-Rad Laboratories). Immunoreactivity was visualized as described above. To control for equal loading, blots were stripped as described above, and reprobed using a p44/42 MAPK primary antibody (1:1000, Cell Signaling Technology). Experiment was performed three times.

4.3.6 Immunocytochemistry (II)

COS-1 cells on 3,5mm coverslips were transfected with HA-tagged WT or mutated PROKR2 cDNA. After 24 h, cells were washed with PBS and fixed with 4% paraformaldehyde for 10 minutes. The cells were blocked with 1% bovine serum albumin in PBS with or without 0.1% Triton-X for one hour. The cells were then treated with anti-HA-antibody (1:1000, H9658, Sigma) and Alexa Fluor 568-conjugated secondary antibody (1:500, Invitrogen) and mounted with mounting solution containing Dapi (Vector Labs). Images were captured with an Axioplan 2 fluorecence microscope (Carl Zeiss Light Microscopy). Experiment was performed twice.

4.4 Statistical and bioinformatical analyses (I – V)

4.4.1 Statistical significance (I, V)

Fisher's exact test was used to compare the frequency of *FGFR1* mutations in males and females (I), the incidence of KS between boys and girls (I), and the frequency of *GNRHR*

mutations in CDGP subjects vs. controls (V). All p-values are two-sided, and $p < 0.05$ was accepted to indicate statistical significance.

4.4.2 Freely available databases (I – IV)

Several databases listing human genetic variation were utilized troughout this thesis: The National center for biotechnology SNP database dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), the 1000 Genomes Project database (http://www.1000genomes.org/), Exome variant server (http://evs.gs.washington.edu/EVS/), and Leiden open variation database (http://grenada.lumc.nl/LOVD2/WS/home.php?select).

PolyPhen (I), PolyPhen2 (Adzhubei et al. 2002, http://genetics.bwh.harvard.edu/pph/; http://genetics.bwh.harvard.edu/pph2/), and MutPred (Li et al. 2009, http://mutpred.mutdb.org/) were used to evaluate the functional significance of the missense variants found. PolyPhen (=Polymorphism Phenotyping) is a tool which predicts possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations. MutPred is a web application tool that is developed to classify amino acid substitutions as disease-associated or neutral in human. MutPred is based upon SIFT (web tool that predicts whether an amino acid substitution affects protein function, http://sift.jcvi.org/) and a gain/loss of 14 different structural and functional properties.

4.5 Ethics

The study was carried out according to the declaration of Helsinki and Finnish legislation and performed with appropriate permissions from each university hospital in Finland and approval from the Ethics Committee of the Hospital for Children and Adolescents and the Department of Psychiatry (E7). Written informed consents were obtained from the participants, and also from their guardian if the participant was less than 16 yrs of age. The genetic analyses of KS patient and his parents from Estonia (III) had the appropriate permissions.

The collection of data and DNA of subjects with CDGP (V) were also approved by the Ethics Committee E7. All participants or their parents or guardians provided their written informed consents.

5 RESULTS

5.1 Incidence of KS and phenotypic features of KS patients in Finland (I)

Altogether 17[1] KS patients (14 boys, 3 girls) were diagnosed between 1996 and 2007. They were born between 1976 and 1987, when a total of 767 778 live-born infants (392 900 boys, 374 878 girls) were born. The overall minimal incidence of KS was 1 in 45 000 new-borns. Incidence estimates differed significantly between boys (1 in 28 000) and girls (1 in 125 000) (p=0.01).

Thirty-four KS patients (6 women, 28 men) were included in the molecular genetic part of this study. The phenotypic features of these patients are presented in Table 4. In short, five female patients had absent pubertal development, whereas one had mild form of congenital HH (secondary amenorrhea, infertility). Reproductive phenotype of KS men varied from severe (cryptorchidism and/or micropenis in childhood) to reversal of HH later in life. Twenty-one (62%) patients had a family history of congenital HH or associated features. Three patients had CHARGE associated features (KS19, KS20 and KS25) and six had a hearing impairment (KS14, KS20, KS24, KS25, KS27, KS_E). Four men were of other than Finnish origin (KS26, KS30, KS37, KS_E).

[1] Incidence calculations have been updated to include one male KS patient, born in 1978, who was identified after publication I was published.

Table 4. Phenotypic features of Kallmann syndrome patients in Finland.

Proband	Sex	Age	Evidence of KS	Family History	MRI	Additional Features	Mutated Gene	Study
KS1	F	16	Absent puberty, anosmia	None	NA	Dental agenesis	*FGFR1*	I
KS2	F	27	Absent puberty, anosmia	KS, nHH	NA	None	*FGFR1*	I
KS3	F	26	Absent puberty, anosmia	nHH, infertility	Absent OB and sulci l.a.	None	*FGFR1*	I
KS4	F	33	Absent puberty, anosmia	KS	Absent OB l.a.	Cleft lip, dental agenesis	*FGFR1*	I
KS5	F	41	Absent puberty, infertility, anosmia	KS	Absent OB l.a.	None	*FGFR1*	I
KS6	F	35	Secondary amennorrhea, infertility, anosmia	Anosmia, infertility	Absent OB l.a.	None		III, IV
KS7	M	48	Bilateral CO, MP, absent puberty, anosmia	KS	NA	Synkinesia, dental agenesis	*KAL1*	I
KS8	M	41	Absent puberty, infertility, anosmia	Anosmia	NA	None		I, III, IV
KS9	M	46	Bilateral CO, MP, absent puberty, infertility, anosmia	KS	NA	Synkinesia, renal agenesis	*KAL1*	I
KS10	M	25	MP, absent puberty, anosmia	None	NA	None		I, III, IV
KS11	M	56	MP, absent puberty, anosmia	None	NA	Cleft lip and palate		I, III, IV
KS12	M	50	Partial puberty, anosmia	Cleft lip	NA	None		I, III, IV
KS13	M	30	Unilateral CO, absent puberty, infertility, anosmia	KS	NA	Dental agenesis		I, III, IV
KS14	M	49	Unilateral CO, absent puberty, infertility, anosmia	KS	NA	Synkinesia, hearing impairment	*KAL1*	I
KS15	M	13	Unilateral CO, MP, anosmia	None	NA	None		I, III, IV
KS16	M	25	Unilateral CO, absent puberty, anosmia	None	NA	None		I, III, IV
KS17	M	41	MP, absent puberty, anosmia	None	NA	None	*FGFR1*	I
KS18	M	61	Partial puberty, inferility, anosmia	KS	absent OB l.a.	None	*FGFR1*	I
KS19	M	48	Bilateral CO, absent puberty, anosmia	None	NA	Cup-shaped ears, upper body muscular atrophy		I, III, IV
KS20	M	37	Partial puberty, anosmia	Coloboma	absent OB l.a.	Reversal of HH, SCC hypoplasia hearing impairment, unspecified atrophicarea in retina	*CHD7*	I

KS21	M	18	Bilateral CO, MP, absent puberty, anosmia	None	NA	High arched palate		I, III, IV
KS22	M	49	Bilateral CO, MP, absent puberty, anosmia	None	Absent OB, hypoplastic sulci l.a.	None		I, III, IV
KS23	M	52	MP, absent puberty, anosmia	nHH, limb anomalies	NA	Cleft lip and palate	*FGFR1*	I
KS24	M	15	Bilateral CO, MP, absent puberty, anosmia	Deafness	NA	Bilateral sensorineural hearing loss		I, III, IV
KS25	M	24	Bilateral CO, absent puberty, anosmia	DP	Rudimentary OB, absent sulci l.a.	Cleft lip and palate, cup-shaped ears, SCC dysplasia, bilateral hearing impairment, unilateral coloboma and microphthalmia		I, III, IV
KS26*	M	22	Absent puberty, anosmia	None	NA	None		I, III, IV
KS27	M	56	Absent puberty, anosmia	Anosmia	Absent OB l.a.	Unilateral hearing loss, color blindness		I, III, IV
KS28	M	28	Unilateral CO, MP, absent puberty, anosmia	Anosmia	Small/absent OB	High arched palate		I, III, IV
KS29	M	41	MP, absent puberty, anosmia	None	Absent OB l.a.	None		I, III, IV
KS30*	M	45	Absent puberty, anosmia	KS, nHH, cleft lip	NA	None	*FGFR1*	I
KS31	M	42	Absent puberty, anosmia	Inner ear anomalies	Absent OB l.a.	None		I, III, IV
KS36	M	17	MP, CO, absent puberty, anosmia	None	Rudimentary OB	None		III, IV
KS37*	M	15	MP, absent puberty, anosmia	KS	None	None	*PROKR2*	II
KS_E*	M	17	Absent puberty, anosmia	None	Absent OB l.a.	Unilateral hearing loss, early grayin, broad nasal bridge, mild dystopia canthorum	*SOX10*	III

KS, Kallmann syndrome; nHH, normosmic HH; F, female; M, male; MRI, magnetic resonance imaging; OB, olfactory bulbs; NA, not assessed; CO; cryptorchidism; MP, micropenis; DP delayed puberty; SCC, semicircular canal. Patient KS_E is diagnosed and treated in Estonia.
* Other than Finnish origin

5.2 Molecular genetic features of KS patients (I-IV)

Out of 34 KS patients in this study, 15 (44%) got a molecular genetic diagnosis. Overview of conclusive mutations is presented in Table 5 and more detailed information about the results of the mutation analyses below.

Table 5. Kallmann syndrome patients with conclusive mutations.

Proband	Gene	Nucleotide change	Amino acid change	Type	Predicted effect	*In vitro* functional studies	Reference	Study
KS1	*FGFR1*	c.246_247delAG	p.E84GfsX26*	frameshift	Leads to premature stop codon		Dode & Hardelin 2009	I
KS2	*FGFR1*	c.142G>A	p.G48S	missense	Impaired MAPK signalling	X	Trabach et al. 2006	I
KS3	*FGFR1*	c.961_962delAA	p.K321RfsX13	frameshift	Leads to premature stop codon		Dode & Hardelin 2009	I
KS4	*FGFR1*	c.1825C>T	p.R609X	nonsense	Premature stop codon		Riley et al. 2007	I
KS5	*FGFR1*	c.1305_1306dupAT	p.S436YfsX3	frameshift	Leads to premature stop codon			I
KS7	*KAL1*	g.2357_2360delAgta		splice site	Abolishes the splice site			I
KS9	*KAL1*	c.784C>T	p.R262X	nonsense	Premature stop codon		Söderlund et al. 2002	I
KS14	*KAL1*	c.471_472delCT	p.S158WfsX45	frameshift	Leads to premature stop codon			I
KS17	*FGFR1*	c.626G>A	p.R209H	missense	?	X		I
KS18	*FGFR1*	c.961_962delAA	p.K321RfsX13	frameshift	Leads to premature stop codon		Dode & Hardelin 2009	I
KS20	*CHD7*	c.151C>T	p.Q51X	nonsense	Premature stop codon			I
KS23	*FGFR1*	c.2009A>C	p.E670A	missense	Impaired MAPK signalling	X		I
KS30	*FGFR1*	c.11G>A	p.W4X	nonsense	Premature stop codon			I
KS37	*PROKR2*	c.701G>A/c.701G>A	p.G234D/p.G234D	missense	Possibly impairs the cell-surface targeting	X		II
KS_E	*SOX10*	c.184G>T	p.E62X*	nonsense	Premature stop codon			III

MAPK, mitogen-activared protein kinase

* confirmed *de novo* mutation

5.2.1 Mutations in FGFR1 (I)

Nine KS patients had an *FGFR1* mutation (9/34, 26%). A significantly higher proportion of women (5/6, 83%) carried an *FGFR1* mutation compared with men (4/28, 14%, p=0.002). All female probands with an *FGFR1* mutation had severe HH (Table 4). Men with *FGFR1* mutation had variable reproductive phenotypes. All *FGFR1* mutations found were heterozygous.

Proband KS1 carried a frameshift mutation c.246_247delAG (p.E84GfsX26) that has been previously reported in KS (Dode & Hardelin 2009). She had no family history of congenital HH and was the only proven carrier of *de novo FGFR1* mutation. Proband KS2 carried a missense mutation c.142G>A (G48S) previously described in a patient with nHH (Trarbach et al. 2006). She had severe HH but her anosmic father carrying the same mutation had had four children during testosterone therapy representing a rather mild reproductive phenotype. Her healthy son with minipuberty was also a carrier (*unpublished result*). Her brother had nHH but he was unavailable for mutation analysis (Figure 7). Proband KS3 with severe HH carried a frameshift mutation c.961_962delAA (p.K321RfsX13) previously reported in KS (Dode & Hardelin 2009). Her sister, mother, aunt and cousin carried the same mutation and had milder phenotypes (Figure 7). This same mutation was also found in the male proband KS18 who had partial puberty, infertility and anosmia. Proband KS4 carried a nonsense mutation c.1825C>T (p.R609X). This mutation has been previously described in a family with KS and clef lip and palate (Riley et al. 2007), and also proband KS4 had cleft lip and palate. Proband KS5 with severe KS carried a novel frameshift mutation c.1305_1306dupAT (p.S436YfsX3). This mutation was passed on to her children born after assisted reproductive techniques, and her daughter had KS and her son had nHH. Proband KS23 with history of micropenis and his brother with nHH and limb anomalies carried a novel missense mutation c.2009A>C (p.E670A). They both also had cleft lip and palate. Another novel missense mutation c.626G>A (p.R209H) was found in male proband KS17. Proband KS30 carried a novel nonsense mutation c.11G>A (p.W4X). He had siblings with KS and nHH but they were unavailable for mutation analysis.

Autosomal dominant mode of inheritance was apparent in almost all families where proband carried an *FGFR1* mutation (7/9, presented in figure 7). Proband KS17 had no previous family history, but unlike with proband KS1, his mutation could not be confirmed as *de novo*

as parents were unavailable for testing. Variable expressivity, typical for *FGFR1* mutations, was seen both within families (KS2, KS3, KS5 and KS23, figure 7), and in unrelated probands carrying the same mutation (KS3 and KS18, figure 7).

All frameshift and nonsense mutations lead to premature stop codons. Missense mutations G48S, R209H and E670A were absent from the dbSNP database, from at least 100 controls, and were further characterized by functional *in vitro* studies (see below). No intragenic aberrations were found in MLPA analysis of *FGFR1*.

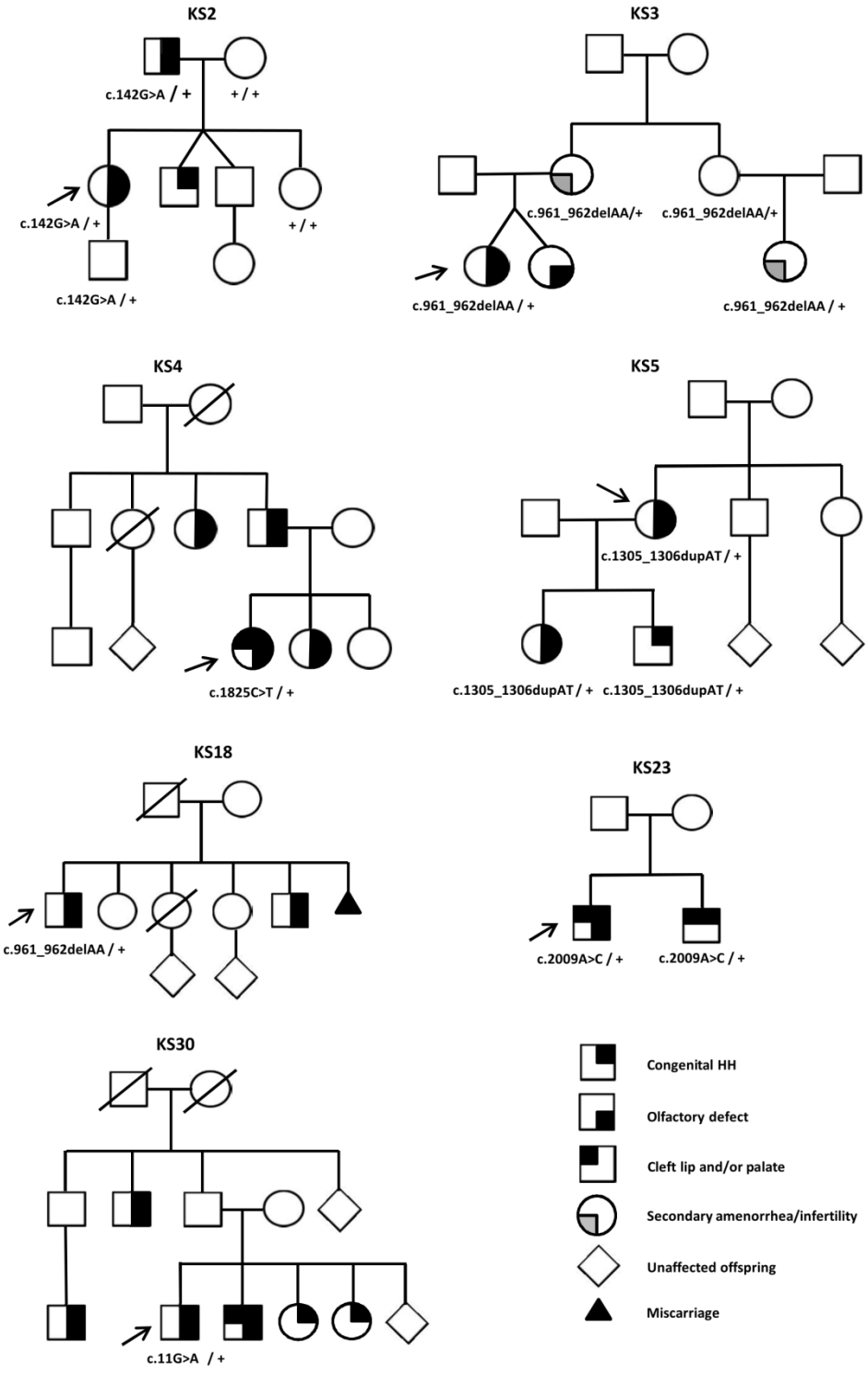

Figure 7. Pedigrees of families where the proband carried an *FGFR1* mutation. Autosomal dominant mode if inheritance and variable expressivity of the mutations is apparent. Families are named according to the proband. Proband is marked with an arrow.

5.2.1.1 Functional characterization of mutant FGFR1s (I)

FGFR1 expression and maturation studies

The overall expression and maturation patterns of FGFR1 mutants G48S, R209H and E670A were analysed in COS-1 cells. Western blot from the cell lysates shows two immunoreactive specific bands for WT FGFR1 at 140 kDa and 120 kDa (Figure 8). PNGase digestion removed all N-linked carbohydrate chains from the receptor and reduced these differently glycosylated bands into a single one of ~100kDa. The overall expression of the mutant receptors as compared to expression of WT receptor was judged from the PNGase treated samples, and no clear difference was seen. EndoHf treatment, which removes only sugars that are typical for immature forms of the receptor, changed the mobility of only the minor 120kDa band (Figure 8), which indicates that it represents the partially processed receptor. The 140kDa band resistant for EndoHf treatment represents the fully glycosylated, mature form of the receptor. Mutant receptors G48S, R209H, and E670A had a similar maturation pattern as the WT receptor.

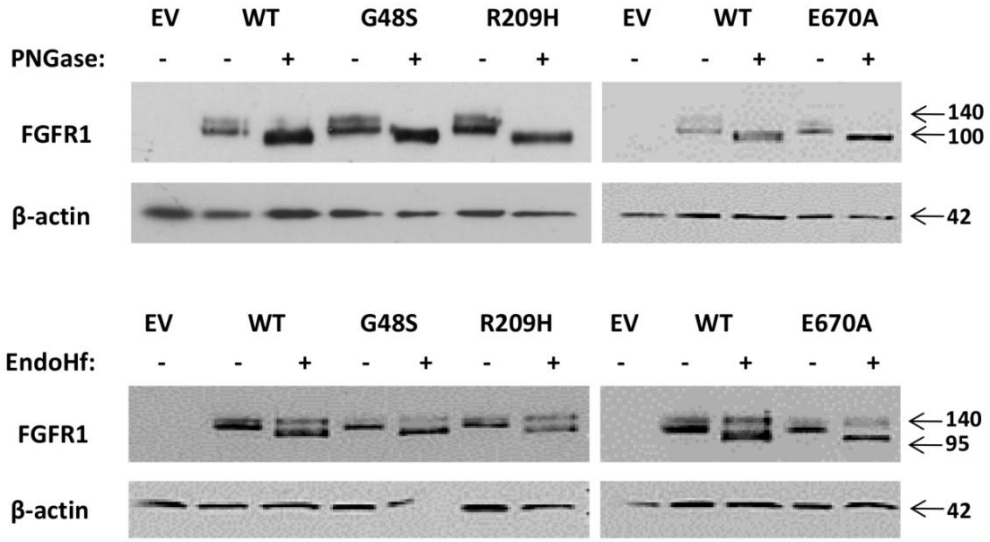

Figure 8. Fibroblast growth factor receptor 1 (FGFR1) expression and maturation studies. COS-1 cells were transiently transfected with wild-type (WT), G48S, R209H, or E670A FGFR1 complementary DNA. Cell lysates were subjected to PNGase or EndoHf treatment. The overall expression of the mutants was not significantly decreased as compared to WT (upper panel, PNGase treated bands) and no difference in maturation pattern was observed (lower panel). EV, empty vector pcDNA3.1+.

FGFR1 cell surface expression

Cell surface expression of the WT receptor and the mutants G48S, R209H, and E670A were also examined in COS-1 cells. Consistent with the results of total expression and maturation studies, the G48S, R209H, and E670A mutants had similar cell surface expression levels as the WT (Figure 9).

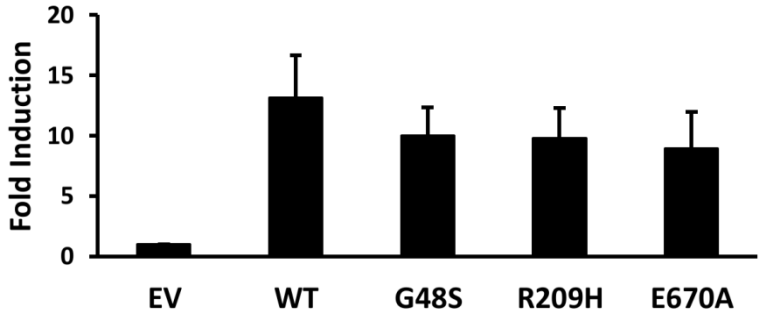

Figure 9. Cell surface expression. COS-1 cells were transiently transfected with wild-type (WT), G48S, R209H, or E670A fibroblast growth factor receptor 1 (FGFR1) complementary DNA. Cell-surface expression levels were determined from fixed cells with enzyme linked immunosorbent assay-based method. The mutant FGFR1s have a similar cell surface expression levels as the WT receptor. EV, empty vector pcDNA3.1+.

FGFR1 MAPK-signaling studies

The signalling activity of the FGFR1 mutants G48S, R209H and E670A as compared to WT receptor was analysed in L6 myoblasts. L6 cells were used as they are largely devoid of endogenous FGFRs and FGFs (Newberry et al. 1996). Cells expressing WT FGFR1 receptor showed a clear phosphorylation of MAPK after 10 and 30 min of FGF2 treatment (50ng/ml) (Figure 10). No ligand-induced phosphorylation of MAPK was seen in any of the time points (2/10/30min) in cells transfected with G48S and E670A mutant receptors, whereas the R209H mutant responded to FGF2 treatment similarly to WT (Figure 10). All untreated samples (0 min) were also run on the same gel, and they did not display differences in MAPK phosphorylation indicating similar base-line activities.

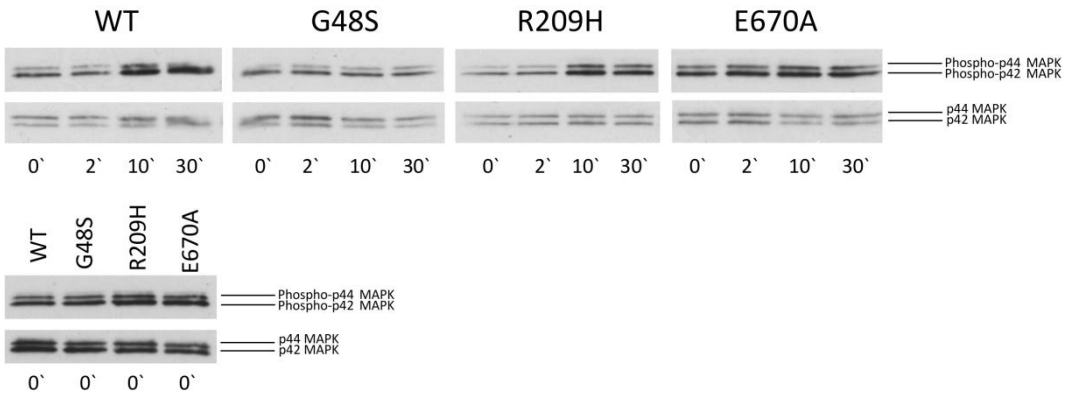

Figure 10. Mitogen-activated protein kinase (MAPK) signalling studies. L6 cells were transiently transfected with wild-type (WT), G48S, R209H, or E670A fibroblast growth factor receptor 1 (FGFR1) complementary DNA and stimulated with fibroblast growth factor 2 for 0/2/10/30 min. WT and mutant receptor R209H show clear phosphorylation of MAPK after 10 minutes. No clear phosphorylation was seen with mutants G48S and E670A.

5.2.2 Mutations in KAL1 (I)

Three male probands (3/34, 9%) had a *KAL1* mutation. They all had severe KS (history of cryptorchidism and micropenis) and also synkenisia (mirror movements). Proband KS7 carried a novel splice site mutation g.2357_2360delAgta. This deletion of four nucleotides in the exon 8 - intron 8 boundary most likely abolishes the splice site, and results in an aberrant transcript. Unfortunately cDNA was not available for testing. Probands nephew and uncle had KS and his sister was an unaffected carrier of the mutation (Figure 11). Proband KS9 carried a previously described nonsense mutation c.784C>T (p.R262X) (Söderlund et al 2002). In addition to severe KS and synkinesia, he also had unilateral renal agenesis. Proband KS14 carried a novel frameshift mutation c.471_472delCT (p.S158WfsX45). His uncle had had KS and his mother and sister were both unaffected carriers of the mutation (Figure 11). Both the nonsense mutation and the frameshift mutation lead to premature stop codons. X-linked recessive mode of inheritance was apparent in families of probands KS7 and KS14 (Figure 11).

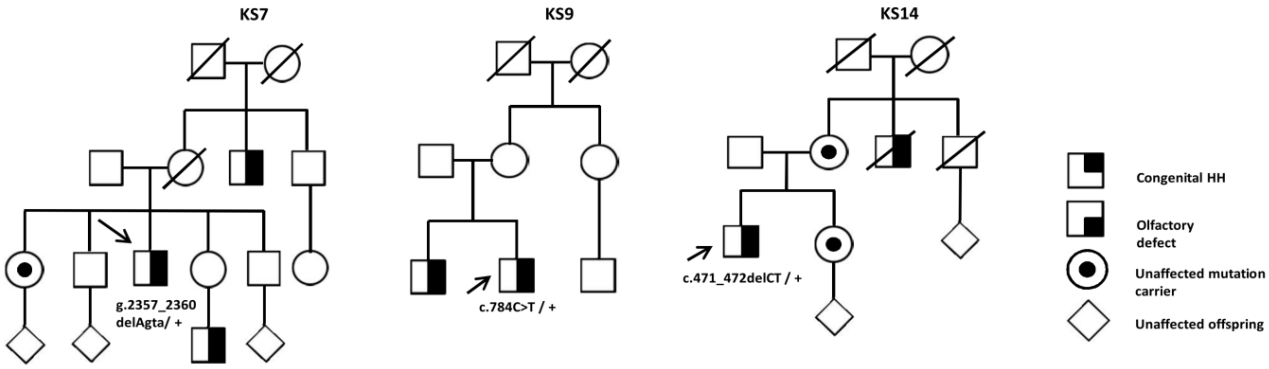

Figure 11. Pedigrees of families where the proband carried a *KAL1* mutation. X-linked recessive mode of inheritance was apparent in families KS7 and KS14. Families are named according to the proband. Proband is marked with an arrow.

5.2.3 Mutations in CHD7 (I)

One male proband (KS20, 1/34, 3%) carried a novel mutation c.151C>T (p.Q51X) in *CHD7*. This nonsense mutation leads to a premature stop codon. Mutation was not reported in the dbSNP database. In addition to KS, proband had semicircular canal hypoplasia, hearing impairment and an unspecified atrophic area in retina, which are CHARGE associated features. He had no previous family history of KS or CHARGE but his daughter had unilateral microphthalmia and bilateral coloboma. Later on, he underwent reversal of HH (Laitinen et al. 2012).

5.2.4 Mutations in PROKR2 (II)

One male proband (KS37, 1/34, 3%) and his brother carried a novel homozygous missense mutation c.701G>A (p.G234D) in *PROKR2*. Both of them had severe KS but no additional phenotypic features. Their parents were healthy heterozygous carriers of the mutation in accordance with recessive mode of inheritance (Figure 12). Brothers had four more siblings but their phenotypic features are unknown. This family was from Iraq.

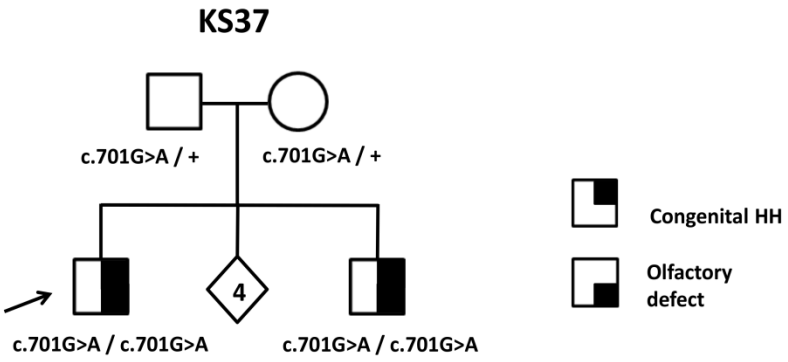

Figure 12. Pedigree of a family with autosomal recessive *PROKR2* mutation. Proband is marked with an arrow.

The c.701G>A (p.G234D) mutation was not reported in the dbSNP database, and was predicted 'probably damaging' by Polyhen2. Mutation was further characterized by immunocytochemistry analysis in COS-1 cells using a HA-tagged *PROKR* cDNA. Compared to the WT receptor, G234D mutant receptor seems to have an impaired cell surface targeting (Figure 13).

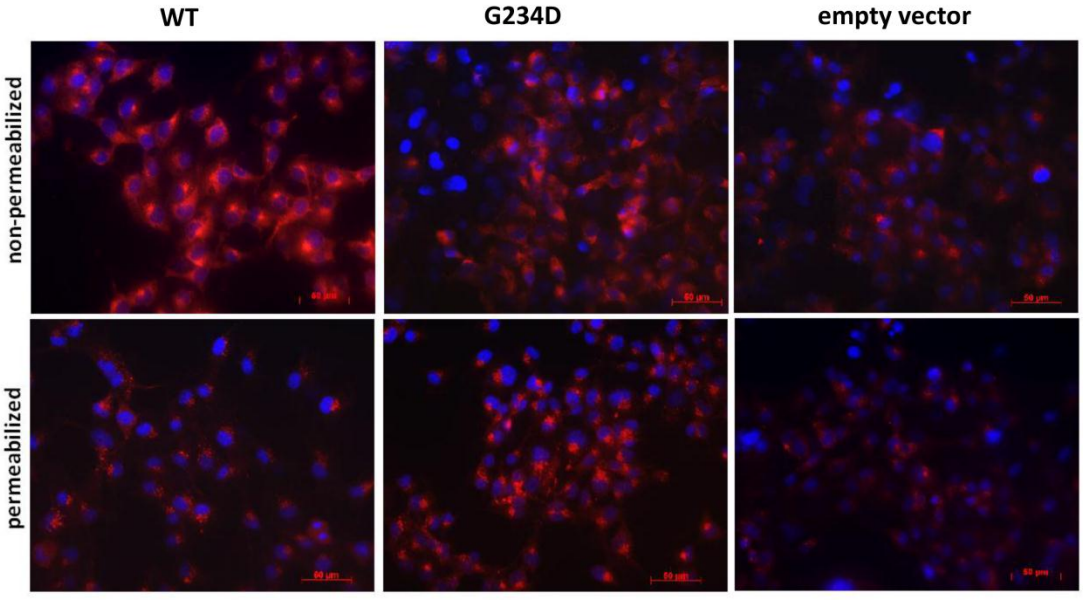

Figure 13. Cell surface and intracellular expression of wild type (WT) and G234D mutant prokineticin receptor 2. Fluorescence immunocytochemistry analyses were performed in COS-1 cells transiently transfected with either the WT or mutant (G234D) constructs. WT receptor was mainly localized at the cell membrane (upper left corner). The mutant receptor appeared to be localized more at the cytoplasmic compartments (middle panels). Empty vector (pcDNA3.1+) was used as a negative control.

In addition to the c.701G>A (p.G234D) mutation, a *PROKR2* polymorphism c.802C>T (R268C) (rs 78861628; MAF (minor allele frequency) 0.01) segregated in the family. Brothers were homozygous and their parents were heterozygous for this variant.

5.2.5 Mutations in SOX10 (III)

Proband KS_E with KS and hearing loss carried a heterozygous nonsense mutation c.184G>T (p.Glu62X) in the first coding exon of *SOX10*. This mutation was not present in 90 control individuals or reported in relevant databases (dbSNP, 1000 Genomes Project database, Exome Variant Server, Leiden Open Variation Database). MRI examination revealed OB aplasia. He had quite many gray hairs, broad nasal bridge, and mild dystopia canthorum. He had complete hearing loss on the right side, present from birth, and mild sensorineural hearing loss in high frequencies was noted in his left ear audiogram. Proband had no family history of KS or WS and the mutation was confirmed as *de novo*.

5.2.6 Additional findings (I)

Two probands with conclusive mutations in *FGFR1* carried also variants in *NELF*, gene suggested to be associated with KS. Proband KS17 (*FGFR1*: c.626G>A (p.R209H)) carried a missense variant c.280 G>A (p.G94S) in *NELF*. This variant was also present in the controls (6/100) and is now reported in the dbSNP database (rs199887535). Proband KS4 (*FGFR1*: c.1825C>T (p.R609X)) carried a missense variant c.1514 C>T (p.T505M) in *NELF*. This variant was also present in the controls (1/100).

Proband KS8 carried a missense variant c.7988C>T (p.A2663V) in *CHD7*. This variant was not present in the controls (0/100) and is not reported in dbSNP database. Proband had no additional phenotypic features. His mother's father had had anosmia. Unfortunately his parents were unavailable for mutation analysis.

5.3 Mutation analysis of HPE genes in KS patients (IV)

Eight genes (*SOX2, SHH, SIX3, TGIF1, TDGF1, FOXH1, GLI2* and *GLI3*) in which mutations are known to cause HPE, SOD, (*SOX2*) and PHS (*GLI3*) were screened in nineteen KS probands without mutations in the known KS genes. Male proband KS22 carried two heterozygous missense variants, one in *SIX3* (c.428G>A, p.G143D) and the other in *GLI2* (c.2509 G>A, p.E837K). These variants were not present in 200 controls, but the *GLI2* E837K was reported in the dbSNP database (rs193090538; MAF 0.001). According to MutPred probability of a deleterious mutation is 0.5 for the *SIX3* variant and 0.284 for the *GLI2* variant, but both of them were predicted to be possibly damaging by PolyPhen2. Probands MRI revealed absent OBs and partially hypoplastic olfactory sulci. He had no additional phenotypic features and no family history of KS. Other found variants were known polymorphisms.

5.4 Mutation analysis of *FGFR1, GNRHR, TAC3* and *TACR3* in subjects CDGP (V)

Altogether 146 subjects (116 males and 30 females) with CDGP were analysed for mutations in *FGFR1* and *GNRHR*. Females were also analyzed for *TAC3* and *TACR3*. One male subject carried a heterozygous missense change c.1307 C>G, (p.S436C) in *FGFR1*. This variant is not reported dbSNP database but it was also present in the subject's mother with normal timing of puberty and in the controls (1/100).

Two heterozygous missense mutations were found in *GNRHR*; c.317 A>G (p.Q106R) in one female and c.785G>A (p.R262Q) in 3 males and in 2 females. Both of these mutations are known to cause autosomal recessive normosmic HH (de Roux et al. 1997). Heterozygous carriers were also found among controls with the same frequencies as the cases (Q106R: 1/146, 0.7% in cases vs. 2/200, 1.0% in controls, p=1.0; R262Q: 5/146, 3.4% in cases vs. 3/200, 1.5% in controls, p= 0.3). Also, a novel deletion mutation, c.924_926delCTT (p.Phe309del), was found in *GNRHR* in one male subject. This deletion was not present in the controls and it segregated with delayed puberty in subjects' family: it was also carried by the father and sister who both had delayed puberty, whereas the mother and brother with normal timing of puberty were not carriers (Figure 14).

In *TACR3* two females carried a synonymous change c.303G>A (p.L101L). No nonsynonymous changes were found in *TAC3* or *TACR3*.

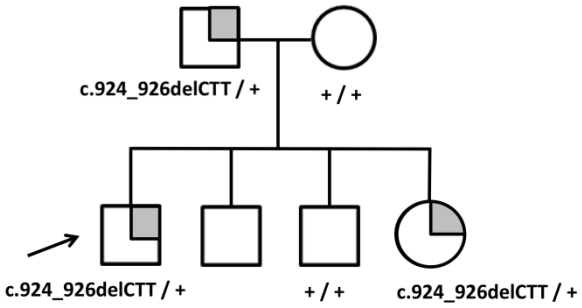

Figure 14. A novel deletion mutation in *GNRHR* segregated with delayed puberty in a male subject's family. Grey colour indicates delayed pubertal development. Index subject is marked with an arrow.

6 DISCUSSION

6.1 Male predominance in KS

The nationwide incidence of KS in Finland was 1:45 000. This is the first nationwide incidence estimate of KS, as previous estimates were made from male military conscripts in France (1:10 000) (Fromantin et al. 1973), and in Sardinia (1:84 000) (Filippi 1986). Military conscript screening is not the optimal method to ascertain the incidence of KS because the sense of smell is not typically asked for, and patients with mild reproductive phenotype may escape detection. Incidence estimates differed significantly between Finnish boys (1:28 000) and girls (1:125 000), which suggests that KS in four times more frequent in men than in women in Finland.

Fourfould difference between sexes was also seen in the patient seriers of this study, with male to female ratio being exactly 4:1 when 4 male patients other than Finnish origin were excluded (24:6). This difference is in line with previous studies showing that the condition is of 4-5 times more frequent in men worldwide (Seminara et al. 1998, Sedlemeyer & Palmert 2002, Hardelin & Dode 2008, Bianco & Kaiser 2009, and Dode & Rondard 2013). The reason for this male predominance is unclear. The X-linked recessive inheritance of *KAL1* mutations can explain only a small fraction of this difference, as *KAL1* mutation are found only in 5-10% of male patients (Bianco & Kaiser 2009). One reason might be the underdiagnosis of female patients, especially in the case of partial pubertal development (Brioude et al. 2010, Shaw et al. 2011). It has also been suggested that for some reason the male HPG axis might be more vulnerable to environmental or genetic disturbances during development. For example, *KAL1* escapes X-inactivation and it has been proposed that higher concentrations of anosmin-1 could somehow protect female embryos (Hardelin & Dode 2008). Also, as more than half of KS patients remain without molecular genetic diagnosis (see below), sex-dependent penetrance of mutations in still unidentified genes could explain, at least in part, this phenomenon.

6.2 Molecular genetic causes of KS in Finland

Overall 44% of KS patients in this study received a molecular genetic diagnosis. This percentage is slightly higher than in previous studies. According to the review by Bianco &

Kaiser (2009), 60-75% of KS patients remain without identified mutation, and in a large U.S. study consisting of 397 congenital HH patients 68% remained without genetic diagnosis (Martin et al. 2011). A clear difference was seen in the distribution of mutations in established KS genes between the patients of this study and those reported in previous studies (Figure 15). The leading molecular genetic cause of KS in this study was a mutation in *FGFR1*, which accounted for 26% of the cases, when the overall estimate of *FGFR1* mutations in KS patients worldwide is 10% (Bianco & Kaiser 2009, Dode & Rondard 2013). Also, mutations in *PROK2* and *PROKR2* that account for 5-10% of KS worldwide (Bianco & Kaiser 2009, Dode & Rondard 2013) were almost completely absent. Only one male patient, originally from Iraq, carried a homozygous *PROKR2* mutation. Lack of mutations in the prokineticin pathway probably reflects the unique genetic heritage of the Finnish population (de la Chapelle 1993, Peltonen et al. 1995, Peltonen et al. 1999, Norio 2003). Of note, quite opposite situation with *PROKR2* mutations is seen in Maghrebian population, where 23% of KS patients carry non-synonymous mutations in this gene, possibly due to a balancing selection of the mutations (Sarfati et al. 2013).

Estimates of *CHD7* mutations in KS patients vary between 1-5%. In this patient series, one KS patient with CHARGE syndrome associated features carried a conclusive *CHD7* mutation. However, two other patients with such features were not found to have a *CHD7* mutation, implying that there may be other genes associated with both syndromes (Bergman et al. 2012). One male patient without any associated features carried a rare missense variant A2663V in *CHD7*. This variant was absent from the controls and is not reported in relevant databases. In contrast to Polyphen, Polyphen2 predicts this variant as probably damaging. However, as patient´s parents were unavailable for testing, the causality of this mutation remains uncertain.

One male patient carried a *de novo* nonsense mutation in *SOX10*. To the best of our knowledge, this is the first study where *SOX10* has been analysed in a series of KS patients since Pingault et al. (2013) identified a high frequency of *SOX10* mutations in patients with KS and deafness. Accordingly, the patient with *SOX10* mutation in this study had KS and hearing loss. The prevalence of deafness in KS patients has been estimated to be approximately 5% (Quinton et al. 2001). To date, leading molecular genetic cause for this association has been a mutation in *CHD7*, but deafness is also seen in patients with mutations in *FGFR1*, *FGF8*, *KAL1*, and *PROKR2* (Costa-Barbosa et al. 2013). In patient series of this

study, six male patients had a hearing impairment, one of them had mutation in *CHD7*, one in *KAL1*, and one in *SOX10* while three remained without an identified mutation(s). The fact that a novel *SOX10* mutation was identified among only six patients with KS and hearing impairment suggests that *SOX10* should be screened whenever KS is associated with deafness. Future studies are needed to assess the true prevalence of *SOX10* mutations among patients with KS.

None of the patients in this series were found to have conclusive mutations in more than one screened gene. Two KS patients with an *FGFR1* mutation carried also variants in *NELF*. Given that the incidence of KS in Finland is 1:45 000, and these variants were also present in the controls (in 1% and 6%), it is apparent that these variants are not causing KS. Absence of rare variants in more than one gene associated with KS is opposite result to those of Sykiotis et al. (2010) and Miraoui et al. (2013), who reported that 2.5 – 19% of patients with congenital HH carried at least two rare-protein altering variants in different genes. Although this study is smaller, the fact that no evidence of di- or oligogenic inheritance was seen among Finnish KS patients suggests that di- or oligogenic inheritance in KS probably involves genes not yet implicated in KS.

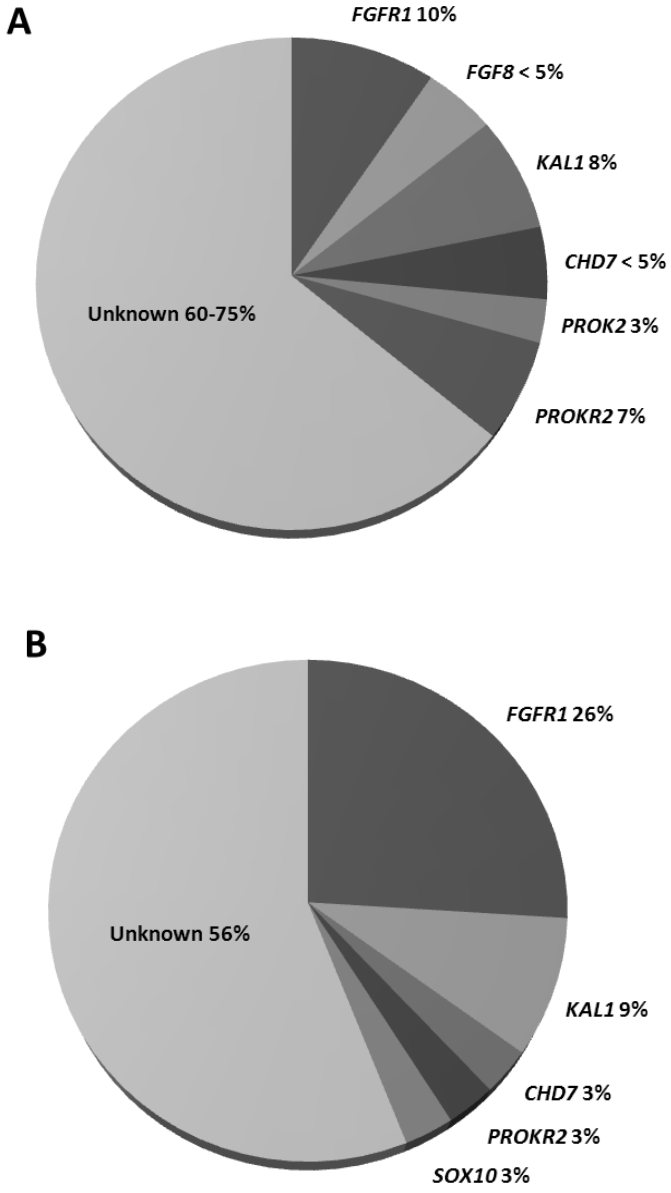

Figure 15. Molecular genetic causes of Kallmann syndrome (KS). A) Distribution of mutations in established KS genes according to reviews by Bianco and Kaiser (2009) and Dode and Rondard (2013). B) Distribution of mutations among 34 KS patients in this study.

6.3 *FGFR1* mutations

6.3.1 Variable expressivity and sex-dependent penetrance

Eight different heterozygous *FGFR1* mutations were found in nine unrelated patients. The reproductive phenotypes varied substantially even within families. In two families (KS2 and KS3, figure 7) the probands had severe KS whereas their family members carrying the same mutation had much more milder reproductive phenotype. Variable expressivity and incomplete penetrance is typical for *FGFR1* mutations (Dode et al. 2003, Pitteloud et al. 2005, Trarbach et al. 2006, Dode et al. 2007, Raivio et al. 2009). In KS, most of the phenotypic variability has been suggested to derive from actions of modifier genes (Sykiotis et al. 2010, Tornberg et al. 2011, Hanchate et al. 2012, Miraoui et al. 2013). However, since the disease phenotype can vary even between monozygotic twins (Hermanussen & Sippel 1985, Hipkin et al. 1990), it suggests that also epigenetic mechanisms and/or environmental factors influence on the development of the disease.

It has been estimated that 30% of *FGFR1* mutations are *de novo* mutations (Dode & Hardelin 2008). This percentage is low considering that *FGFR1* mutations are dominant and cause infertility (Strachan & Read 2011), but transmission to the next generation is possible because of the incomplete penetrance of the mutations. Accordingly, in this series only one of the nine *FGFR1* mutations was confirmed as *de novo* (KS1), whereas in 3 out of seven familial cases it was confirmed that *FGFR1* mutation was passed from parent to child (KS2, KS3, KS5, figure 7). However, in two cases the familial transmission occurred via assisted reproductive techniques.

A significantly higher proportion of women (5/6, 83%) carried an *FGFR1* mutation compared with men (4/28, 14%, p=0.002). A similar sex-specific difference was also found among a large series of nHH patients from US with heterogeneous genetic background (Raivio et al. 2009). The reason for this is unclear. It has been suggested that since anosmin-1 is directly involved FGFR1 signalling, the higher dose in female embryos would compensate for impaired FGFR1 signaling (Cadman et al. 2007), and this could explain the overall higher male to female ratio of KS. Results of this study do not support this theory: if some mechanism exists that protects female embryos, it is not able to compensate for deficient *FGFR1* signaling, at least not in Finnish females. Dode et al. (2003, 2007) have suggested

that sex-dependent penetrance of *FGFR1* mutations lead to a more severe phenotype in male offspring of unaffected female carriers. On the contrary, 83% of the Finnish female KS patients had an *FGFR1* mutation and tended to have more severe reproductive phenotypes than men with an *FGFR1* mutation. Especially apparent this was in family KS2, where the female proband had severe KS, her father had rather mild reproductive phenotype, and her healthy son had had normal minipuberty. However, comparison of reproductive phenotypes between sexes is not straightforward, as in females there are no suggestive phenotypic features in infancy, such as cryptorchidism or micropenis. Also, normal minipuberty in males is not necessarely indicative of normal pubertal development (Quinton et al. 2001, Pitteloud et al. 2002a).

6.3.2 Functional consequences

All eigh *FGFR1* mutations identified in this study are located in different domains of the receptor (Figure 16). Four of them were previously undescribed (W4X, R209H, E670A, and S436YfsX3). Both the nonsense mutations and the frameshift mutations lead to premature stop codons and the messenger ribonucleic acids are therefore predicted to undergo nonsense mediated decay (Nicholson et al. 2010), although this was not verified. Effects of missense mutations G48S (Trarbach et al. 2006), R209H, and E670A on receptor function were studied *in vitro* with COS-1 and L6 cells. In these studies, no difference between the WT and the mutant receptors was seen in expression or in maturation patterns (Figures 8 and 9). However, both missense mutations G48S and E670A displayed weakened downstream signaling as assessed by MAPK phosphorylation (Figure 10). G48S is located in the first immunoglobulin-like domain, which is involved in the receptor autoinhibition, and interacts with the second and the third immunoglobulin -like domains. These interactions alter the affinity for FGFs and HSPGs (Groth & Lardelli 2002, Olsen et al. 2004). E670A, located in the intracellular tyrosine kinase domain of the receptor, is anticipated to disrupt autophosphorylation of the tyrosinase kinase domain (Groth & Lardelli 2002). These results were consistent with loss-of-function mutations. On the other hand, R209H, located also in the tyrosine kinase domain, displayed relatively normal p42/44 MAPK signaling, which suggests that another signaling pathway may be impaired by this mutation. Also, relatively simple functional *in vitro* studies also have limitations in predicting consequences of the mutations, because most proteins have multiple functions that can contribute to entirely different processes and are often cell or tissue-dependent (Zaghloul and Katsanis, 2010).

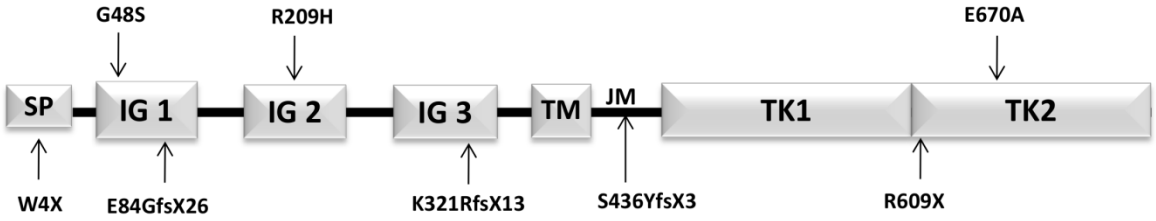

Figure 16. Schematic of the *FGFR1* mutations at protein level. SP, signal peptide; IG 1-IG 3, immunoglobulin-like domains; TM, transmembrane domain; JM, juxtamembrane domain; TK1-2, tyrosine kinase domains.

6.4 Overlap between KS and other rare syndromes

KS and CHARGE. KS can be considered as a milder allelic variant of CHARGE syndrome (Kim et al. 2008, Jongmans et al. 2009, Bergman et al. 2012). CHARGE syndrome belongs to a class of neurocristopathies, which are a diverse class of pathologies that arise from defects in the development of tissues containing cells derived from NC (Bolande 1974). All tissues involved in CHARGE syndrome are derived from cranial NC, and Siebert et al. (1985) suggested almost three decades ago that the anomalies seen in CHARGE result from abnormal development, migration or interaction of NC cells. More recently, it has been suggested that KS may also in part be a neurochristopathy (Forni et al. 2011), as patients with KS can also present with NC-associated defects such as cleft palate, dental agenesis, and deafness (Seminara et al., 1998, Forni et al. 2011). Indeed, it has been shown that Chd7 is one of the key genes for controlling the formation of cranial NC (Bajpai et al. 2010, Patten et al. 2012), Fgf8 is required for early NC survival and differentiation (Chen et al. 2012), and Fgfr1 signalling in cranial NC is essential for palatogenis (Wang et al. 2013). Moreover, it has been recently shown that NC gives rise to a subpopulation (30%) of GnRH neurons (Barraud 2010, Forni 2011, Katoh 2011). Reduced number GnRH cells due to disrupted development of NC might explain some of the milder reproductive phenotypes seen in KS patients. For example, the male KS patient in this study with CHARGE associated features and a conclusive *CHD7* mutation later on underwent reversal of HH (Laitinen et al. 2012). Screening of *CHD7* has been recommended for those KS patients with CHARGE-associated features (Jongmans et al. 2009, Bergman et al. 2012). However, as KS patients without these features may also carry

CHD7 mutations (Kim et al. 2008, Costa-Barbosa et al. 2013, this study) and even have children with CHARGE syndrome (Feret et al. 2010), should *CHD7* mutation analysis be considered to all KS patients.

KS and WS. Another link between KS and NC was offered by Pingault et al. when they discovered that mutations in *SOX10*, transcription factor essential for NC development and differentiation, cause KS with deafness (Pingault 2013). Involvement of *SOX10* mutations also in KS was suspected when MRI revealed a high frequency (88%) of OB agenesis among patients with WS (Pingault et al. 2013). Previously, there have been very few reports of anosmia and hypogonadism among WS patients with *SOX10* mutations, but these features might be underestimated in WS since ability to smell is not typically asked for and WS is often diagnosed before puberty (Pingault et al. 2013). Deafness is a known associated feature of KS (Dode et al. 2003, Costa-Barbosa et al. 2013), but to our knowledge, no pigmentation abnormalities have been reported. However, features like early greying of hair could have been easily overlooked before this association between WS and KS was discovered. Indeed, one the KS patients in the study by Pingault et al. had early greying in addition to deafness, and in retrospect fulfilled the diagnostic criteria for WS. The KS patient in this thesis study with deafness and *SOX10* mutation had also quite many grey hairs, broad nasal bridge, and mild dystopia canthorum, which could also be considered as subtle WS associated features.

Sox10 is widely expressed during early inner ear development (Breuskin et al. 2009, Breuskin et al. 2010). Besides deafness, a proportion of WS individuals with *SOX10* mutation have an enlarged vestibule, agenesis or hypoplasia of semicircular canals, and an abnormally shaped cochlea (Elmaleh-Berges et al. 2013). These features were also seen in three of the KS patients with a *SOX10* mutation (Pingault et al. 2013). As the association between ear anomalies, OB agenesis and HH is also found in CHARGE, it might be difficult to differentiate individuals affected with mild forms of CHARGE from those with WS or KS and a *SOX10* mutation. Thus, the existence of semicircular canal hypoplasia or agenesis in KS patient should be considered as an indication for both *CHD7* and *SOX10* molecular genetic analyses (Pingault et al 2013). Also, in case of identified *SOX10* mutation the possibility of more severely affected future children should be taken under consideration and offer genetic testing and counseling, as with *CHD7* mutations.

KS, HPE and SOD. Midline defects of variable severity are common in all three syndromes (Seminara et al. 1998, Dode et al. 2003, Dattani et al. 1998, Dobourg et al. 2007). Mutations in *FGFR1*, which are the leading identified molecular genetic cause of KS (Bianco & Kaiser 2009, this study), have been identified in also patients with SOD (Raivio et al. 2012) and in Harstfield syndrome, which is a rare association of HPE and ectrodactyly (Simonis et al. 2013). Ectrodactyly involves deficiency or absence of central digits and is also known as split hand/split foot malformation, or lobster claw syndrome (Moerman et al. 1998). Of note, limb anomalies have also been reported in patients with KS (Dode et al. 2003), and in this study brother of proband KS23 had an *FGFR1* mutation, nHH and fusion of 3rd and 4th metatarsal bones and lack of the 2nd and 3rd toes in both feet.

Due to the phenotypic and genotypic overlap between these three syndromes, we screened 19 KS patients without mutations in the known KS genes for mutations in *SHH, SIX3, TGIF1, TDGF1, FOXH1, GLI2* and *SOX2*, in which mutations are known to cause HPE and SOD (*SOX2*). We found 2 heterozygous missense changes, one in *GLI2* and one in *SIX3* in one male KS patient. These rare variants were predicted possibly damaging by PolyPhen2, and they were absent from 200 controls. The patient had severe KS, but no additional features, and the MRI did not reveal any signs consistent with HPE. Although there is no direct evidence that these rare variants are cause of patients KS, it cannot be ruled out as *GLI2* mutations have been described in families with variable pituitary hormone deficiencies and midline defects without signs of HPE (Franca et al. 2010), and *SIX3* mutations causing variable phenotypes including subtle microform of HPE have been reported (Solomon et al. 2010). No mutations were found in *SOX2*. Overall these results suggest that mutation in *SHH, SIX3, TGIF1, TDGF1, FOXH1, GLI2* and *SOX2* are not a common cause for KS, but as one patient harbored a novel missense change in *SIX3* and another rare missense variant in *GLI2*, a genetic overlap also in these genes between KS and HPE might exist. However, this finding needs to be confirmed by studies in different patient groups and populations.

6.5 *FGFR1, GNRHR, TAC3* and *TACR3* in CDGP

CDGP, a variant of the normal spectrum of pubertal timing, is the most common cause for delayed puberty (Sedlemeyer & Palmert 2002). In one large series of adolescents with delayed puberty, approximately 65% of boys and 30% of girls had CDGP (Sedlemeyer et al. 2002). Overall, CDGP is seen more often in boys than in girls, the male-to-female ratio has

been reported to range from 2:1 to 5:1 (Crowne et al. 1991, Sedlemeyer et al. 2002, Sedlemeyer & Palmert 2002). No physiological explanation for this exists. One reason could be the vulnerability of male HPG axis during development, as suggested in KS. Another explanation might be that boys request further investigation for their delayed puberty more easily than girls (Crocket & Petersen 1987).

The genetic background of CDGP is unknown. As delayed puberty is also seen among pedigrees with congenital HH, especially in those where the underlying genetic cause is *FGFR1* or *GNRHR* mutation(s) (Pitteloud et al. 2006b, Raivio et al. 2009, Lin et al. 2007), we hypothesized that variation in *FGFR1* and *GNRHR* could underlie some cases with CDGP.

Thesis publication V is the first study where *FGFR1* has been sequenced among patients with CDGP. Out of 146 patients, one male with CDGP and his mother with normal timing of puberty carried a previously undescribed heterozygous missense change (S436C) in *FGFR1*. The S436C missense was also present in one female control (1/30, 100 controls in total), suggesting that S436C may be a rare Finnish variant. However, it is also possible that S436C is a mutation underlying the male patients CDGP, but not sufficient to cause the CDGP phenotype in females. This would be consistent with the theory of vulnerable male HPG axis during development.

In *GNRHR*, 3 different heterozygous mutations were identified (in 7/146 cases, ~5%). Two of these (Q106R and R262Q) have been previously described in patients with autosomal recessive nHH, and shown to partially inactivate the GnRH receptor (de Roux et al. 1997). However, as only heterozygous carriers of Q106R and R262Q were found among the CDGP subjects, and both mutations were present in the controls with same frequencies as in the cases, it is evident that these heterozygous mutations are not underlying CDGP. In contrast, one male carried a previously undescribed heterozygous deletion in *GNRHR* (Phe309del), not present in 200 controls, which segregated with delayed puberty in his family (Figure 14). Phe309, located in the seventh and last transmembrane domain of the receptor, is highly conserved across species, and Phe309del has been found in a Finnish nHH patient in compound heterozygous state (Laitinen et al. 2012) providing evidence that this mutation is deleterious when accompanied by another mutation in *GNRHR*. Since *GNRHR* mutations causing nHH are recessive, and all heterozygous mutation carriers reported so far have normal reproductive phenotype (de Roux et al. 1997, Layman et al. 1998, Kottler et al. 2000,

Beranova et al. 2001, Lin et al. 2006) it is questionable whether this heterozygous mutation alone is sufficient to cause CDGP. However, we cannot completely rule out this possibility, as it has been suggested that some *GNRHR* mutations can regulate WT receptor in a dominant-negative fashion (Leanos-Miranda et al. 2003).

NKB signaling appears indispensable for HPG axis function in boys during minipuberty, as evidenced by high frequency of microphallus among male nHH probands with *TACR3* mutations (Gianetti et al. 2010). Because microphallus is not a phenotypic feature of CDGP, it seems unlikely that *TAC3* or *TACR3* mutations would underlie CDGP in males. In females, there are no phenotypic features relating to early infancy that would help to differentiate between CDGP and nHH. As reversal of HH has been reported in females with nHH and a homozygous frameshift mutation in *TAC3* (Gianetti et al. 2010), *TAC3* and *TACR3* were sequenced from females with CDGP. However, no mutations were found, suggesting that mutations in these genes are not a common cause of CDGP in females.

7 CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

The ever growing locus heterogeneity in KS presents a considerable challenge in prioritizing of genetic screening and providing optimal genetic counselling to patients and their families (Costa-Barbosa et al. 2013). Due to large number of isolated cases, small families and incomplete penetrance and variable expressivity of the mutations, mode of inheritance is often difficult or impossible to determine. Therefore, there is a need for identifying those phenotypic features that can guide prioritization of mutation analyses. In the large study of Costa-Barbosa et al. (2013) this need was addressed by performing a detailed phenotypic evaluation in a study group of over 200 KS patients from US, carrying rare sequence variants (RSVs) (MAF <1% in controls) in genes implicated in KS. In US, dental agenesis and limb anomalies in patients with *FGFR1* or *FGF8* RSVs, synkinesia in patients with *KAL1* RSVs, and hearing impairment in patients with *CHD7* RSVs stood out as discriminatory features (Table 6) (Costa-Barbosa et al. 2013). Based on the result of this thesis study, in Finland, female KS patients should be screened for *FGFR1* mutation, and presence of synkinesia is a strong indicator of *KAL1* mutation (Table 6, figure 16). Other additional features such as cleft lip and/or palate, limb anomalies, renal agenesis, and hearing impairment should also guide mutation analyses (Table 6, figure 16), although presence of additional features in general is not indicative of mutation in known KS gene (42% of patients without identified molecular genetic cause had additional features). A step-wise mutation analyses algorithm for congenital HH patiens has already been proposed previously (Dode & Hardelin 2009, Young 2012, Laitinen 2012), and with small modifications and utilizing the result of this thesis study it is applicable for Finnish KS patients (Figure 16).

Futhermore, this thesis study addressed the role of congenital HH genes *FGFR1*, *GNRHR*, *TACR3* and *TACR3* in CDGP, and although mutations in these genes are not a common cause of CDGP it is possible that variation in *FGFR1* and *GNRHR* could contribute to the phenotype (Figure 16). Overall, genetic backgroud of CDGP remains elusive.

Table 6. Prevalence of nonreproductive phenotypes across genetic groups in Finnish Kallmann Syndrome patients of this study* (Fin) and in a large U.S. study (Costa-Barbosa et al. 2013).

	***FGF*[1]**		***KAL1***		***CHD7***		**No identified mutation / RSV**	
	Fin	**US**	**Fin[2]**	**US**	**Fin**	**US**	**Fin**	**US**
Number of cases	**9**		**6**		**1**		**19**	
Sex (F/M)	**5/4****	NA	0/6	NA	0/1	NA	1/18	NA
No additional features	66% (6/9)	NA	17% (1/6)	NA	0	NA	58% (11/19)	NA
Cleft lip/palate	22% (2/9)	11% (5/47)	0	0 (0/30)	0	9% (2/22)	11% (2/19)	6% (4/64)
Limb anomalies[3]	10% (1/10)[4]	9-23% (3-8/35)	0	5-10% (1-2/20)	0	5-10% (1-2/20)	0	4-13% (2-7/55)
Dental agenesis	22% (2/9)	39% (13/33)	17% (1/6)	0 (0/9)	0	13% (2/15)	3% (1/19)	3% (3/36)
Synkinesia	0	7% (3/44)	**83%*** (5/6)**	43% (13/30)	0	5% (1/20)	0	11% (6/53)
Renal agenesis	0	0 (0/20)	17% (1/6)	18% (3/17)	0	0	0	17% (4/23)
Deafness	0	16% (7/43)	17% (1/6)	14% (3/21)	100% (1/1)	40% (8/20)	16% (3/19)	11% (6/55)
CHARGE features	0	3% (1/34)	0	0 (0/11)	100% (1/1)	5% (1/20)	11% (2/19)	2% (1/52)

1) In U.S. study, patients with *FGFR1* or *FGF8* RSVs were grouped together.
All Finnish patients in this group had an *FGFR1* mutation.
2) This group includes three additional KS males with *KAL1* mutation (*unpublished observation*). Their genotypic or phenotypic features are not included in other parts of this thesis.
3) Clinodactyly, syndactyly/polydactyly/camptodactyly, short limb bones, and short fourth metatarsal.
4) Brother of proband KS23 with limb anomalies and an *FGFR1* mutation included.
KS, Kallmann syndrome; RSV, rare sequence variant; F, female; M, Male; CHARGE features: coloboma, heart defects, choanal atresia, or ear anomalies such as cup shaped ears or semicircular canal hypoplasia/dysplasia.

* In addition, one male from Estonia, with KS, deafness and pigmentation defects carried a *SOX10* mutation, and one male KS patient, originally from Iraq, with no additional features carried a homozygous *PROKR2* mutation.

** A significantly higher proportion of females (5/6) carried an *FGFR1* mutation compared with males (4/28, p=0.002)

*** Presence of synkinesia in patients with *KAL1* mutation was statistically significant (present in 5/6 males with *KAL1* mutation vs. in 0/25 males without *KAL1* mutation, $p < 0.0001$)

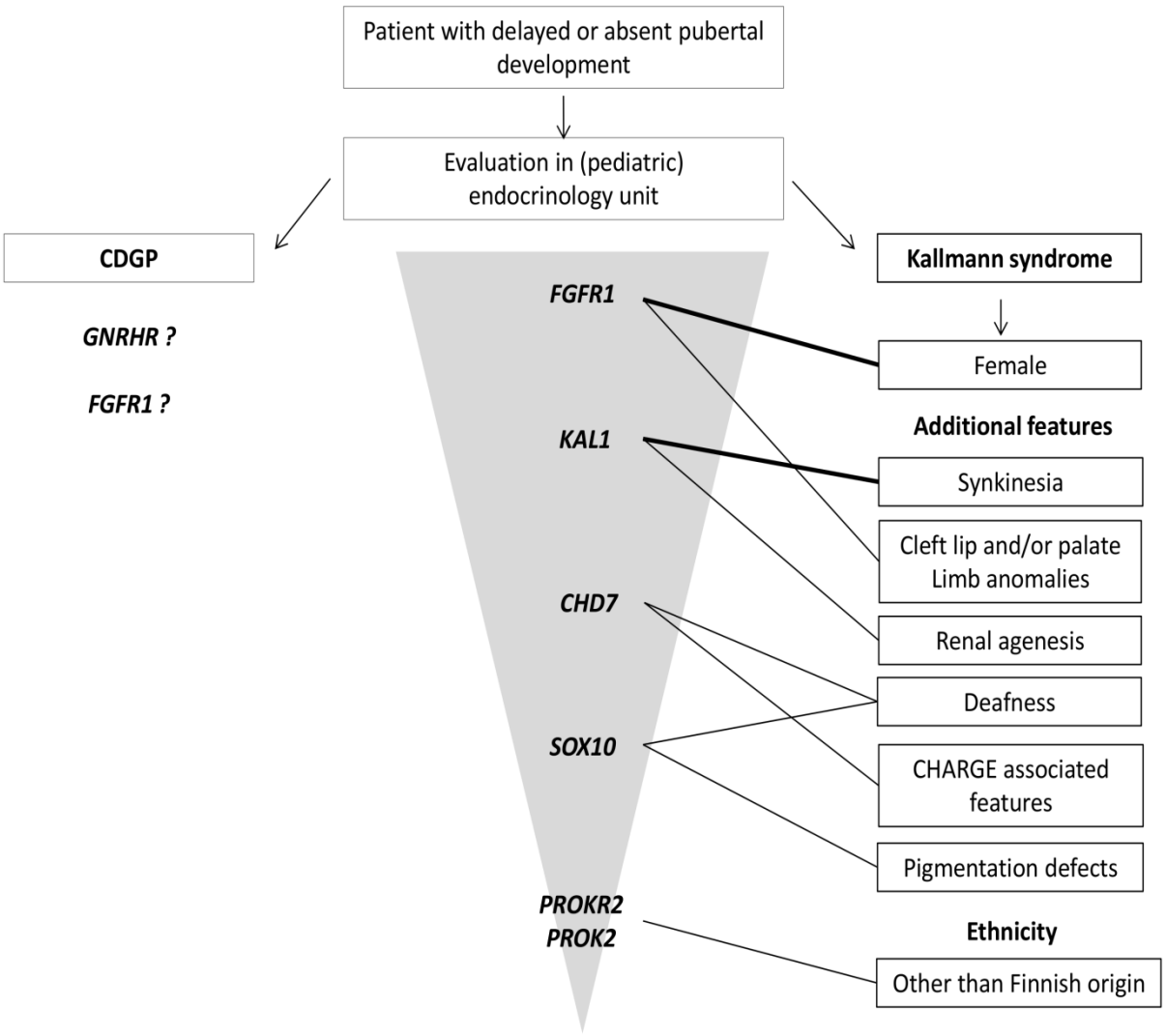

Figure 16. Proposed model for first-line molecular genetic analyses in Finnish patients diagnosed with Kallmann syndrome or constitutional delay of growth and puberty (CDGP) based on the results of this thesis and the data presented in Table 6 (Finnish patients). The triangle symbolizes the prevalence of mutations in this study. Thick lines indicate statistically significant findings (Table 6). In case of CDGP, the genetic background remains elusive. Updated and modified from Laitinen 2012.

Despite of growing locus heterogeneity, still more than 50% of KS patients remain without identified molecular genetic cause. The recent observation that NC gives rise to a subpopulation of GnRH neurons, and the precence of NC associated defects in KS patients suggest that novel genes underlying KS may be found amongst those that are involved in the development and differentiation of NC. Causal mutations may also exist in the (distant) promoter and regulatory regions of the known KS genes, and those may be identified as our knowledge about the organization of the human genome increases. In the future, next-generation sequencing (NGS) techniques will propably reveal novel genes underlying KS. Methods such as targeted exome sequencing will facilitate mutation analyses, as established KS genes, suggested KS genes and additional candidate genes can be simultaneously sequenced. Indeed, NGS techniques have proven to be powerful tools for detecting underlying genetic defects in other rare Mendelian diseases (Ng et al. 2010, Baple et al. 2013, Sousa et al. 2013). However, as mentioned above, in KS, the mode of inheritance varies and is not easy to determine, and mutations may have imcomplete penetrance and variable expressivity. Therefore, filtering sequencing data in order to identify disease causing mutations is extremely challencing. Whether mutation is identified with traditional method or with NGS technique the pathogenicity must still be proven.

So far there has been little success in identifying genes that contribute to the variation in age at pubertal onset. Screening of candidate genes in patients with CDGP has identified mutations only in rare instances. Although in the past five years genome-wide association studies have identified numerous loci that associate with timing of puberty, the actual causal variants remain unidentified. Moreover, in most of these studies identified loci associate with age of menarche, which indicates the completion of puberty in females. It remains to be seen, if causal variants are identified, whether these loci also influence timing of other pubertal phenotypes, especially in CDGP boys.

8 CONCLUSIONS

KS is a male predominant condition, with male to female ratio being 4:1 among patients of Finnish origin. The reason for this male predominance is unknown. Overall 44% of KS patients received a molecular genetic diagnosis. A clear difference was seen in the distribution of molecular genetic diagnoses in this study and in those reported previously, as the leading molecular genetic cause of KS, mutation in *FGFR1*, accounted for 26% of the cases, and mutations in *PROK2* and *PROKR2* were completely absent among Finnish patients. A significantly higher proportion of women (83%) carried an *FGFR1* mutation compared with men (14%). Female KS patients with an *FGFR1* mutation also tended to have more severe reproductive phenotypes than men with an *FGFR1* mutation, a result opposite to previous studies. One KS patient with CHARGE syndrome associated features carried a conclusive *CHD7* mutation. Two other patients with such features were not found to have a *CHD7* mutation, implying that there may be other genes associated with both syndromes. One male with KS and deafness carried a *de novo* nonsense mutation in *SOX10*, gene recently implicated in KS with hearing impairment. Three men had a mutation in *KAL1*, and they all had synkinesia. No evidence of di-or oligogenic inheritance was observed.

Considerable genotypic and phenotypic overlap is seen between KS, WS and CHARGE syndrome. From now on, hearing impairment and/or the existence of semicircular canal hypoplasia or agenesis in KS patient should be considered as an indication for both *CHD7* and *SOX10* molecular genetic analyses and, in case of identified mutation the possibility of more severely affected future children should be taken under consideration and offer genetic testing and counselling.

Mutations in known HPE genes are not a common cause for KS in Finland. However, one male with severe KS carried heterozygous missense changes, one in *GLI2* and one in *SIX3*, suggesting the possibility of additional genetic overlap between KS and HPE.

Finally, mutations in *FGFR1*, *GNRHR*, *TAC3* or *TACR3* are not a common cause of CDGP, although one male carried a previously undescribed heterozygous deletion in *GNRHR*, which segregated with delayed puberty in his family.

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