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MOLECULAR GENETICS OF RARE GROWTH AND PUBERTY DISORDERS IN FINLAND

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ACADEMIC DISSERTATION

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ABSTRACT

Growth and pubertal development are complex and interconnected processes, disruption of which leads to abnormal development of the adult height or secondary sexual characteristics or both, and often causes notable distress and even adverse health effects for the individual. Growth and pubertal development are both dependent on hormones secreted from the pituitary gland.

Growth hormone (GH), secreted from the pituitary somatotropes, is required for growth of the bones and cartilage and achievement of the adult height. Formation of GH-secreting pituitary tumors, somatotropinomas, leads to excessive GH secretion and acromegaly or gigantism, which are both characterized by exaggerated growth, either at peripheral body parts or at the long bones depending on the onset of GH excess. In a proportion of cases, a germline gene defect can predispose to somatotropinoma formation.

The ability to reproduce is achieved in puberty once the sex organs and other sexual characteristics mature into the adult form. The onset of pubertal development occurs upon the reactivation of the hypothalamic-pituitary-gonadal axis after quiescency following the previous activation phase in infancy. In the pubertal reactivation, increased gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus triggers the secretion of gonadotropins from the pituitary. Premature activation of gonadotropin secretion leads to central precocious puberty (CPP), where the pubertal development begins before the age of eight in girls or the age of nine in boys. In turn, deficient gonadotropin secretion leads to congenital hypogonadotropic hypogonadism (CHH), characterized by delayed, absent, or partial puberty. If defective sense of smell co-occurs with CHH, the condition is named Kallmann syndrome (KS).

Despite multiple genes are implicated in the disorders of pituitary hormone secretion, a great proportion of patients miss a molecular genetic diagnosis. The aim of this thesis was to discover defects in specific genes and evaluate their roles in disorders of growth and pubertal development, which originate from aberrant GH or gonadotropin secretion. The disorders were gigantism, acromegaly, CPP, and CHH.

Variants in the potassium channel gene *KCNQ1* have been previously implicated in growth hormone deficiency, and co-expression of the mutated KCNQ1 with the potassium channel subunit KCNE2 has decreased adrenocorticotropin secretion from a pituitary tumor cell line. *KCNQ1* and *KCNE2* were screened for germline variants in 49 Finnish patients and four patients of other ethnicities with acromegaly, who represented the phenotypic model opposite to growth hormone deficiency. In *KCNQ1*, deep intronic and common synonymous variants were identified, and one heterozygous variant with unknown significance in *KCNE2* was found in three patients. The frequency of the *KCNE2* variant was significantly higher among the patients compared to controls.

Two Polish and one Finnish CPP patient as well as their family members were screened for variants in *MKRN3*, a maternally imprinted gene suggested to function as a pubertal brake. Novel, deleterious variants segregating with CPP in a paternally inherited manner were identified in both families. The first *MKRN3* variant in Finnish CPP patients and the first long-term effects of a variant in this gene in a boy with CPP are described.

Twenty-four Finnish patients with normosmic CHH or KS were screened for variants in the microRNA genes *MIR7-3, MIR141, MIR429*, and *MIR200A-C*, which were predicted to regulate CHH-related genes based on evidence from animal models, literature, or bioinformatic analyses or all. A common, heterozygous variant in *MIR200A* was detected in one patient.

The genetic basis of KS in a Finnish patient with a *de novo* 2.38 Mb deletion in 9q31.2 and no likely pathogenic variants in a KS gene targeted sequencing panel was investigated with whole genome linked-read sequencing, whole exome sequencing, and RNA sequencing. In the whole genome linked-read sequencing, the deletion was found to encompass six protein-coding genes, including *ZNF462*, consistent with his Weiss-Kruszka syndrome. The deletion did not cover the nearby KS candidate gene *PALM2AKAP2,* expression of which was not suppressed by the deletion. The patient carried no rare variants in thirty-two known KS genes in the whole exome sequencing and displayed no abnormal splicing of fifteen KS genes expressed in peripheral blood leukocytes. He is the first reported patient with a 9q31.2 deletion, KS, and Weiss-Kruszka syndrome. Screening of sixteen other Finnish KS patients for variants in *PALM2AKAP2* revealed no likely pathogenic defects in this gene.

In conclusion, the thesis produced new information on the association of *KCNQ1, KCNE2*, and the selected microRNA gene variants with disorders of aberrant pituitary hormone secretion. The results demonstrate that germline variants in *KCNQ1* or *KCNE2* do not seem to account for somatotropinoma formation and that variants in the microRNA genes are unlikely causes of CHH. In turn, deletions in 9q31.2 appear to underlie KS, but based on the results, variants in the KS candidate gene *PALM2AKAP2* do not seem to contribute to the condition in the investigated cohort. In addition, novel variants in *MKRN3* were identified, and they were found to underlie CPP in Finnish patients for the first time. Finally, an interesting finding was that male carriers of *MKRN3* variants may reach their target height without treatment.

TIIVISTELMÄ
Kasvu ja murrosiän kehitys ovat monimutkaisia ja keskenään vuorovaikutteisia tapahtumasarjoja. Niiden häiriintyminen johtaa aikuispituuden, sekundaaristen sukupuoliominaisuuksien tai molempien poikkeavaan kehitykseen ja aiheuttaa usein huomattavaa ahdistusta ja jopa haitallisia terveysvaikutuksia yksilölle. Kasvu ja murrosiän kehitys ovat molemmat riippuvaisia aivolisäkkeen erittämistä hormoneista.

Aivolisäkkeen somatotrooppisolujen erittämää kasvuhormonia tarvitaan luiden ja ruston kasvuun sekä aikuispituuden saavuttamiseen. Kasvuhormonia erittävien aivolisäkkeen kasvainten eli somatotropinoomien muodostuminen johtaa liialliseen kasvuhormonin erityksen sekä gigantismiin tai akromegaliaan. Molemmille sairauksille on tunnusomaista liiallinen kasvu joko kehon ääreisosissa tai pitkissä luissa riippuen kasvuhormonin liikaerityksen alkamisajankohdasta. Osalla sairastavista ituradan geenivirhe voi altistaa somatotropinooman muodostumiselle.

Sukukypsyys saavutetaan murrosiässä, jolloin sukuelimet ja sekundaariset sukupuoliominaisuudet kypsyvät aikuisiin muotoihinsa. Murrosikäkehitys alkaa hypotalamus-aivolisäke-sukurauhanen –akselin aktivoituessa uudelleen imeväisiän lyhyttä aktivaatiota seuraavan hiljaisen vaiheen jälkeen. Murrosiän uudelleenaktivoitumisessa gonadotropiinien vapauttajahormonin eritys hypotalamuksesta lisääntyy, mikä käynnistää edelleen gonadotropiinien erityksen aivolisäkkeestä. Gonadotropiinien erityksen käynnistyminen liian aikaisin johtaa sentraaliseen ennenaikaiseen murrosikään (engl. central precocious puberty, CPP) eli murrosikäkehityksen alkamiseen tytöillä ennen kahdeksaa ja pojilla ennen yhdeksää ikävuotta. Gonadotropiinien puutteellinen eritys puolestaan johtaa synnynnäiseen hypogonadotrooppiseen hypogonadismiin (engl. congenital hypogonadotropic hypogonadism, CHH), jolle on tunnusomaista viivästynyt, puuttuva tai osittainen murrosiän kehitys. Jos CHH:n yhteydessä esiintyy vajavainen hajuaisti, sairautta kutsutaan Kallmannin oireyhtymäksi (engl. Kallmann syndrome, KS).

Siitä huolimatta, että useita geenejä on liitetty aivolisäkkeen hormonierityksen sairauksiin, suuri osuus niitä sairastavista jää vaille molekyyligeneettistä diagnoosia. Tämän väitöstyön tavoitteena oli selvittää valittujen geenien vaihtelun merkitystä poikkeavasta kasvuhormonin tai gonadotropiinien erityksestä johtuvissa kasvun ja murrosikäkehityksen sairauksissa. Nämä sairaudet olivat gigantismi, akromegalia, sentraalinen ennenaikainen murrosikä ja synnynnäinen hypogonadotrooppinen hypogonadismi.

Virheet kaliumkanavageeni *KCNQ1*:ssä on aiemmin liitetty kasvuhormonivajeeseen, ja virheellisen KCNQ1-proteiinin ilmentymisen yhdessä kaliumkanavan alayksikön KCNE2:n kanssa on osoitettu vähentävän aivolisäkehormonin eritystä aivolisäkkeen kasvainsoluista. *KCNQ1*- ja *KCNE2*-geeneistä seulottiin ituradan variantit 49 suomalaiselta ja neljältä muiden kansallisuuksien akromegaliapotilaalta, jotka edustivat kasvuhormonivajeelle vastakkaisen ilmiasun mallia. *KCNQ1*:stä löydettiin syvällä intronеissa sijaitsevia ja yleisiä synonyymisiä variantteja. *KCNE2*:sta puolestaan löydettiin kolmelta potilaalta yksi merkitykseltään tuntematon heterotsygoottinen variantti, jonka frekvenssi oli merkittävästi korkeampi tutkittujen potilaiden joukossa verrattuna kontrolleihin.

MKRN3 on äidiltä perityssä kromosomissa hiljentynyt geeni, jonka on ehdotettu toimivan murrosiän jarruna. *MKRN3* seulottiin kahdelta puolalaiselta ja yhdeltä suomalaiselta CPP-potilaalta sekä heidän perheenjäseniltään. Kummaltakin perheeltä löydettiin ennen raportoimaton ja haitallinen, isältä peritty sentraalisen ennenaikaisen murrosiän kanssa segregoiva variantti. Työssä raportoidaan ensimmäistä kertaa CPP:tä aiheuttava *MKRN3*-geenin variantti suomalaisilla sekä geenistä löytyneen variantin pitkäaikaisvaikutukset pojalla, jolla on sentraalinen ennenaikainen murrosikä.

Yhteensä kahdeltakymmeneltäneljältä suomalaiselta CHH- tai KS-potilaalta seulottiin mikroRNA:ita koodaavat *MIR7-3, MIR141, MIR429* ja *MIR200A-C,* joiden ennustettiin säätelevän CHH-geenejä eläinmallien, kirjallisuuden tai bioinformaattisten analyysien tai näiden kaikkien perusteella. Yleinen, heterotsygoottinen variantti *MIR200A*-geenissä löydettiin yhdeltä potilaalta.

Suomalaiselta KS-potilaalta ei ollut aiemmin löytynyt todennäköisesti tautia aiheuttavia KS-geenien variantteja kohdistetussa sekvensointipaneelissa, mutta hänellä oli havaittu *de novo* 2,38 megaemäksen deleetio kromosomissa 9q31.2. Hänen sairautensa geneettistä perustaa tutkittiin työssä koko genomin linked-read-, koko eksomin- ja RNA-sekvensoinneilla. Deleetion 9q31.2:ssa selvitettiin koko genomin linked-read sekvensoinnilla kattavan kuusi proteiineja koodaavaa geeniä, mukaan lukien *ZNF462*:n, jonka puutos selitti potilaan Weiss-Kruszkan oireyhtymän. Lähellä sijaitseva Kallmannin oireyhtymän ehdokasgeeni, *PALM2AKAP2,* jäi deleetion ulkopuolelle, eikä deleetio hiljentänyt sen ilmenemistä. Potilaalta ei löytynyt harvinaisia variantteja kolmessakymmenessäkahdessa tunnetussa KS-geenissä koko eksomin sekvensoinnissa, eikä silmukointi viidessätoista veressä ilmenneessä KS-geenissä ollut hänellä poikkeava. Potilas on ensimmäinen, jolla on raportoitu sekä 9q31.2-kromosomin deleetio, Kallmannin oireyhtymä että Weiss-Kruszkan oireyhtymä. *PALM2AKAP2*-geenin seulonnassa kuudellatoista muulla suomalaisella KS-potilaalla ei löytynyt todennäköisesti tautia aiheuttavia variantteja.

Kaiken kaikkiaan väitöstyö tuotti uutta tietoa *KCNQ1-, KCNE2*- ja tutkittujen mikroRNA-geenien varianttien yhteydestä poikkeaviin aivolisäkehormonien erityksen sairauksiin. Tulokset osoittavat, että *KCNQ1*- tai *KCNE2*-geenin ituradan variantit eivät näytä olevan syynä somatotropinooman muodostumiselle, ja että tutkittujen mikroRNA-geenien variantit ovat epätodennäköisiä synnynnäisen hypogonadotrooppisen hypogonadismin aiheuttajia. Sen sijaan deleetiot 9q31.2-kromosomissa näyttävät voivan aiheuttaa Kallmannin oireyhtymää, mutta ehdokasgeeni *PALM2AKAP2*:n variantit eivät tulosten perusteella liittyneet oireyhtymään tutkitulla potilasjoukolla. Lisäksi työssä löydettiin uusia *MKRN3*-geenin variantteja, ja niiden havaittiin voivan aiheuttaa sentraalista ennenaikaista murrosikää suomalaisilla ensimmäistä kertaa. Niin ikään mielenkiintoinen havainto oli, että *MKRN3*-varianttien miespuoliset kantajat voivat joskus saavuttaa aikuispituutensa myös ilman hoitoa.

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LIST OF ORIGINAL PUBLICATIONS

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

I. Iivonen AP, Känsäkoski J, Karppinen A, Kivipelto L, Schalin-Jäntti C, Karhu A, Raivio T. Screening for germline KCNQ1 and KCNE2 mutations in a set of somatotropinoma patients. *Endocr Connect.* 2018 May;7(5):645-652. doi: 10.1530/EC-18-0123.

II. Iivonen AP, Känsäkoski J, Vaaralahti K, Raivio T. Screening for mutations in selected miRNA genes in hypogonadotropic hypogonadism patients. *Endocr Connect.* 2019 May 1;8(5):506-509. doi: 10.1530/EC-19-0080.

III. Varimo T, Iivonen AP, Känsäkoski J, Wehkalampi K, Hero M, Vaaralahti K, Miettinen PJ, Niedziela M, Raivio T. Familial central precocious puberty: two novel MKRN3 mutations. *Pediatr Res.* 2021 Aug;90(2):431-435. doi: 10.1038/s41390-020-01270-z.

IV. Iivonen AP, Kärkinen J, Yellapragada V, Sidoroff V, Almusa H, Vaaralahti K, Raivio T. Kallmann syndrome in a patient with Weiss-Kruszka syndrome and a de novo deletion in 9q31.2. *Eur J Endocrinol.* 2021 May 21;185(1):57-66. doi: 10.1530/EJE-20-1387.

ABBREVIATIONS AND DEFINITIONS

3PAs	pituitary adenoma with paraganglioma/pheochromocytoma
AC	adenylyl cyclase
ACMG	The American College of Medical Genetics and Genomics
ACTH	adrenocorticotropin
AKAP2	A-kinase anchoring protein 2
AKT	AKT serine/threonine kinase
ALS	acid labile subunit
AMP	The Association for Molecular Pathology
AMPK	AMP-activated protein kinase
ANNOVAR	Annotate Variation
ANOVA	analysis of variance
AVP	arginine vasopressin
BAMS	Bosma arhinia micropthalmia syndrome
BDGP	Berkeley Drosophila Genome Project
BLASTN	Nucleotide Basic Local Alignment Search Tool
BLAT	BLAST-like alignment tool
cAMP	cyclic adenosine monophosphate
CDGP	constitutional delay of growth and puberty
CGA	glycoprotein hormones, alpha polypeptide
CHARGE	CHARGE syndrome (coloboma, heart anomaly, choanal atresia, retardation, genital and ear anomalies)
CHH	congenital hypogonadotropic hypogonadism
CL57BL6	mouse reference strain
CPP	central precocious puberty
CREB	cAMP responsive element-binding protein
CRH	corticotropin-releasing hormone
DA	dopamine
dbSNP	Single Nucleotide Polymorphism database
DECIPHER	Database of genomic variation and phenotype in humans using Ensembl Resources
DGCR8	DGCR8 microprocessor complex subunit
DICER	endoribonuclease DICER
DROSHA	drosha ribonuclease III
ERK	extracellular signal-regulated kinase
ESP	Exome Sequencing Project
ExAC	Exome Aggregation Consortium
FATHMM	Functional Analysis through Hidden Markov Models
FATHMM-MKL	FATHMM-Multiple Kernel Learning
FIPA	familial isolated pituitary adenoma
FKTN	fukutin
FSHβ	follicle stimulating hormone subunit beta
FSH	follicle stimulating hormone

FSHR	follicle stimulating hormone receptor
GABA	gamma-aminobutyric acid
GATK	Genome Analysis Toolkit
GH1	growth hormone 1 (gene)
GH	growth hormone
GHD	growth hormone deficiency
GHBP	growth hormone–binding protein
GHR	growth hormone receptor
GHRH	growth hormone–releasing hormone
GHRHR	growth hormone-releasing hormone receptor
gnomAD	Genome Aggregation Database
GnRH	gonadotropin-releasing hormone
GNRH1	gonadotropin releasing hormone 1 (gene)
GNRHR	gonadotropin releasing hormone receptor
GWAS	genome-wide association study
HSD	Honestly Significant Difference
IGF-1	insulin–like growth factor 1
IGF-1R	insulin–like growth factor 1 receptor
IGF-2	insulin–like growth factor 2
IGFBP	insulin–like growth factor binding protein
IGV	Integrative Genomics Viewer
ISO-BMI	age- and sex-adjusted body mass index
JAK	Janus kinase
KCNQ1	potassium voltage-gated channel subfamily Q member 1
KCNE2	potassium voltage-gated channel subfamily E regulatory subunit 2
KISS1	KiSS-1 metastasis suppressor
KLF4	Kruppel like factor 4
KNDy	kisspeptin/neurokinin B/dynorphin
KS	Kallmann syndrome
LH	luteinizing hormone
LHβ	luteinizing hormone subunit beta
LHR	luteinizing hormone receptor
LRT	Likelihood Ratio Test
MAF	minor allele frequency
MAPK	mitogen-activated protein kinase
MBD3	methyl-CpG binding domain protein 3
M-CAP	Mendelian Clinically Applicable Pathogenicity
MEN1	multiple endocrine neoplasia type 1
MEN2A	multiple endocrine neoplasia type 2A
MEN2B	multiple endocrine neoplasia type 2B
MetaSVM	meta-analytic support vector machine
MetaLR	meta-analytic logistic regression
miRNA	microRNA

MIR141	microRNA 141
MIR200A	microRNA 200a
MIR200B	microRNA 200b
MIR200C	microRNA 200c
MIR429	microRNA 429
MIR30B	microRNA 30b
MIR7-3	microRNA 7-3
MIR8081	microRNA 8081
MISO	Mixture-of-Isoforms
MKRN3	makorin ring finger protein 3
mTOR	mechanistic target of rapamycin kinase
NF1	neurofibromatosis type 1
NFAT	nuclear factor of activated T-cells
NKB	neurokinin B
OMIM	Online Mendelian Inheritance in Man
OXT	oxytocin
PABP	poly(A) binding proteins
PACAP	pituitary adenylate cyclase-activating polypeptide
PALM2	paralemmin 2
PALM2AKAP2	PALM2 and AKAP2 fusion
PDYN	prodynorphin
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PKA	protein kinase A
PolyPhen-2	Polymorphism Phenotyping v2
POU1F1	POU class 1 homeobox 1
PPNAD	primary pigmented nodular adrenal disease
PRL	prolactin
PROVEAN	Protein Variation Effect Analyzer
RAD23B	RAD23 homolog B, nucleotide excision repair protein
RFRP	RFamide-related peptide
RIMBP3C	RIMS binding protein 3C
RISC	RNA-induced silencing complex
SARS1	seryl-tRNA synthetase 1
SHC1	SHC adaptor protein 1
SIFT	Sorting Intolerant From Tolerant
SISu	Sequencing Initiative Suomi
SST	somatostatin
STAR	Spliced Transcripts Alignment to a Reference
STAT	signal transducer and activator of transcription
TAC3	tachykinin precursor 3
TAL2	TAL bHLH transcription factor 2
TMEM38B	transmembrane protein 38B
TSH	thyroid-stimulating hormone

TRBP	trans-activation response element RNA-binding protein
TRH	thyrotropin-releasing hormone
VUS	variant of unknown significance
WES	whole exome sequencing
WGS	whole genome sequencing
WNT	Wnt family
XLAG	X-linked acrogigantism
ZNF462	zinc finger protein 462

1. INTRODUCTION

The hormones of the pituitary gland, secreted under the control of hypothalamus, are vital in the maturation of the human body into its adult form. In this process, two essential hormonal axes are interconnected: the hypothalamic-pituitary-insulin like growth factor 1 (IGF-1) axis and the hypothalamic-pituitary-gonadal axis (HPG). Growth hormone (GH) secretion from the somatotrope cells in the anterior pituitary is stimulated by the hypothalamic growth hormone-releasing hormone (GHRH), and GH itself provokes IGF-1 production from the liver. Both GH and IGF-1 are necessary for achieving the adult height, as they participate in stimulating linear growth by acting in growth plates at the end of long bones. GH and IGF-1 are also vital in gaining adult bone mass (Dixit et al., 2021). The ability to reproduce is achieved in puberty when the sex organs and secondary sexual characteristics mature into the adult form. The onset of puberty is dependent on the HPG axis reactivation after quiescency following the previous short activation phase in infancy, minipuberty. At the onset of puberty, gonadotropin-releasing hormone (GnRH) secretion, which also IGF-1 seems to stimulate (Dees et al., 2021), begins with elevated frequency from the hypothalamic GnRH neurons. The elevated pulsatile GnRH secretion activates the secretion of gonadotropins, luteinizing hormone, and follicle-stimulating hormone, from the pituitary, which stimulate gamete formation and the gonadal secretion of sex steroids. During puberty, the hypothalamic-pituitary-IGF-1 axis is regulated by sex steroids, which drive the pubertal growth spurt and skeletal maturation (Ağırdil, 2020).

GH-secreting pituitary tumors, or somatotropinomas, result from abnormal activation of pituitary secretory cells. The tumors lead to excessive GH and IGF-1 secretion, and cause acromegaly in adults and gigantism in children and adolescents. In both conditions, excessive GH manifests as exaggerated growth; enlargement of the facial features, hands, and feet in acromegaly, and accelerated bone growth in gigantism before the fusion of the growth plates. Apart from these changes, complications risking health and reducing life quality, such as visual impairment and increased risk for cancer, can follow (Asa & Ezzat, 2021). While the majority of somatotropinomas form sporadically, a familial gene defect predisposes to somatotropinoma formation in a proportion of cases (Vasilev et al., 2020). However, in half of the familial somatotropinoma cases, the possible underlying genetic defect cannot be identified, implying that genes responsible for the tumor formation remain undiscovered (Rostomyan et al., 2015). Variants in the potassium channel gene *KCNQ1* have been implicated in autosomal dominant growth hormone deficiency, and co-expression of the mutant KCNQ1 with the potassium channel auxiliary unit KCNE2 has resulted in reduced pituitary hormone secretion in functional experiments (Tommiska et al., 2017). Given that *Kcnq1* is expressed in GHRH neurons and somatotrope cells, and that KCNQ1 and KCNE2 form potassium channels in secretory or excitable cells in several tissues (Tommiska et al., 2017; Abbott, 2015), these two genes are interesting candidates for somatotropinoma formation.

In turn, central precocious puberty (CPP) and congenital hypogonadotropic hypogonadism (CHH) are manifestations of premature or deficient pituitary hormone secretion, respectively. CPP results from premature activation of GnRH- and thus pituitary gonadotropin secretion and reactivation of the HPG axis, leading to the appearance of first signs of puberty before the age of 8 years in girls or the age of 9 years in boys (Maione et al., 2021). In turn, CHH is a rare disorder with delayed, absent, or

partial puberty as its cardinal feature. The condition originates from defects in GnRH secretion or signaling, or development of the GnRH neurons (Grinspon, 2022). Sometimes, CHH can manifest as a part of a syndrome, such as Kallmann syndrome (KS), in which patients have an absent or defective sense of smell in addition to CHH. Syndromic forms of CHH are in some cases associated with large genomic copy-number variants (Xu et al., 2015; Xu et al., 2013).

Approximately 50-80% of the variation in the timing of puberty is explained by genetic factors (Cousminer & Grant, 2020; Parent et al., 2015). However, the genetic factors controlling the onset of puberty are incompletely understood. Identifying genetic defects underlying the early and late ends of pubertal timing can reveal new components contributing to the event. Findings in animal models indicate that not only protein-coding genes but also non-coding RNAs, such as microRNAs, are key players in the onset of puberty (Cao et al., 2018). The detection of variants in *MKRN3* (Abreu et al., 2013), which appears to function in preventing premature HPG axis reactivation through ubiquitination (Li et al., 2020; Li et al., 2021; Abreu et al., 2020), has provided a significant addition to the otherwise poorly comprehended genetic causes of CPP. Indeed, defects in *MKRN3* are currently considered the most frequent genetic causes of CPP in Western countries (Soriano-Guillén et al., 2022). In Finland, however, the presence of *MKRN3* variants in CPP patients is largely unknown. Increasing comprehension of the genetic complexity of CHH and the simultaneous rise of high-throughput sequencing methods, which may reveal multiple potentially disease-causing gene defects in a single patient, have brought new challenges in discerning the relevant defects affecting the phenotype in each case. Moreover, little is still known about the role of microRNAs in human CHH. Dozens of genes have already been associated with CHH, but in approximately 50% of patients, the genetic cause remains unknown (Louden et al., 2021). The high percentage of patients missing a detected genetic cause suggests the existence of more disease genes, variants in which underlie CHH in the Finnish and other populations.

The aim of this thesis was to discover genetic defects in disorders where the normal growth or timing of puberty is disrupted due to aberrant pituitary hormone secretion: gigantism, acromegaly, CPP, and CHH.

2. REVIEW OF THE LITERATURE

2.1 Development and function of the pituitary and hypothalamus

2.1.1 Hypothalamus

The hypothalamus maintains body homeostasis by coordinating the endocrine and autonomic functions, as well as behavior, in response to internal and external stimuli. The hypothalamus is a part of the diencephalon in the brain, located under the thalamus and the third ventricle and above the pituitary gland. It receives and integrates information from interconnections to the surrounding brain parts, including the brainstem, cerebral cortex, hippocampus, amygdala, thalamus, pituitary, and retina, as well as circulation (Bear et al., 2021). The hypothalamus itself can be rostrocaudally divided into four regions: preoptic, anterior, tuberal, and mammillary hypothalamus, each of which has lateral, medial, and periventricular zones. Furthermore, each hypothalamic region consists of nuclei that serve the different hypothalamic functions (Xie & Dorsky, 2017).

The hypothalamus coordinates the endocrine system through the pituitary gland, to which it is connected via the pituitary stalk, or infundibulum. Hypothalamic control of pituitary hormone release is regulated by two types of neuroendocrine neurons, the magnocellular and parvocellular neurons, which convert the stimuli, received by the hypothalamus, into endocrine output. The magnocellular neurons lie in the paraventricular and supraoptic nuclei in the anterior hypothalamus, and they regulate the hormonal release of the posterior pituitary (neurohypophysis). The magnocellular neurons and the posterior pituitary form the hypothalamic-neurohypophyseal system, where the magnocellular neurons produce oxytocin and arginine vasopressin, and project, through the pituitary stalk, to the posterior pituitary, where they release these hormones into the bloodstream through the capillary bed and hypophyseal veins (**Figure 1**) (Xie & Dorsky, 2017). Proportions of oxytocin and arginine vasopressin are also released from parvocellular neurons in the paraventricular nucleus (Polito et al., 2011; Poisbeau et al., 2018).

The parvocellular neurons, which participate in the hormonal regulation of the anterior pituitary (adenohypophysis), lie in the preoptic nuclei; paraventricular and periventricular nuclei in the anterior hypothalamus, and in the ventromedial, dorsomedial, and arcuate nuclei in the tuberal hypothalamus (Musumeci et al., 2015; Xie & Dorsky, 2017; Sanchez Jimenez & De Jesus, 2021). The parvocellular neurons send projections to the median eminence at the base of the hypothalamus, where the neurons discharge the thyrotropin-releasing hormone (TRH), gonadotropin-releasing hormone (GnRH), growth hormone-releasing hormone (GHRH), corticotropin-releasing hormone (CRH), somatostatin (SST), and dopamine into the capillary bed which connects to the anterior pituitary via the pituitary stalk portal veins (**Figure 1**) (Chapman et al., 2020). The TRH stimulates the secretion of thyroid-stimulating hormone (TSH) and prolactin in the anterior pituitary. GnRH stimulates the release of gonadotropins (follicle-stimulating hormone and luteinizing hormone), and CRH the release of adrenocorticotrophin (ACTH). GHRH stimulates and SST inhibits the release of growth hormone and TSH, and dopamine inhibits the secretion of prolactin (Musumeci et al., 2015; Jimenez & De Jesus, 2021).

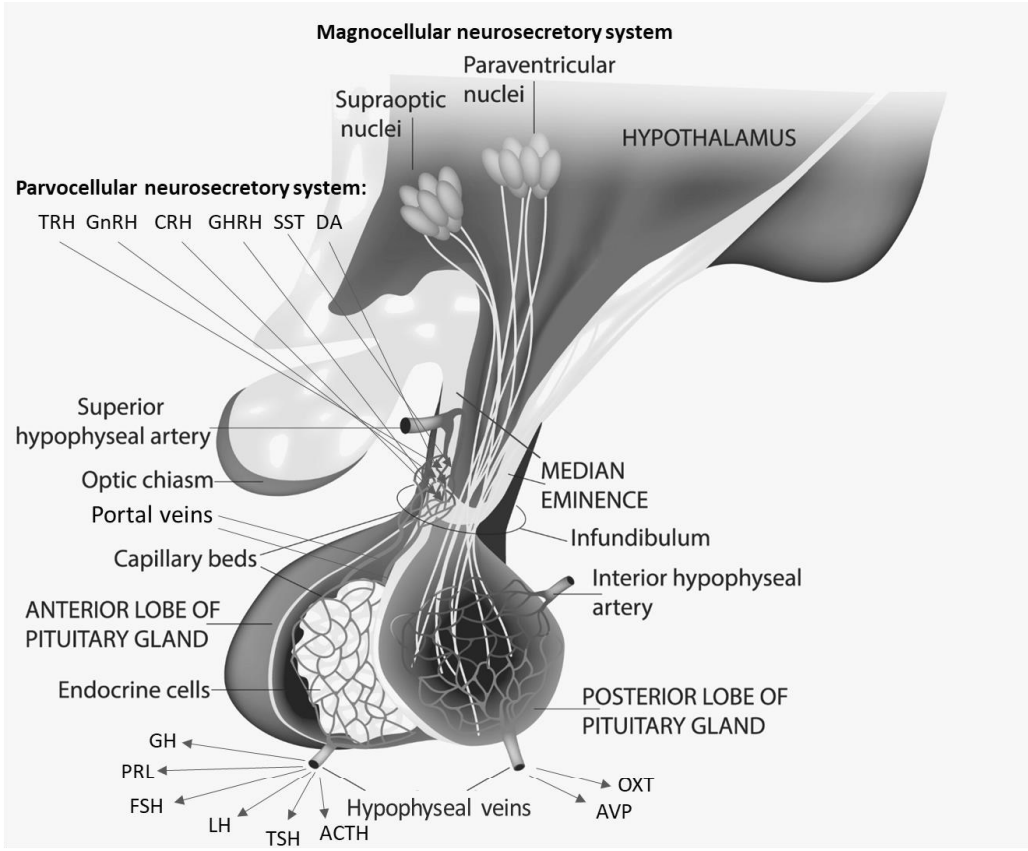


Figure 1. The neuroendocrine system of the hypothalamus and pituitary gland.
The hypothalamus connects to the anterior and posterior lobes of the pituitary via the infundibulum. The magnocellular neurons of the hypothalamic paraventricular and supraoptic nuclei project to the posterior pituitary where they release OXT and AVP to the capillary bed. OXT and AVP further flow to the circulation through the hypophyseal veins. The parvocellular neurons, which regulate the anterior pituitary hormone secretion, lie in the hypothalamic preoptic, paraventricular, periventricular, ventromedial, dorsomedial, and arcuate nuclei (nuclei not shown). The parvocellular neurons project to the capillary bed in the median eminence, where they release TRH, GnRH, GHRH, CRH, SST, and DA to the anterior pituitary via the portal veins. The TRH stimulates the secretion of TSH and PRL, GnRH stimulates the secretion of gonadotropins (FSH and LH), CRH stimulates the secretion of ACTH, and GHRH stimulates the secretion of GH from the anterior pituitary endocrine cells. SST inhibits the secretion of GH and TSH, and DA inhibits the secretion of PRL. The anterior pituitary hormones flow to the circulation from the capillary bed via the hypophyseal veins. The intermediate lobe, which secretes melanocyte-stimulating hormone and endorphins, lies between the anterior and posterior pituitary lobes (intermediate lobe not shown). OXT: oxytocin, AVP: arginine vasopressin, TRH: thyrotropin-stimulating hormone, GnRH: gonadotropin-releasing hormone, GHRH: growth hormone-releasing hormone, CRH: corticotrophin-releasing hormone, SST: somatostatin, DA: dopamine, TSH: thyroid-stimulating hormone, PRL: prolactin, FSH: follicle-stimulating hormone, LH: luteinizing hormone, ACTH: adrenocorticotrophin, GH: growth hormone. The image is modified from Sakurra/Shutterstock.com/standard license.

The hypothalamus originates from the anterior-most ventral portion of the neural tube in early development. The preoptic area rises from the telencephalon, and other parts of the hypothalamus from the diencephalon (Qin et al., 2018). The hypothalamic primordium is induced during neural plate formation, and it comprises three subregions and a cell band, which are the alar plate, basal plate, floor plate, and the intrahypothalamic diagonal (Qin et al., 2018). The alar plate gives rise to the supraoptic and paraventricular nuclei, and the basal plate generates the arcuate, dorsomedial, and ventromedial nuclei (Qin et al., 2018). WNT-, NOTCH-, Sonic hedgehog-, fibroblast growth factor-, and bone morphogenetic signaling proteins participate in the early morphogenesis of the hypothalamus and formation of the distinct hypothalamic nuclei (Qin et al., 2018; Gao & Sun, 2016). The magnocellular neurons in the paraventricular and supraoptic nuclei originate from a small neuronal progenitor cell group; one patch of these cells remains close to the third ventricle to generate the paraventricular nucleus, and the other patch migrates ventrolaterally to constitute the supraoptic nucleus (Qin et al., 2018). Similarly, the parvocellular neurons originate from distinct domains of progenitor cells, from which they migrate to their places under the guidance of a complex network of transcription factors. Among the important protein families participating in neuronal migration and axonal navigation are, in addition to the WNT-, Sonic hedgehog-, fibroblast growth factor-, and bone morphogenetic proteins, the semaphorins, neuropilins, and plexins (Alvarez-Bolado, 2019).

The parvocellular nature of the GnRH neurons is somewhat controversial as their developmental steps differ from those of other hypothalamic parvocellular neurons, yet their axons project to the median eminence. The GnRH neurons originate outside the central nervous system, from the olfactory placode, and migrate along vomeronasal nerves to the cribriform plate (Wierman et al., 2011). At the cribriform plate, the GnRH neurons turn caudally into the forebrain. After crossing the cribriform plate, the GnRH neurons start to extend long processes through the basal forebrain towards the median eminence. Finally, the neurons stop migrating and settle in the central nervous system in the preoptic and anterior hypothalamic regions (Wierman et al., 2011; Alvarez-Bolado, 2019). Specific proteins regulate the different stages of GnRH neuron migration; these proteins range from transcription factors, multiple transmembrane tyrosine kinases, G-protein-coupled receptors and their ligands to extracellular matrix proteins, including some of the same proteins that regulate parvocellular neuron migration, such as fibroblast growth factors, semaphorins, neuropilins, and plexins (Wierman et al., 2011). However, many of the GnRH migration regulatory proteins remain unknown.

2.1.2 Pituitary

The pituitary was first described by the ancient Greek physician, Claudius Galenus (Galen of Pergamon) in the 2nd century AD. He hypothesized that the pituitary gland is a receptacle for mucus (or waste products of the "animal spirit") passing from the brain ventricular structures to the nasopharynx. Furthermore, he suggested that the pituitary is surrounded by a capillary network that he named "rete mirabilis", where the "vital spirit", or energy of the body, was transformed into the "animal spirit", or a sensation and impulse that was eventually transferred to the periphery of the body and its "glands" (Toni, 2000). Galen's view was proven false by the discoveries made in the 17th and 18th centuries. By the mid-20th century, the fundamental principles of the neuroendocrine regulation of the pituitary gland had been established (Toni, 2000).

Although not a receptacle, Galen was on the right track, as indeed, the pituitary participates in the maintenance of the animal's vitality and controls its "glands". Today, the pituitary gland is known as a master endocrine organ that regulates other endocrine organs throughout the body. It lies in the sella turcica, a saddle-shaped depression in the central sphenoid bone, and is covered by diaphragma sellae (Chapman et al., 2020). The pituitary can be divided into anterior, posterior, and intermediate lobes. The anterior lobe constitutes 75% of the whole pituitary volume and encompasses the pars distalis, which contains hormone-secreting cells, and the pars tuberalis, which surrounds the pituitary stalk, contains the hypophyseal portal veins, and merges to the pars distalis (Morgan & Williams, 1996). The pars intermedia, or intermediate lobe, stands between the pars distalis and the posterior pituitary, and it contains Rathke's pouch cleft remnants. The posterior lobe is an anatomic extension of the hypothalamus and the site for oxytocin and vasopressin secretion from axon terminals of the magnocellular neurons (Chapman et al., 2020). The pituitary hormones flow to the circulation via the hypophyseal veins (**Figure 1**).

The anterior pituitary stores and secretes prolactin from lactotropes, growth hormone (GH) from somatotropes, ACTH and other proteolytic products of proopiomelanocortin (endorphins, melanocyte-stimulating hormone, and lipotropic hormone) from corticotropes; TSH from thyroptropes, and follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from gonadotropes. Broadly, lactotropes constitute 15%, somatotropes 40-50%, corticotropes 15-20%, thyrotropes 5%, and gonadotropes 10% of the anterior pituitary cells (Perez-Castro et al., 2012). In addition to the secretory cells, non-secretory folliculostellate cells constitute 5-10% of the anterior pituitary cells. The intermediate lobe secretes melanocyte-stimulating hormone, which regulates the production and distribution of melanin, as well as endorphins (Perez-Castro et al., 2012).

Prolactin stimulates milk production in mammary glands. Growth hormone stimulates insulin-like growth factor (IGF-1) production in the liver and regulates growth and nutrient metabolism. ACTH stimulates corticosteroid secretion from the adrenal gland cortex, which is crucial in adaptation to stress. TSH induces secretion of the thyroid hormones (T4 and eventually T3), which regulate energy metabolism and growth, from the thyroid gland. FSH stimulates the growth of ovarian follicles and estradiol production in ovaries in women, and testicular growth and sperm production in men, whereas LH triggers ovulation and progesterone production in women, and stimulates testosterone production in Leydig cells in men. In addition, FSH and LH participate in the maintenance of lean body mass and bone density (Hong et al., 2016; Murray & Clayton, 2013). Production of anterior pituitary hormones is mainly regulated via negative feedback loops, in which raising target organ hormone levels in the blood suppress hypothalamic and/or anterior pituitary hormone secretion. Additionally, other but direct target organ hormones, autocrine and paracrine factors, circulating growth factors and cytokines, cell-to-cell communication, as well as pathophysiological conditions, and diurnal, lifespan, and pathophysiological changes contribute to the regulation of different hypothalamic-pituitary hormonal axes (Perez-Castro et al., 2012).

Oxytocin and arginine vasopressin are secreted from the posterior pituitary. Oxytocin promotes milk secretion and uterine contraction, and its secretion is mainly regulated by reflex circuits during childbirth and breastfeeding. Oxytocin also modulates the sensation of pain (Poisbeau et al., 2018;

Qin et al., 2018). Arginine vasopressin regulates water reabsorption in the kidneys in response to increasing plasma osmolality, and thus fluid and electrolyte balance. Furthermore, arginine vasopressin can induce moderate vasoconstriction. Oxytocin and arginine vasopressin also participate in social behaviors, emotional regulation, gastrointestinal motor activity (Qin et al., 2018), as well as in ACTH secretion (Bao et al., 2008).

During the embryonic development, the anterior and intermediate lobes form from the ectodermal cells of the Rathke's pouch which develops from an invagination of the oral ectoderm (Kelberman et al., 2009). These events are controlled by a complex network of transcription factors and signaling molecules, including members of the SIX homeodomain-, paired-like homedomain-, LIM homeodomain-, and SOX transcription factor families, many of which interact with the NOTCH and WNT/β-catenin signaling molecules (Kelberman et al., 2009). In turn, the posterior lobe and the pituitary stalk originate from the midline of the ventral diencephalon in the developing central nervous system, above the Rathke's pouch. The Rathke's pouch lumen remains as the pituitary cleft and separates the anterior and intermediate lobes in the mature pituitary (Kelberman et al., 2009). Signals from the diencephalon and mesenchyme are essential for the induction and maintenance of Rathke's pouch, and for the regionalization within the pouch, allowing the emergence of the different endocrine cell types. These signals are mediated by proteins involved in bone morphogenetic protein-, fibroblast growth factor-, Sonic hedgehog-, WNT/β-catenin- and NOTCH signaling networks (Kelberman et al., 2009).

2.2 General concepts of human growth

The growth and final height of a child are regulated by complex interactions among several factors, including genes, hormones, sex, ethnic background, nutrition, prenatal environment, socioeconomic conditions, as well as physiological and psychological health in childhood. The heritability (fraction of phenotype variability that is attributable to genetic variation between individuals) estimates of height vary depending on age, being lowest in infancy (0.40 in boys and 0.38 in girls) and increasing to its greatest in adolescence, when the height of boys shows greater heritability (up to 0.83) than the height of girls (up to 0.76) (Jelenkovic et al., 2016). This suggests that environmental effects on height are strongest in early childhood, and that part of the genetic variation is sex-specific, and possibly confounded with genetic variance in pubertal events. On average, men are taller than women in human populations, particularly in the wealthiest countries, which might be partly explained by the trend of girls reaching the age at menarche earlier than boys (Jelenkovic et al., 2016; Perkins et al., 2016).

Although variation in height is largely determined by genes, it is also reflected by childhood living conditions. Nutrition, especially lack of protein, is a crucial factor in reducing height, but also diseases, particularly infections, can have an effect (Perkins et al., 2016; Bozzoli et al., 2009). Especially in wealthy European countries, an increasing trend in average height has been observed in the 20th century in parallel with decreasing infant mortality, an indicator of childhood disease (Bozzoli et al., 2009; Batty et al., 2009; Perkins et al., 2016). Inhabitants of richer and more equal countries are taller than those of poorer and less equal countries, which might reflect genetic variation, socioeconomic well-being, or gene-environment interactions, or all (Jelenkovic et al., 2016; Bozzoli

et al., 2009; Perkins et al., 2016). Indeed, socioeconomic conditions of the family, such as occupational social class, education, income, and living conditions, represent access to resources, exposure to risk factors, and health behaviors of the mother, which are all intertwined with nutrition and disease in childhood (Batty et al., 2009; Perkins et al., 2016). Finally, emotional stress or deprivation in childhood may have reducing effects on growth and height (Batty et al., 2009).

2.2.1 The hypothalamic-pituitary-IGF-1 axis

The hypothalamic-pituitary-IGF1 axis is the main hormonal regulator of growth in humans, GH and IGF-1 being its key components. Anterior pituitary somatotropes produce GH in a pulsatile and sexually dimorphic manner, a peak in secretion occurring at night (Murray & Clayton, 2013). Hypothalamic GHRH binds to a G-protein coupled receptor, GHRHR, and triggers GH gene (*GH1*) transcription via CREB and POU1F1 in response to adenylyl cyclase (AC)-cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) signaling, and it induces cAMP-mediated somatotrope proliferation (Cohen et al., 1999; Peverelli et al., 2014). Moreover, GHRH induces GH secretion to circulation through membrane depolarization by opening sodium and calcium channels (Peverelli et al., 2014; Murray & Clayton, 2013). SST, in turn, inhibits AC and cAMP synthesis and prevents GH secretion through the opening of potassium channels and membrane hyperpolarization (Murray & Clayton, 2013). Along with hypothalamic control, GH secretion is inhibited by IGF-1 and GH circulatory levels and glucocorticoids, whereas estrogen, testosterone (via aromatization to estrogen), thyroid hormones, and ghrelin stimulate GH synthesis; leptin and insulin are also thought to participate in GH regulation, yet their effects are less clear (Murray & Clayton, 2013; Bergan-Roller & Sheridan, 2018).

In circulation, secreted GH binds to GH-binding proteins (GHBPs) that are cleaved forms of growth hormone receptors (GHRs). GHBPs increase the half-life of GH in circulation and prevent GH from binding to membrane-bound GHRs. Once GH binds to a GHR on a target cell membrane, it results in dimerization of the GHR and activation of growth-promoting JAK-STAT signaling pathway (Bergan-Roller & Sheridan, 2018). In brief, in this signaling pathway, JAK2 is inactively bound to each GHR, and GH binding to GHR induces phosphorylation of both, JAK2 and GHR. The activated GHR-JAK2 complex then recruits STAT5A and STAT5B, or, to a lesser extent STAT1 and STAT3, which are phosphorylated, homo- or heterodimerized, and translocated into the nucleus to activate transcription of GH-dependent genes. Besides STATs, JAK2 can interact with other signaling molecules and activate alternative signaling pathways, such as insulin receptor substrate-PI3K-AKT, and SHC1-MAPK-mediated pathways (Bergan-Roller & Sheridan, 2018; Murray & Clayton, 2013).

GHRs are present in several tissues, being most abundant in the liver, where GH binding to GHR leads to the synthesis and secretion of IGF-1, particularly via JAK2-STAT5B pathway. IGF-1 is bound by an IGF-binding protein (IGFBP) and an acid labile subunit (ALS) in circulation which restrict its bioavailability. In the target cells, IGF-1 binds to IGF-1R receptors, and its actions are mediated via insulin receptor substrate-PI3K-AKT and SHC1-MAPK pathways (Racine & Serrat, 2020).

GH and IGF-1 regulate linear growth by binding to their receptors, GHR and IGF-1R respectively, in the cartilaginous growth plates at the ends of long bones. In the growth plates, GH induces paracrine/autocrine manner-acting IGF-1 secretion in addition to the circulatory IGF-1 from the liver (Dixit et al., 2021). GH and IGF-1 stimulate proliferation and differentiation of the cartilage cells, chondrocytes, in the growth plate, thus inducing bone growth. In this process, GH has both independent and IGF-1-dependent proliferative effects, and IGF-1 also has autocrine/paracrine effects independent of GH (Racine & Serrat, 2020). In addition to GH and IGF-1, IGF-2 is a vital growth regulator, particularly during the prenatal period, although it is not commonly included in the classical hypothalamic-pituitary-IGF-1 axis (Racine & Serrat, 2020). Finally, GH and IGF-1, together with sex hormones, contribute to the gain and maintenance of bone mineral mass. During puberty, the hypothalamic-pituitary-IGF-1 axis is upregulated by sex hormones, and bone mass is also significantly increased (Dixit et al., 2021). Longitudinal growth is completed upon estrogen-regulated fusion of the growth plates after the pubertal growth spurt (Murray & Clayton, 2013).

In addition to inducing bone growth, growth hormone has a variety of other physiological functions, some of which are mediated by IGF proteins. For instance, GH stimulates muscle growth, gonadal steroid, and thyroid hormone synthesis, and it regulates lipid metabolism (Bergan-Roller & Sheridan, 2018).

2.2.2 Genetics of growth disorders

Height is a polygenic trait following a normal distribution, with several genetic variants contributing to the final height of a healthy individual. Short and tall stature are defined as more than two standard deviations (SD) below or above the mean height for a given age, sex, and population, or the predicted midparental target height. In most cases, short or tall stature have no obvious pathological causes, and they fall in the normal genetic variation of growth and development. In other words, the underlying causes are often familial short or tall stature, constitutional delay, or an advance of growth and puberty (Pedicelli et al., 2009; Corredor et al., 2019). However, in a minority of short and tall children, a disorder underlies the condition; the more severe the deviation of height is, the more likely it is due to a disorder (Kärkinen et al., 2020; Corredor et al., 2019).

Disorders of short stature can be classified into primary and secondary disorders (Wit et al., 2007). The primary disorders of short stature include syndromes, the child being small for gestational age without catch-up growth, and skeletal dysplasias, among others. In turn, the secondary disorders of short stature include malnutrition, disorders in organ systems (such as muscular, neurological, and cardiac disorders, chronic renal failure, and celiac disease); growth hormone deficiency, and other disorders of the hypothalamic-pituitary-IGF-1 axis. The secondary disorders of short stature also include other endocrine disorders, such as hypothyroidism or combined pituitary hormone deficiency, as well as metabolic disorders, and psychosocial and iatrogenic causes (for instance, adoption) (Wit et al., 2007; Kärkinen et al., 2020). Similarly, disorders of tall stature include several types of syndromes, obesity, GH and IGF-1 excess, as well as other endocrine disorders, such as hypogonadism, precocious puberty, aromatase deficiency, and estrogen receptor α deficiency. A referral for assessment of tall stature is sought less frequently than for short stature, which reflects the wider social acceptance of tallness (Corredor et al., 2019).

Height has a complex genetic architecture. Recently, Yengo et al. (2018) performed a meta-analysis of genome-wide association studies and concluded that even 3290 single nucleotide polymorphisms (SNPs), 712 loci, and 610 genes are associated with height. Furthermore, the authors demonstrated that genes associated with height were enriched among genes that participate in skeletal growth as well as connective tissue and cartilage development (Yengo et al., 2018). Up to 19 jointly significant signals for height were particularly clustered to the locus on chromosome 12q23.2 (hg19), which contained the *IGF1* gene (Yengo et al., 2018). Indeed, defects in genes that are associated with the hypothalamic-pituitary-IGF-1 axis, and development of the hypothalamus and pituitary, are remarkable modulators of growth, and defects in them may lead to either short or tall stature. In fact, growth hormone deficiency is the most common endocrinological cause of short stature, being present in approximately 11.8% of all children fulfilling the criterion of short stature (Hussein et al., 2017). Growth hormone excess, in turn, underlies approximately 19% of cases with tall stature; yet, this estimate is based on a small cohort, thus possibly being an overestimate (Goyal et al., 2020).

2.2.3 Growth hormone deficiency

Growth hormone deficiency (GHD) can be congenital or acquired, and it can occur isolated or with other pituitary hormone deficiencies. Midline tumors, infiltrative disorders, infection, psychosocial deprivation, or damage secondary to trauma, surgery, or irradiation can cause acquired GHD, whereas genetic or structural defects in the brain cause congenital GHD. Congenital GHD is most commonly sporadic, around 30% of cases being familial, and the condition can be inherited in autosomal recessive or dominant, or X-linked manners (Alatzoglou et al., 2014; Gregory & Dattani, 2020). In recent years, digenic and oligogenic modes of inheritance have also been suggested (Vasques et al., 2020).

During the neonatal period and infancy, GHD can manifest as a short birth length and a progressive decline in length standard deviation score (SDS) during the first year of life (Boguszewski, 2020). Jaundice and prolonged hypoglycemia may also occur, and especially in the presence of combined pituitary hormone deficiency, micropenis and cryptorchidism in males can be found. Pituitary defects, such as cysts, hypoplastic or absent pituitary, anterior pituitary or pituitary stalk, or ectopic posterior pituitary, might be present, referring to a congenital condition (Collett-Solberg et al., 2019; Blum et al., 2018). Later in childhood, the characteristic features of GHD are short stature, delayed growth velocity and skeletal maturation, frontal bossing, mid-facial hypoplasia, and truncal obesity (Boguszewski, 2020). Some children with GHD may develop other pituitary hormone deficiencies over time, and various other features, related to the etiology of GHD and other possible hormone deficiencies, can manifest (Collett-Solberg et al., 2019). In both children and adults with GHD, levels of GH, IGF-1, and IGF-binding protein 3 (IGFBP-3) in circulation can be low, and psychosocial well-being, cognitive function, insulin sensitivity, endothelial and cardiovascular function, body composition, and bone mineral density can be impaired (Alatzoglou et al., 2014; Gupta, 2011).

The most common genes implicated in congenital GHD comprise *GH1* and *GHRHR,* encoding growth hormone and GHRH receptor (Blum et al., 2018). Other known genes implicated in GHD often encode transcription factors or signaling molecules that control the development of the hypothalamus and pituitary. Those genes comprise, for instance, *HESX1, HMGA2, PAX6, OTX2,*

GLI2, GLI3, GPR161, TCF7L1, PROKR2, SOX3, LHX3, SOX2, PITX2, LHX4, PROP1, and *POU1F1*. Defects in some of these genes associate with other pituitary hormone deficiencies and co-occurring developmental abnormalities, such as septo-optic dysplasia or ocular abnormalities, skeletal defects, and intellectual impairment (Alatzoglou et al., 2014; Gregory & Dattani, 2020; Vasques et al., 2019). The gene defect underlying congenital GHD is rarely found, however. One large cohort study found the underlying genetic defect in only 6.5% of patients with congenital isolated GHD and in 14.7% of patients with GHD and other pituitary hormone deficiencies (Blum et al., 2018).

A growing number of new genetic defects underlying GHD are being identified. Recent studies have indicated new genes in GHD etiology, including components of the minor spliceosome (*RNPC3*), immunoglobulins (*IGSF1*), translation initiation factors (*EIF2S3*), as well as genes involved in cell membrane integrity (*PNPLA6*), ciliary function (*IFT172*, *ALMS1*), and formation of ion channels (*KCNQ1*) (Gregory & Dattani, 2020). Tommiska et al. (2017) found missense variants in *KCNQ1* in patients with autosomal dominant GHD (which expanded to combined pituitary hormone deficiency in some patients), co-occurring mild craniofacial dysmorphic features, and maternally inherited gingival fibromatosis. The authors showed that *Kcnq1* was expressed in the murine hypothalamic GHRH neurons and somatotropes, and that co-expression of the mutant KCNQ1 protein with KCNE2 led to reduced hormone secretion from a mouse pituitary tumor cell line (Tommiska et al., 2017). *KCNQ1*, a paternally imprinted gene, encodes a potassium voltage-gated channel subfamily Q member 1, and is previously implicated in cardiac arrhythmia syndromes (Hedley et al., 2009). *KCNE2,* in turn, encodes a potassium voltage-gated channel subfamily E regulatory subunit 2, and it is an auxiliary subunit that forms functional potassium channels with KCNQ1 in secretory or excitable cells in the pituitary (Tommiska et al., 2017) and other organs (Abbott, 2015). However, the exact mechanism of how *KCNQ1* and *KCNE2* contribute to GHD remains unknown. In addition to their potential role in hormone secretion, KCNQ1 might participate in somatotrope proliferation and tumorigenesis through the Wnt/β-catenin signaling, in which *KCNQ1* is a key regulator and a target gene (Rapetti-Mauss et al., 2017). Thus, a possibility exists that defects in *KCNQ1* (and *KCNE2*) might, in some cases, lead to GH excess, discussed in the next subchapter.

2.2.4 Gigantism and acromegaly

Excessive GH and IGF-1 secretion causes acromegaly in adults and (acro-)gigantism in children and adolescents. Most patients with acromegaly or gigantism carry a benign GH-secreting adenoma, somatotropinoma, which overproduces GH (and, occasionally another pituitary hormone, often prolactin). Somatotropinomas are the third most common type of all pituitary adenomas following prolactinomas and non-functioning pituitary adenomas, and their formation can occur due to inherited and acquired genetic defects as well as epigenetic changes (Marques & Korbonits, 2017). In rare cases, excessive GHRH secretion from a hypothalamic or neuroendocrine tumor leading to somatotrope hyperplasia, or ectopic GH secretion is the cause of GH excess (Vilar et al., 2016).

Development of acromegaly is typically insidious, most patients being diagnosed between the ages of 40 and 50 years old, with an average delay of 8 years after the actual disease onset. Gigantism, in turn, occurs before fusion of the growth plates, causing excessive linear growth in children (over 3

SD above the mean height for age, or more than 2 SD above the mid-parental target height). Clinical features of acromegaly and gigantism include coarsening of facial features and voice deepening, enlargement of hands and feet due to soft tissue swelling, excessive sweating, thickened skin, headaches, and visual field disturbances (Vilar et al., 2016). Hyperprolactinemia, sleep apnea, muscle and joint pain, hypertension and cardiovascular complications, impaired libido and fertility, and insulin resistance may also occur, and the risk to certain malignant tumors is increased. In contrast to GHD, circulatory GH and IGF-1 levels are often high (Rostomyan et al., 2015). Furthermore, acromegaly and gigantism may remarkably reduce the quality of life (Vilar et al., 2016).

The great majority of somatotropinomas form sporadically and most commonly due to somatic *GNAS* gene variants, which are found in up to 40% of cases. In only around 5% of cases, a familial gene defect predisposes to somatotropinoma formation. Familial isolated pituitary adenoma (FIPA) is the commonest cause of familial acromegaly or gigantism, and it manifests as an isolated pituitary tumor without syndromic features (Bogusławska & Korbonits, 2021). Known genes implicated in FIPA comprise *AIP* and *GPR101*. In a minority of familial cases, acromegaly or gigantism can be a part of a syndrome, such as multiple endocrine neoplasia type 1 or 4, Carney complex, pheochromocytoma/paraganglioma and pituitary adenoma association, neurofibromatosis type 1, or McCune-Albright syndrome. Genes implicated in these syndromes comprise *MEN1, MEN4, PRKAR1A, PRKACB*, members of the *SDH* gene family, *NF1, MAX*, and *GNAS* (Bogusławska & Korbonits, 2021) (**Table 1**). In the end, in over a half of the cases with somatotropinomas, the underlying gene defect remains unidentified (Rostomyan et al., 2015).

Table 1. Genes implicated in familial or syndromic gigantism and acromegaly.

Gene	Full name	HGNC code	Chromosomal location (hg38)	Disease	Additional phenotype	OMIM code	Mode of inheritance
MEN1	menin 1	7010	11q13	multiple endocrine neoplasia type 1	different endocrine tumors, skin lesions	131100	autosomal dominant, sporadic (mosaicism possible)
CDKN1B	cyclin dependent kinase inhibitor 1B	1785	12p13.1	multiple endocrine neoplasia type 4	different endocrine tumors	610755	autosomal dominant, sporadic
GNAS	GNAS complex locus	4392	20q13.32	McCune-Albright syndrome	fibrous dysplasia, precocious puberty, café-au-lait skin lesions, hyperprolactinemia, endocrine organ hyperactivity	174800	sporadic, mosaic
AIP	aryl hydrocarbon receptor interacting protein	358	11q13.2	FIPA	isolated pituitary tumor	102200	autosomal dominant with incomplete penetrance, sporadic
PRKAR1A, PRKACB	protein kinase cAMP-dependent type I regulatory subunit alpha, protein kinase cAMP-activated catalytic subunit beta	9388, 9381	17q24.2, 1p31.1	Carney complex	myxomas, PPNAD, endocrine hypersecretion, schwannomas, skin pigmentation	160980	autosomal dominant, sporadic (*PRKAR1A*), sporadic (*PRKACB*)
GPR101	G protein-coupled receptor 101	14963	Xq26.3	FIPA/X-linked acrogigantism	isolated pituitary tumor, hyperprolactinemia	300943	X-linked dominant, sporadic (mosaicism possible)
SDH gene family, *MAX*	succinate dehydrogenase subunits, MYC associated factor X	641, 6913	multiple, 14q23.3	pheochromocytoma/paragan glioma and pituitary adenoma association	phaeochromocytoma, paraganglioma, optic glioma	-	autosomal dominant (*SDH* genes), sporadic (*MAX, SDH* genes)
NF1	neurofibromin 1	7765	17q11.2	neurofibromatosis type 1	neurofibromas, central precocious puberty,café-au-lait pigmentation, osseous lesions, freckling, Lisch nodules	162200	autosomal dominant, sporadic, mosaic

FIPA: familial isolated pituitary adenoma, PPNAD: primary pigmented nodular adrenal disease

MEN1

MEN1 is a tumor suppressor gene and encodes menin. The tumor suppressor role of menin is restricted to the tissues where the MEN1-associated tumors (see below) typically develop. Menin seems to perform its tumor suppressor activity through multiple processes, as it participates at least in cell division and adhesion, genome stability, cell signaling, cytoskeletal structure, and transcriptional regulation with its interaction partners (Brandi et al., 2021). The protein is known to interact with several nuclear receptors, β-catenin, JUND, SMAD family members, and MLL-histone methyltransferases, and affect the expression of cell proliferation regulators, such as p18 and p27 in murine cells (Brandi et al., 2021). Furthermore, menin and histone methyltransferase KMT2A (MLL1) regulate the expression *CDKN1B* (see ***CDKN1B***) (Schernthaner-Reiter et al, 2016). Nevertheless, menin's role in tumorigenesis is incompletely understood (Bogusławska & Korbonits, 2021).

After the gene's discovery in 1997, nearly 800 different *MEN1* germline variants have been reported (Concolino et al., 2016; Brandi et al., 2021). Inactivating *MEN1* variants associate with highly penetrant autosomal dominant (or rarely sporadic) MEN1. Approximately 10% of *MEN1* variants arise *de novo*, and mosaic variants can also occur (Thakker, 2014; Beijers et al., 2019). A single *MEN1* variant may lead to different phenotypes in different patients, which might reflect the functional versatility of menin in different tissues. However, *MEN1* variants are unidentified in 10-30% of all MEN1 patients (Schernthaner-Reiter et al, 2016; Brandi et al., 2021).

MEN1 has an estimated prevalence of 3-20:100 000 (Brandi et al., 2021). Classical features of this syndrome comprise pituitary, duodenopancreatic neuroendocrine, and parathyroid tumors or hyperplasia of the latter. Tumors of the skin, adrenal and mammary glands, stomach, lungs, thymus, and uterus may also occur, and some of these can be malignant (Brandi et al., 2021). Somatotropinomas occur in approximately 10% of MEN1 patients (Thakker, 2014). However, according to one estimation, only 3% of MEN1 patients with confirmed *MEN1* variants manifest with acromegaly (Ellard et al., 2005). In isolated acromegaly without typical MEN1 features, the occurrence of *MEN1* variants is even rarer: around 1.2% of patients under or 30 years old with isolated (sporadic) acromegaly carry a *MEN1* variant (Cuny et al., 2013). Among individuals with gigantism, around 1% carries a *MEN1* variant (Rostomyan et al., 2015).

Of note, the combination of pituitary adenomas and paragangliomas or pheochromocytomas (3PAs, see ***SDH* genes and *MAX*** below) can occur as a part of MEN1 (Xekouki et al., 2019).

CDKN1B

CDKN1B is a tumor suppressor gene, yet an atypical one, as both of its alleles are not always lost in tumors (instead, its expression can be compromised) (Frederiksen et al., 2019; Pellegata et al., 2006; Alrezk et al., 2017). *CDNK1B* encodes cyclin-dependent kinase inhibitor 1B, also known as p27 or Kip1, which regulates cell cycle progression and arrest via inhibition of several cyclins and cyclin-dependent kinases. p27 itself is regulated by ubiquitin-mediated proteasomal degradation through the MAPK and PI3K pathways (Alrezk et al., 2017). Inactivating *CDKN1B* variants associate with autosomal dominant or sporadic multiple endocrine neoplasia type 4 (MEN4). The *CDKN1B* variants

reported in MEN4 patients have altered the p27 expression in different tissues, and its translation, subcellular localization, stability, or ability to interact with partner proteins (Agarwal, 2009; Pellegata, 2006; Occhi et al., 2013).

MEN4 was initially described in rats, in whom the syndrome was named MENX, in the early 21st century. In 2006, Pellegata and colleagues identified the murine *Cdkn1b* gene and reported a *CDKN1B* variant (in the absence of *MEN1* variants) in a patient with MEN1-like features. Like in MEN1, typical features in MEN4 comprise parathyroid and pituitary neuroendocrine tumors. Other findings include gastric, intestinal, adrenal, and malignant neuroendocrine tumors, and occasional non-endocrine tumors (Frederiksen et al., 2019; Alrezk et al., 2017). Approximately 3% of patients with MEN1 phenotype harbor *CDKN1B* variants (Thakker, 2014). By the time of writing, literature has reported under 60 cases with confirmed *CDKN1B* variants (Frederiksen et al., 2019; Alrezk et al., 2017; Chevalier et al., 2020; Chasseloup et al., 2020; Pellegata et al., 2006; Tichomirowa et al., 2012, Occhi et a., 2013; Sambugaro et al., 2015; Mamedova et al., 2015), of which 6 had acromegaly or gigantism (Pellegata et al., 2006; Tichomirowa et al., 2012; Occhi et al., 2013; Sambugaro et al., 2015; Mamedova et al., 2015). Two of these cases with acromegaly or gigantism were sporadic with MEN4-like features (Occhi et al., 2013; Mamedova et al., 2015), three isolated acromegaly or gigantism without MEN4 features (Tichomirowa et al., 2012; Sambugaro et al, 2015), and one familial somatotropinoma with MEN4 features (Pellegata et al., 2006). Given the small number of reported MEN4 cases so far, complete genotype-phenotype association, as well as the penetrance and expressivity of the syndrome warrant further elucidation.

GNAS

McCune and Albright with their colleagues, independent of each other, described McCune-Albright syndrome in 1937 (McCune et al., 1937; Albright et al., 1937). They both noted in their patients the bone fibrous dysplasia, café-au-lait spots on the skin, and precocious puberty. Albright stated about the co-occurrence of these features, referring to McCune's work: "If pathologic manifestations, which at first seem to be totally disconnected, are found to occur together in a sufficient series of patients, some relation between them is apparent."

Indeed, the "pathologic manifestations" arise from mosaic activating variants in the *GNAS* (GNAS complex locus) gene. The variants emerge post-zygotically in early embryogenesis, and the extent and location of the variant-bearing tissue determine the phenotype of McCune-Albright syndrome (Spencer et al., 2019). A *GNAS* variant that produced McCune-Albright syndrome-mimicking phenotypes has been heritable in transgenic mice (Saggio et al., 2014). In humans, however, the syndrome is generally considered non-heritable in the absence of familial cases (Javaid et al., 2019; Spencer et al., 2019).

GNAS shows variable imprinting and encodes multiple alternatively spliced transcripts from both parental alleles. One of the gene products is the stimulatory G protein alpha subunit (Gsα), which is biallelically expressed in most tissues; however, in some, including the hypothalamic paraventricular nucleus, thyroid, gonads, and pituitary, it shows maternal expression (Turan & Bastepe, 2015). Gsα attaches to G-protein-coupled receptors on the cell surface. Upon ligand binding to the receptor, Gsα

dissociates and stimulates cAMP production via adenylyl cyclase. Normally, Gsα has intrinsic GTPase activity, and it converts itself back to an inactive state after a short period of action (Turan & Bastepe, 2015). The activating *GNAS* variants lead to constitutive activation of the cAMP pathway, which is central in affected tissues in McCune-Albright syndrome. The cAMP pathway activation translates into the syndrome phenotype through increased secretion of melanocyte-stimulating hormone, LH, FSH, GHRH, and ACTH, and further melanin, estradiol, testosterone, thyroxine, GH, and cortisol (Dumitrescu & Collins, 2008). In bone, constitutive Gsα activation leads to impaired differentiation of skeletal stem cells and consequent replacement of normal bone and marrow with immature bone and fibrous stroma (Spencer et al., 2019).

In addition to the bone fibrous dysplasia, café-au-lait spots on the skin, and precocious puberty that McCune and Albright described, McCune-Albright syndrome may manifest with gastrointestinal diseases, acromegaly or gigantism, hearing or vision loss, hyperprolactinemia, bone marrow failure, platelet dysfunction, and malignancies of the affected tissues (Spencer et al., 2019). The estimated prevalence of McCune-Albright syndrome is 1/100 000 to 1/1 000 000 (Dumitrescu & Collins, 2008).

In patients with McCune-Albright syndrome, acromegaly or gigantism is present in those who harbor a variant in the maternal allele (Turan & Bastepe, 2015) Acromegaly affects 20-30% of McCune-Albright patients in total, being more common in males (Yao et al., 2017). Over 80% of patients with acromegaly can have hyperprolactinemia (Salenave et al., 2014). In patients with gigantism, McCune-Albright syndrome is present in 4.9% (Rostomyan et al., 2015). Of note, *GNAS* variants are also the commonest cause of isolated somatic somatotropinomas (Bogusławska & Korbonits, 2021).

AIP

A study from Finland first described the association of FIPA with inactivating variants in the *AIP* gene (encoding aryl hydrocarbon receptor interacting protein) in 2006 (Vierimaa et al., 2006). Thereafter, *AIP* founder mutations have appeared in several countries, including Finland (Vierimaa et al., 2006), Italy (Occhi et al., 2010), the United Kingdom (Salvatori et al., 2017), and Northern Ireland (Radian et al., 2017). In fact, the Northern Irish founder mutation may provide a genetic background to the legends of giants in the Irish folklore (Radian et al., 2017).

In real life, gigantism and acromegaly are indeed common in FIPA, a condition where pituitary adenomas, whether same or different type, occur in two or more family members without syndromic manifestations. The exact prevalence of FIPA is unknown (Bogusławska & Korbonits, 2021). In a cohort of 1231 FIPA families, acromegaly or gigantism was present in 46.6%, and *AIP* variants occurred in 39% of the cohort (Hernández-Ramírez et al., 2015). However, *AIP* variants are heritable with low penetrance, and thus, may occur in apparently sporadic cases with isolated pituitary adenomas. Hernández-Ramírez et al. (2015) reported *AIP* variants to be present in 17% of sporadic cases.

Among both sporadic and familial *AIP* variant carriers, somatotropinoma is the commonest tumor type, yet prolactinomas, corticotropinomas, thyrotropinomas, gonadotropin-secreting, or non-functioning adenomas are other possible manifestations (Marques et al., 2020; Hernández-Ramírez

et al., 2015). Moreover, Marques and colleagues (2020) noted that *AIP* variant carriers exhibit specific features compared to those with isolated pituitary adenomas, in whom *AIP* variants are absent: the variant carriers are taller, more commonly males, and have an earlier disease onset (typically before the age of 30 years old), and age at diagnosis. Their condition shows more often suprasellar extension and pituitary apoplexy and requires more extensive treatment. Moreover, *AIP* variant carriers more commonly have GH excess and gigantism as the predominant diagnosis than non-carriers (Marques et al., 2020). *AIP* variants are present in 29% of patients with gigantism (Rostomyan et al., 2015).

AIP shows tumor suppressor activity in pituitary tumors. It is a co-chaperone that forms a complex with the aryl hydrocarbon receptor, stabilizes the receptor and enhances its cytoplasmic localization (Schernthaner-Reiter et al., 2020). The aryl hydrocarbon receptor regulates the intracellular response to halogenated aromatic hydrocarbons, and it has been suggested that exposure to certain environmental toxins may increase the risk for GH excess. In addition, abnormalities in the aryl hydrocarbon receptor partners, such as loss of aryl hydrocarbon receptor nuclear translocator (ARNT) expression, have been detected in AIP-associated pituitary tumors. However, the role of aryl hydrocarbon receptor interactions in somatotropinoma formation is unclear (Schernthaner-Reiter et al., 2020).

AIP interactions are not limited to those with the aryl hydrocarbon receptor but include multiple chaperones, such as those involved in mitochondrial protein import, and members of the AC-cAMP-PKA pathway. Namely, AIP interacts with the PKA pathway via inhibitory G-protein α-subunit (Giα), phosphodiesterases PDE4A4 and PDE2A3, and with PKA itself directly or via the heat-shock protein HSP90. In turn, PKA regulates *AIP* transcription. Reduction in AIP expression has been noted to associate with altered levels of its interaction partners in the AC-cAMP-PKA pathway, particularly with increased cAMP levels and PKA activity. To conclude, the putative AIP-dependent somatotropinoma formation mechanisms are complex and may involve several pathways, the roles of which in tumorigenesis remain to be elucidated (Schernthaner-Reiter et al., 2020).

GPR101

Trivellin et al. (2014) noted the association of genomic microduplications in the Xq26.3 region, which contained the *GPR101* gene, with pediatric non-syndromic gigantism. Accordingly, they named the condition X-linked acrogigantism (XLAG). The microduplication (~500 to 600 kb long) carriers were more often girls than boys, and their accelerated growth started in infancy. Furthermore, the carriers presented with increased GH, prolactin, and IGF-1 levels, aggressive adenoma or pituitary hyperplasia, and increased *GPR101* expression in their pituitaries. Beckers et al. (2015) later confirmed and elaborated these clinical findings: the accelerated growth started at a younger age in girls than in boys, some patients had elevated GHRH levels, and many showed acromegalic features, such as coarsening of facial features and acral enlargement, as well as increased appetite and acanthosis nigricans. Under 40 confirmed XLAG patients had been described by 2020 (Trivellin et al., 2020). However, some of the historical extremely tall people probably suffered from XLAG. Such an example is Robert Wadlow, the tallest person in history (272 cm): Wadlow's abnormal growth started before 3 years of age, and he had a diagnosis of pituitary pathology (Beckers et al., 2015).

XLAG is considered a specific form of FIPA (Stiles et al., 2020). In most cases, XLAG appears sporadically due to *de novo* germline microduplications. The condition can also occur in a somatic mosaic state (in boys) or be inherited in an X-linked dominant manner. By the time of writing, three XLAG families have been described, and in all of them, the Xq26.3 microduplication was inherited from an affected mother to an affected son (Vasilev et al., 2020). According to one estimate, around 3% of FIPA families manifest with XLAG (Stiles et al., 2020). Among individuals with gigantism, approximately 10% carry Xq26.3 microduplications (Rostomyan et al., 2015). Furthermore, somatic or germline *GPR101* variants of unknown significance have been found in sporadic cases of acromegaly (Trivellin et al., 2014; Trivellin et al., 2020).

GPR101 encodes the class A orphan G protein-coupled receptor 101, 'orphan' meaning that it has no known endogenous ligand (however, some potential ligands have been suggested, such as GnRH). GPR101 is expressed, in addition to the pituitary, in hypothalamic arcuate nucleus, in which GHRH neurons locate, and in nucleus accumbens, which participates in reward-seeking eating behavior (Trivellin et al, 2020). Expression in these tissues might imply that *GPR101* potentially regulates GHRH secretion and appetite, which might explain the elevated GHRH levels and increased appetite in XLAG patients. Moreover, GPR101 shows an age-dependent expression pattern, the expression being highest during fetal development, which might imply that it plays a role in pituitary development (Trivellin et al., 2020).

Finally, GPR101 activates the AC-cAMP signaling pathway in GH-secreting cells (Trivellin et al., 2014), drives GH secretion through the G-proteins Gs and Gq/11, and their downstream pathways (AC-cAMP-PKA, and phospholipase Cβ-protein kinase C, respectively) in transgenic mice (Abboud et al., 2020). Trivellin et al. (2020) hypothesized that the Xq26.3 microduplications affect the local interactions of non-coding regulatory genes, which might lead to *GPR101* overexpression in the pituitary, and perhaps other tissues. The exact mechanism of *GPR101* overexpression in pituitary tumorigenesis, however, is incompletely understood. Many additional aspects of *GPR101* function remain to be explored, such as how *GPR101* expression is regulated, in which pituitary cells the gene is expressed, and how GPR101 regulates growth and appetite, and potentially reproduction, in concert with its putative ligands (Trivellin et al., 2020).

PRKAR1A* and *PRKACB

In 1985, Carney and colleagues reported on "the complex of myxomas, spotty pigmentation and endocrine overactivity" (Carney et al., 1985), which was later named Carney complex. Kirschner et al. later discovered the association between heterozygous *PRKAR1A* variants and Carney complex (Kirschner et al., 2000). However, even as early as 1914, Dr. Cushing treated the first patient with Carney complex and a pathogenic *PRKAR1A* variant, later confirmed from a century-old specimen (Tsay et al., 2017). Cushing's patient had complained: "I grew all over", and he had manifested with acromegaly. Further examinations revealed that he had also presented with tumors in endocrine organs (the pituitary, pancreas, parathyroids, and thyroid), lentigines, pigmented, pedunculated moles, and "adrenocortical hyperplasia with pigmented nodules", referring to primary pigmented nodular adrenocortical disease (PPNAD) (Tsay et al., 2017).

Today, primary pigmented nodular adrenocortical disease (PPNAD), is the most frequent Carney complex manifestation. Other manifestations include Cushing's syndrome, acromegaly, thyroid and gonadal tumors, lentigines and other pigmented skin lesions, cardiac, skin, bone, and breast myxomas, schwannomas, and malignant endocrine or non-endocrine tumors (Bertherat et al., 2009; Espiard et al., 2020). Carney complex is more prevalent in women than in men, women are diagnosed earlier, and more often have PPNAD (Bertherat et al., 2009). Some genotype-fenotype correlation in Carney complex has been noted: *PRKAR1A* variant carriers are more likely to have myxomas, schwannomas, pigmented skin lesions, and thyroid and gonadal tumors, and they develop these tumors earlier than non-carriers. Acromegaly is present in 12% of *PRKAR1A* (especially exonic) variant carriers (Bertherat et al., 2009). Carney complex is most commonly a highly penetrant autosomal dominant disease, yet one-third of patients have no obvious family history (Bertherat et al., 2009). Of all Carney complex patients, 70% to 80% carry a variant in *PRKAR1A* (Bertherat et al., 2009; Espiard et al., 2020). The prevalence of Carney complex remains unknown (Bouys & Bertherat, 2021).

PRKAR1A encodes the regulatory subunit 1α of PKA. PKA consists of two regulatory and two catalytic subunits. Upon G protein-coupled receptor activation, the cAMP, synthesized by AC, binds to these regulatory subunits, which then dissociate from the catalytic subunits, and the catalytic subunits are activated (Correa et al., 2015). The *PRKAR1A* defects in Carney complex lead to *PRKAR1A* haploinsufficiency and uncontrolled catalytic subunit activity. Consequently, the AC-cAMP-PKA signaling increases, triggering tumor formation in affected tissues (Correa et al., 2015; Kirschner et al., 2000; Yin et al., 2008).

Only one Carney complex patient with acromegaly, lentigines, myxomas, and a 1.6 Mb triplication in 1q31.1 including *PRKACB* has been reported (Forlino et al., 2014). *PRKACB* encodes the protein kinase cAMP-activated catalytic subunit beta. In the reported patient, PRKACB levels and cAMP activity were increased in lymphocytes, and the authors suggested that *PRKACB* gain of function is responsible for the patient's phenotype (Forlino et al., 2014). However, the role of *PRKACB* in Carney complex remains to be further elucidated.

SDH* genes and *MAX

The *SDHA, -B*, *-C,* and *-D* genes encode the four subunits of the multimeric enzyme succinate dehydrogenase (SDH), also known as mitochondrial complex II. *SDHAF2*, in turn, encodes SDH complex assembly factor 2. SDHAF2 participates in SDHA flavination, which is essential for the integrity of the SDH complex. The SDH complex binds to the inner mitochondrial membrane and participates in the tricarboxylic acid/Krebs cycle and the respiratory electron transfer chain (Gill, 2018). SDHA and SDHB constitute the catalytic core of the enzyme, whereas SDHC and SDHD anchor the enzyme to the membrane and act as a ubiquinone-binding site (Gill et al., 2018). If any component of the SDH is lost, the complex becomes unstable or unable to form, and SDHB is released to the cytoplasm where it degrades (thus, SDHB immunohistochemical staining is used as an indicator of pathogenic *SDH* complex genes (*SDH*x) variants) (Gill et al., 2018). In turn, *MAX* encodes the ubiquitously expressed MYC-associated factor X, and is involved in cell differentiation, proliferation, and apoptosis via members of the MYC transcription factor network (Carroll et al., 2018).

The first patient with pheochromocytoma and acromegaly was described in 1952 (Iversen, 1952). In 2012, Xekouki et al. reported the first patient with a germline pathogenic variant in *SDHD,* multiple paragangliomas, acromegaly, and a pheochromocytoma. Some of the patient's family members carried the same variant and manifested with paragangliomas without somatotropinomas (Xekouki et al., 2012). Xekouki et al. (2015) later named the association of pituitary adenomas with paragangliomas or pheochromocytomas or both the 3 P association (3PAs). By the time of writing, the 3PAs genetic spectrum has expanded from *SDH*x to other candidate genes (Xekouki et al., 2019).

3PAs can be familial with low penetrance or sporadic. Among the 82 3PAs patients reported by 2019, a genetic defect was found in nearly 40% (Xekouki et al., 2019). Acromegaly as a part of 3PAs has been reported in patients with *SDHD, SDHC, SDHAF2, SDHB, MAX,* and *MEN1* inactivating variants, including exon deletions (Xekouki et al., 2012; Dénes et al., 2015; Papathomas et al., 2014; de Sousa et al., 2017; Xekouki et al., 2015; Daly et al., 2018). However, the *SDHAF2* and *SDHC* variants were of unknown significance (Dénes et al., 2015; de Sousa et al., 2017), and the role of *MAX* in somatotropinoma formation remains to be shown (Daly et al., 2018). Mougel et al. (2020) investigated whether *SDH*x and *MAX* variants were present in patients with isolated pituitary adenomas and noted its rarity: 3 patients in 263 carried an *SDH*x variant, and none of the variant carriers harbored a somatotropinoma.

The pituitary adenomas in 3PAs are typically aggressive macroadenomas requiring multimodal treatment, and they may present with loss of *SDHB* immunohistochemical staining and intracytoplasmic vacuoles (Xekouki et al., 2019; Dénes et al., 2015; Daly et al., 2018). Pituitary adenomas are more common in familial cases, and the affected family members may harbor different types of adenomas. The reported types of pituitary adenomas include somatotropinomas, prolactinomas, corticotropinomas, and non-functioning adenomas (Xekouki et al., 2019). A clear genotype-phenotype correlation between *SDH*x defects and pituitary adenomas is lacking (MacFarlane et al., 2020). Acromegaly due to a GHRH-secreting pheochromocytoma may rarely occur (Dénes et al., 2015). Of note, *SDH*x variants are also associated with other tumors, including gastrointestinal stromal tumors and renal cell carcinoma, which can occasionally be present in patients with 3PAs (MacFarlane et al., 2020).

Xekouki et al. (2012) noted an increased HIF-1α (hypoxia inducible factor 1 subunit alpha) expression and indications of reduced respiratory chain function in the pituitary of their patient with an *SDHD* defect. In 2015, they reported increased HIF-1α expression, as well as hyperplasia, poorly defined heterochromatin, and enlarged mitochondria in the pituitaries of $Sdhb^{+/-}$ mice, all indicating hypoxia. Furthermore, Dénes et al. (2015) noted intracytoplasmic vacuoles, potentially due to hypoxia, in *SDH*x-related pituitary adenomas. It has been speculated that *SDH*x variants lead to activation of the hypoxia pathway, which causes a pseudohypoxic state, and triggers cell proliferation and eventually tumor formation (Xekouki et al., 2019; Dénes et al., 2015; Vandeva et al., 2019; Pepe et al., 2019). *MAX* inactivation in pheochromocytomas may lead to tumor formation through uncontrolled MYC activity (Maltais et al., 2017). However, the role of *MAX* in pituitary tumorigenesis remains to be investigated.

NF1

Neurofibromatosis type 1 (NF1) is an autosomal dominant, childhood-onset RASopathy with a prevalence of 1:3000 (Cambiaso et al., 2017; Ly & Blakeley, 2019). The condition is characterized by optic pathway and other central nervous system gliomas, cognitive and behavioral problems, café-au-lait spots on the skin, freckling, neurofibromas, Lisch nodules (iris hamartomas), bone abnormalities and scoliosis, hypertension, and endocrine disorders, such as central precocious puberty, diencephalic syndrome, and growth hormone excess (Santoro et al., 2020; Ly & Blakeley, 2019). Furthermore, NF1 patients have an increased risk for benign and malignant tumors. The type and severity of NF1 manifestations are highly variable between individuals and even members of the same family (Ly & Blakeley, 2019). *NF1* germline or mosaic, postzygotic variants are associated with NF1. Nearly one-half of the germline pathogenic variants are inherited, and the other half are sporadic. The mosaic variants can be inherited, and their carriers may show a narrower phenotype. Some genotype-phenotype correlations have been noted: microdeletions and pathogenic missense variants in certain codons may result in greater disease severity (Ly & Blakeley, 2019). In particular, *NF1* microdeletions have been associated with childhood overgrowth (Stewart et al., 2019)

The endocrine disorders in NF1 are often secondary to optic pathway gliomas. In a cohort of 166 NF1 children, Santoro et al. (2020) reported growth hormone excess in 6% of those who presented with an optic pathway glioma. Cambiaso et al. (2017) reported a higher percentage of GH excess (11%) in a cohort of 64 NF1 patients with optic gliomas. The children with GH excess showed increased height velocity and IGF-1 and IGFBP-3 levels. In all of them, the optic pathway glioma had reached the optic chiasm.

GH excess in NF1 seems to be a transient phenomenon for an unknown reason. Some patients have received no treatment for GH excess, and in others, GH excess has not returned after cessation of treatment (Santoro et al., 2020; Cambiaso et al., 2017; Josefson et al., 2016; Stewart et al., 2019). It has been suggested that the optic pathway gliomas, which have extended to the optic chiasm or beyond, may interfere with the hypothalamic control of GH secretion. The transient nature of GH excess may reflect inconstant behavior of the optic pathway gliomas, or activation of new SST pathways (Santoro et al., 2020; Cambiaso et al., 2017). Although GH excess in NF1 usually occurs without a somatotropinoma, occasional patients with NF1 and a somatotropinoma have been reported in the 21st century. In these cases, however, the co-occurrence of these manifestations might have been a coincidence (Hozumi et al., 2019; Checa Garrido et al., 2013).

NF1 encodes the ubiquitously expressed neurofibromin, which inactivates the proto-oncogene *RAS* and its downstream signaling, including MAPK and PI3K-mTOR pathways. Furthermore, neurofibromin is involved in cAMP signaling. The RAS-MAPK pathway is a crucial regulator of cell growth, differentiation, and senescence (Kiuru & Busam, 2017). Due to the shared mechanism of RAS-MAPK pathway dysregulation, NF1 has overlapping clinical features with other RASopathies, such as Noonan and Legius syndromes. Furthermore, the mTOR-PI3K pathway and cAMP signaling are deregulated in certain *NF1*-deficient tumors (Kiuru & Busam, 2017). In addition to cAMP signaling, the involvement of some RAS-dependent pathways (Raf- mitogen-activated protein kinase kinase – ERK and PI3K-mTOR) has been suggested in pituitary adenoma formation (Dworakowska

et al., 2009). Whether RAS-dependent pathways are associated with somatoropinoma formation in the context of NF1 remains at the level of speculation (Hozumi et al., 2019; Checa Garrido et al., 2013; Stewart et al., 2019).

Germline variants in genes with a potential association with GH excess

IGSF1 encodes a transmembrane immunoglobulin superfamily glycoprotein, which is highly expressed in the pituitary and hypothalamus (Sun et al., 2012; Joustra et al., 2020). Inactivating hemi- or heterozygous variants in the X-chromosomal *IGSF1* are associated with IGSF1 deficiency syndrome, which predominantly affects boys and men. The syndrome is characterized by congenital central hypothyroidism, hypoprolactinemia, late adrenarche, delayed puberty with adulthood macro-orchidism, partial, often transient GHD, and adulthood GH excess with acromegalic features (in up to 52.4% of patients). The adult heights of the patients are usually normal (Sun et al., 2012; Joustra et al., 2016; Joustra et al., 2020).

Faucz et al. (2015) reported a family with central hypothyroidism and somatomammotroph hyperplasia or tumor or both, and a segregating missense variant in *IGSF1*. The authors noted increased IGSF1 staining in a variant-bearing patient's tumor. However, the variant was detected in two healthy controls and it had no effect on GH production *in vitro*. Today, the variant's minor allele frequency is up to 1.6%, and its clinical classification is "benign" according to gnomAD v2.1.1 (https://gnomad.broadinstitute.org/). Recently, Joustra et al. (2020) demonstrated somatotrope hyperfunction in *IGSF1*-deficient humans and mice, yet the mechanism was unexamined. The authors suggested that IGSF1 may participate in either hypothalamic or somatotrope-specific GH regulation, likely in a modulatory manner. They noted that more information is required on the *IGSF1* interactome, functional domains, and normal function in the first place to propose a mechanism for *IGSF1*-related GH excess.

DICER1 encodes dicer 1, which processes precursors into mature microRNAs. Inactivating germline *DICER1* variants predispose to the variably penetrant DICER1 syndrome, also known as pleuropulmonary blastoma-familial tumor and dysplasia syndrome (Pepe et al., 2019; de Kock et al., 2020). Main manifestations of the syndrome include, in addition to pleuropulmonary blastomas, cystic nephroma, ovarian Sertoli-Leydig cell tumors, multinodular goiter, sarcomas, other rare malignant or benign tumors, as well as central nervous system tumors, such as pituitary blastomas (de Kock et al., 2020). The pituitary blastomas in DICER1 syndrome are rare with a prevalence below 1%, and they appear during the first two years of life (de Kock et al., 2014; Liu et al., 2021). The pituitary blastomas may manifest as Cushing's syndrome, ophthalmoplegia, diabetes insipidus, or all, and levels of other pituitary hormones may be altered (de Kock et al., 2020).

The pituitary blastomas in patients with *DICER1* pathogenic variants can include secretory cells showing GH immunoreactivity. Liu et al. (2020) reviewed all known, properly investigated cases of pituitary blastoma (excluding the report by Chhuon et al. (2020)) and reported GH-positive immunohistochemistry in 10 of the 14 tested pituitary blastomas. Despite the presence of GH-secreting cells, the GH and IGF-1 levels suggested no GH excess, nor the patients exhibited accelerated growth (Liu et al., 2020). The report by Chhuon et al. (2020) described a 19-year-old

woman with a likely pituitary blastoma and elevated GH and IGF-1 levels but absent signs of acromegaly or gigantism. The possibility of another type of tumor could not be fully excluded, and *DICER1* was unexamined in her (Chhuon et al., 2020). Future cases are needed to conclude whether acromegaly or gigantism can be manifestations of DICER1-related pituitary blastomas.

VHL encodes von Hippel-Lindau tumor suppressor, also known as VHL protein (pVHL). Inactivating germline variants in *VHL* predispose to autosomal dominant von Hippel-Lindau disease (Gläsker et al., 2020). The incidence of the disease is 1:36 000, and the common manifestations include highly vascularized tumors, such as hemangioblastomas in the central nervous system or retina, renal cell carcinomas, pancreatic and endolymphatic sac tumors, pheochromocytomas, epididymal cystadenomas, and paragangliomas. The patients have a risk for malignant tumors, and the disease typically manifests in young adulthood (Gläsker et al., 2020).

Pituitary adenomas or excessive pituitary hormone secretion are not classical manifestations in von Hippel-Lindau disease, although hyperprolactinemia (or symptoms referring to it) is occasionally seen in von Hippel-Lindau-related pituitary stalk hemangioblastomas (Lonser et al., 2009; Lee et al., 2015; Tudorancea et al., 2012; Shimoda et al., 2012; Cassol & Mete, 2015; Chevalier et al., 2021; Dénes et al., 2015). However, a few reports with a pituitary adenoma and von Hippel-Lindau disease or a *VHL* pathogenic variant exist: Tudorancea and colleagues (2012) described a 15-year-old boy with familial von Hippel-Lindau disease, a pituitary adenoma containing GH- and prolactin-immunoreactive cells, and two cerebellar hemangioblastomas. His prolactin levels were elevated and GH levels normal. Shimoda et al. (2012) reported a patient with a cerebellar hemangioblastoma and a pituitary adenoma showing negative immunostainings for all anterior pituitary hormones. Variants in *VHL* were unexamined, and the authors hesitated to diagnose the patient with von Hippel-Lindau disease. Dénes et al. (2015) reported one patient with a prolactinoma, pheochromocytoma, a pathogenic *VHL* variant, and a variant of unknown significance in *SDHA*. This patient (nor the patient reported by Shimoda et al. (2012) had no family history of von Hippel-Lindau disease.

The suggested disease mechanism in *VHL*-related tumors is the induction of a pseudohypoxic state (Chevalier et al., 2021), which has also been indicated in conditions, such as 3PAs, occasionally manifesting with somatotropinomas (see ***SDH* genes and *MAX***). Moreover, pVHL is potentially involved in pituitary adenoma aggressiveness and vascularization (Shimoda et al., 2013; Vidal et al., 1999). In the end, it cannot be excluded that the occasional pituitary adenomas in patients with von Hippel-Lindau disease (or a *VHL* variant) are merely coincidental (Xekouki et al., 2019; Tudorancea et al., 2012; Shimoda et al., 2012; Dénes et al., 2015).

TMEM127 encodes transmembrane protein 127, the biological role of which is incompletely understood. In tumorigenesis, TMEM127 potentially functions as an inhibitor of the mTOR signaling, which might explain the trend of increased mTORC1 activation in TMEM127-related tumors (Armaiz-Pena et al., 2021; Deng et al., 2018). The tumors associated with inactivating germline variants in *TMEM127* include pheochromocytomas, paragangliomas, and renal cell carcinomas (Armaiz-Pena et al., 2021). In addition, one patient with a *TMEM127* variant of unknown significance, acromegaly due to a somatotropinoma, and a neck paraganglioma have been reported

(Stütz et al. 2020). Activation of the mTOR upstream signaling has been implicated in pituitary adenoma formation, yet only future cases may show whether *TMEM127* can be added to the list of 3PAs genes.

RET encodes ret proto-oncogene, also known as rearranged during transcription. RET is a transmembrane glycoprotein receptor-tyrosine kinase, and the gene has been implicated in several human cancers (Drilon et al., 2018). Germline activating variants in *RET* associate with multiple endocrine neoplasia types 2A and 2B (MEN2A and MEN2B). The classical manifestations of these syndromes include medullary thyroid carcinoma, pheochromocytoma, occasional pituitary adenomas, and specifically, hyperparathyroidism in MEN2A, and mucosal ganglioneuromas and marfanoid habitus in MEN2B (Tatsi & Stratakis, 2019; Alrezk et al., 2017). In these syndromes, constitutive RET activity leads to increased PI3K-AKT-, Jun N-terminal kinase-, and RAS-ERK-mediated signaling, as well as STAT3 activation (Redaelli et al., 2018).

RET variant carriers with somatotropinomas are rare. Guerrero-Pérez et al. (2019) reported one patient with a somatotropinoma, paraganglioma, multinodular goitre, and a germline variant of unknown significance in *RET,* whereas Saito et al. (2010) described a patient with a history of somatotropinoma, current medullary thyroid carcinoma, and a variant in *RET,* which his mother with MEN2A also carried. Brauer et al. (2004), in turn, reported a family with an MEN2A-associated *RET* variant and hypothyroidism. One of the variant carriers presented with actual MEN2A and a somatotropinoma. Furthermore, Heliövaara et al. (2010) and Vargiolu et al. (2009) investigated the presence of *RET* pathogenic variants in somatotropinoma patients but found no carriers.

In normal somatotropes, RET can induce either apoptotic or antiapoptotic pathways depending on the presence of specific ligands. In addition, RET is expressed in somatotropinomas, in which it induces GH secretion via AKT in the presence of its ligand GDNF, which is required to prevent RET-dependent apoptotic pathway. If GDNF is absent, RET activates an apoptotic pathway via increased PIT-1 expression (Chenlo et al., 2019). Moreover, RET is an interaction partner of AIP in the pituitary gland (Vargiolu et al., 2009). Heliövaara et al. (2010) found no *RET* expression in *AIP* mutation-positive somatotropinomas and suggested that *RET* underexpression might be involved in *AIP*-mediated tumorigenesis. Finally, Cañibano et al. (2007) noted somatotrope hyperplasia (probably due to enhanced survival in the differentiation process) in *Ret* newborn knockout mice before their natural death due to the absence of kidneys. *RET* activation, rather than loss*,* is the classical mechanism in MEN2-tumorigenesis, whereas RET inactivating variants usually associate with Hirschsprung disease. However, cases with both MEN2 and Hirschsprung disease exist, and they associate with specific variants in *RET* (Coyle et al., 2014). The molecular mechanism of this association is poorly understood, and these specific variants were absent in the patients above (Guerrero-Pérez et al., 2019; Saito et al., 2012; Brauer et al., 2004; Coyle et al., 2014) nor they had Hirschsprung disease. It remains elusive whether these somatotropinomas were linked to pathogenic variants in *RET*.

The cyclin-dependent kinase inhibitor genes ***CDKN2B, CDKN2C, CDKN1A***, as well as ***CASR***, encoding calcium sensing receptor, and ***CDC73,*** encoding cell division cycle 73, have been screened for pathogenic variants as MEN(1)-like syndrome candidate genes (Nachtigall et al., 2020; Agarwal

et al., 2009; Turner et al., 2010; Bogusławska & Korbonits, 2021). Several cohort studies have failed to detect variants in *CDKN2B, CDKN2C,* or *CDKN1A* in patients with acromegaly and somatotropinomas (Nachtigall et al., 2020; Backman et al., 2020; Agarwal et al., 2009; Carvalho et al., 2018). In turn, at least two MEN1 phenocopies with GH excess have been reported: one patient with a likely pathogenic *CDC73* variant, acromegaly due to a somatotropinoma, and a pancreatic neoplasm (Lines et al., 2020), as well as one patient with a loss-of-function *CASR* variant, somatotropinoma and clinical MEN1 with familial hypocalciuric hypercalcemia (Turner et al., 2010).

The cyclin-dependent kinase inhibitors *CDKN2B, CDKN2C,* and *CDKN1A* are involved in cell cycle inhibition, and variants in them have been found in patients with familial primary hyperparathyroidism, or MEN1 with a prolactinoma (Agarwal et al., 2009; Brewer et al., 2019). Furthermore, *Cdkn1a*- and *Cdkn2c*-knockout mice have shown MEN1-like phenotype with pituitary adenoma or hyperplasia (Franklin et al., 2000). Inactivating variants in the G protein-coupled receptor *CASR* are associated with familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism, which are due to impaired CASR signaling, trafficking and cell surface expression in the parathyroid glands and kidneys. Pituitary adenomas are generally not considered symptoms of these syndromes (Leach et al., 2020). *CASR* is also expressed in somatotropinomas, in which it regulates intracellular Ca^{2+} and cAMP levels, as well as GH-secretory response to GHRH (Romoli et al., 1999). However, the patient reported by Turner et al. (2010) was screened only for three genes (*MEN1, CASR*, and *CDC73*), so an alternative genetic cause could not be excluded. In turn, inactivating variants in *CDC73* are associated with hyperparathyroidism-jaw tumor syndrome, which manifests as parathyroid tumors, ossifying fibromas of the jaw, and tumors in the uterus, kidneys, testes, thyroid, as well as in pituitary lactotropes. CDC73 has been implicated in the regulation of transcription and cell proliferation (possibly via the Wnt/β-catenin signaling), yet the mechanism of how *CDC73* participates in tumorigenesis (or somatotropinoma formation) is unclear (Lines et al., 2020; Brewer et al., 2019).

TSC1 encodes TSC complex subunit 1 (previous name hamartin), and ***TSC2*** encodes TSC complex subunit 2 (previous name tuberin). TSC1 and TSC2 form a complex that inhibits the mTORC1 complex activity. Inactivating variants in *TSC1* and *TSC2* associate with autosomal dominant tuberous sclerosis complex, incidence of which is 1:6000 to 1:10 000 (Wataya-Kaneda et al., 2017). As a result of the pathogenic variants, mTORC1-related pathways and HIF-1α are activated, triggering tumor formation. Clinical features of tuberous sclerosis complex include abnormally pigmented skin lesions, fibromas, cortical dysplasia and tumors, retinal and cardiac hamartomas, epilepsy, neurocognitive disorders, as well as benign and malignant tumors in the kidneys and lungs (Wataya-Kaneda et al., 2017). Neuroendocrine tumors in different organs, including the pituitary, are occasional manifestations. Patients with corticotropinomas, non-functioning pituitary adenomas, and somatotropinomas have been reported, including two patients with acromegaly or gigantism (Dworakowska & Grossman, 2009; Bogusławska & Korbonits, 2021). Although the mTORC1 signaling activation has been implicated in both pituitary adenomas and tuberous sclerosis complex, the association between the disease and pituitary adenomas is debated (Bogusławska & Korbonits, 2021; Chevalier et al., 2021; Chen et al., 2017; Dworakowska & Grossman, 2009; Dworakowska et al., 2009).

CDH23 encodes cadherin related 23, an atypical cadherin. Zhang et al. (2017) reported that rare germline missense variants in *CDH23*, which were identified by whole exome sequencing (WES), associate with sporadic and familial isolated pituitary adenomas, including somatotropinomas. The authors presented one family, in whom a *CDH23* variant was found in two symptomatic and two young, still asymptomatic members, and reported *CDH23* variants in other probands and sporadic cases with pituitary adenomas. All variants were rare (MAF under 0.05%), pathogenic according to at least one *in silico* prediction tool, and some of them lied at conserved sites in the gene. Among the sporadic cohort, up to 12% carried a *CDH23* variant, whereas among the healthy controls, they were present in 0.8% (one pathogenic variant was present among the healthy). This frequency difference yielded a statistical significance.

Generally, variants in *CDH23* associate with Usher syndrome 1D with deafness, vestibular areflexia, and vision loss, as well as non-syndromic autosomal recessive deafness (of note, the patients reported by Zhang et al. (2017) had neither disease) (Schultz et al., 2011). Furthermore, CDH23 has been implicated in tumor progression via regulation of cell-cell adhesion in the early stages of tumor invasion (Apostolopoulou & Ligon, 2012). Zhang and colleagues (2017) noted that *CDH23*-mutated pituitary adenomas were smaller and less invasive than the wild-type ones and suggested that the identified variants could be inactivating and impair cell-cell adhesion. Furthermore, some cadherins are observed in pituitary adenomas, and their altered expression and activity are implicated in tumor invasion (Chauvet et al., 2016). However, the study by Zhang et al. (2017) is the only study on *CDH23*-related familial pituitary adenomas so far, and the mechanism of adenoma formation in such cases warrants further functional studies.

2.3 Factors regulating the onset of puberty

Puberty is the stage of sexual maturation and achievement of fertility, during which the sex organs and secondary sexual characteristics develop into the adult form. Marshall & Tanner presented the sequence of physical changes in puberty in girls in 1969 and in boys in 1970, and their classification of different pubertal stages is still in use. In girls, the first sign of puberty is typically the onset of breast development (Tanner stage B2) (Marshall & Tanner, 1969), whereas in boys, the first sign is the enlargement of the scrotum and testes (Tanner stage G2) (Marshall & Tanner, 1970). Puberty starts upon reactivation of the hypothalamic-pituitary-gonadal axis with increased pulsatile secretion of GnRH from the hypothalamus. GnRH activates LH and FSH secretion from the pituitary and, via FSH and LH, the production of gametes and sex hormones from the gonads (Alotaibi, 2019). Genetic, epigenetic, and environmental factors, which interact with each other in a complex manner, are involved in the activation of the hypothalamic-pituitary-gonadal axis (Parent et al., 2015).

The timing of the pubertal onset shows great variability, approximately 5 years, among individuals. The onset of puberty occurs between 9 and 14 years of age in most boys and between 8 and 13 years of age in most girls (Farello et al., 2019). The timing of puberty correlates among ethnic groups, twins, and families, genetic factors explaining 50-80% of the variation (Cousminer & Grant, 2020; Parent et al., 2015; Gajdos et al., 2010). GWAS studies have identified up to 389 genetic loci in ~250 genes significantly associated with the age at menarche, and 76 loci in or near single genes with the age at voice breaking (Day et al., 2017; Hollis et al., 2020). Loci associated with these traits overlap,

and some fall near or in genes implicated in monogenic disorders of aberrant pubertal timing, such as *TACR3*, *GNRH1, KISS1*, *CYP19A1*, *KAL1, LEPR,* and *FGF8* (Day et al., 2017; Hollis et al., 2020). Of note, 9q31.2 appears among the loci significantly associated with both age at menarche and voice break (Day et al., 2017; Perry et al., 2009; Hollis et al., 2020). In the end, according to Day et al. (2017), the identified loci explain only ~25% of the estimated heritability of pubertal timing.

In addition to genes, pubertal timing is regulated by epigenetic factors, which potentially play a role in the adaptation of pubertal timing to environmental conditions. Upon activation of the hypothalamic-pituitary-gonadal axis, the hypothalamic epigenome changes, leading to GnRH secretion. For instance, promoters and enhancers of *Kiss1*, a driver of GnRH secretion, are released from transcriptional repressors and undergo gene-activating histone and chromatin modifications at least in rodents (Shalev & Melamed, 2020). In turn, Kiss1, along with other factors, induce activating histone modifications and demethylation at the *Gnrh1* regulatory regions. Similar epigenetic cascades (histone modifications, demethylation, chromatin modification) occur in the gonadotropin-coding genes in the pituitary upon exposure to GnRH (Shalev & Melamed, 2020).

Interestingly, some imprinted genes are significantly associated with pubertal timing, and function as potential regulators of pubertal onset through epigenetic mechanisms (Day et al., 2017; Shalev & Melamed, 2020). Such an example is the maternally imprinted *MKRN3*, which acts as a GnRH secretion brake through transcriptional repression of *KISS1* and *TACR3* promoters (Abreu et al., 2020). Regulation of *MKRN3* further provides an example of the importance of non-coding RNAs in the epigenetic regulation of pubertal onset: the microRNA mirR-30b represses *Mkrn3* in rats, and increasing hypothalamic levels of miR-30b correlate with decreasing *Mkrn3* levels towards rodent pubertal ages. Moreover, disturbance of the *Mkrn3*-miR-30b interaction during the juvenile period resulted in altered pubertal timing (Heras et al., 2019). Likewise, *Lin28b* acts as a blocker of *let-7* family miRNAs and shows decreasing hypothalamic expression levels towards the pubertal ages in rodents, whereas levels of *let-7* show the opposite trend. Cruciality of the *Lin28(b)/let-7* axis as a regulator of pubertal onset is evidenced by GWAS studies, in which the *LIN28B* locus, as well variants in *let-7* miRNA target genes, are significantly associated with the age at menarche (Avendaño et al., 2017; Day et al. 2017).

A secular trend towards earlier puberty has occurred during the past century: the average age at menarche has dropped from ~16 years of age in the early 1900s close to 13 years of age in the early 2000s (Brix et al., 2019; Parent et al., 2015). Similarly, the average age at voice break has declined at least between the 1970s and 2020s from the average of 15.5 to ~13 years (Brix et al., 2019). Earlier timing of puberty is a concern because it is associated with an increased risk for breast and ovarian cancer, cardiometabolic diseases, health risk behaviors, and shorter life span (Day et al., 2017; Hollis et al., 2020). The trend has been explained by improved health and socioeconomical conditions, endocrine-disrupting chemicals, and childhood obesity. Sufficient weight and fat mass are indeed required for pubertal development, and leptin (a hormone mainly produced by adipocytes) is one factor which gives information on body composition and energy status to the hypothalamus (Parent et al., 2015). In addition, various stress factors, malnutrition, acute and chronic illnesses, and fetal environment are suggested to modulate the reproductive axis and pubertal timing (Parent et al., 2015).

All in all, some insight has been gained on the system of driving and restraining factors involved in the activation of the hypothalamic-pituitary-gonadal axis. However, the mechanism that originally triggers the onset of puberty remains a mystery.

2.3.1 The hypothalamic-pituitary-gonadal axis

The hypothalamic-pituitary-gonadal axis is the central controller of the onset of puberty and maintenance of adult reproductive capacity. In infancy, the axis is temporarily activated, and secretion of GnRH, gonadotropins, and sex hormones is increased. This short activation phase is named minipuberty, and it is associated with the development of reproductive organs and germ cells at least in boys. After minipuberty, the hypothalamic-pituitary-gonadal axis remains dormant until puberty, when it undergoes the final reactivation (Lanciotti et al., 2018).

The pubertal increase of the hypothalamic GnRH secretion is regulated by several neuromodulators, briefly touched in section 2.3. Kisspeptin (encoded by *KISS1*), secreted from the kisspeptin neurons in the arcuate nucleus and preoptic area, is one of the most powerful GnRH secretagogues (Spaziani et al., 2021). In addition to kisspeptin, the kisspeptin-secreting neurons in the arcuate nucleus, named KNDy neurons, secrete neurokinin B (NKB, encoded by *TAC3*), as well as dynorphin (encoded by *PDYN*); NKB stimulates kisspeptin release, whereas dynorphin prevents it. The KNDy neurons are integral GnRH pulse generators, and they set the pace of GnRH secretion to the pituitary via the median eminence (Livadas & Chrousos, 2016; Nagae et al., 2021). In turn, the kisspeptin neurons in the preoptic area, which receive positive feedback from estradiol in the prepubertal period, are vital for the preovulatory LH surge and permissive for the pubertal onset (Livadas & Chrousos, 2016; Nagae et al., 2021; Clarkson et al., 2009; Smith et al., 2005; Smith et al., 2005). Nitric oxide resets the GnRH neuron electrical activity between the secretory bursts (Constantin et al., 2021). Other neuromodulators or -transmitters upstream of the KNDy neurons, such as GABA, glutamate, neuropeptide Y, PACAP, and RFRP participate in the regulation of GnRH release. Finally, MKRN3 plays an inhibitory role in GnRH secretion, whereas leptin and ghrelin regulate GnRH release in response to energy and metabolic signals; specifically, leptin allows GnRH secretion once a sufficient mass of adipose tissue has been achieved (Spaziani et al., 2021; Livadas & Chrousos, 2016).

At the start of puberty, pulsatile secretion of GnRH gradually increases, first during sleep, and later throughout the day as puberty progresses (Herbison, 2016). GnRH, encoded by *GNRH1*, binds to the G-protein coupled receptor GnRHR on the surface of the pituitary gonadotropes, and triggers synthesis and secretion of FSH and LH. FSH and LH consist of a common α subunit and a specific β subunit (FSHβ or LHβ). The binding of GnRH to its receptor on gonadotropes activates transcription of FSH and LH subunit genes via multiple potential pathways, including the protein kinase C-MAPK-, calcium/calmodulin-dependent kinase II-, Calcineurin-NFAT-, and cAMP-PKA-CREB pathways (Stamatiades & Kaiser, 2018). Rodent models have shown that *Fshb* production is primarily driven by low GnRH pulse frequencies and *Lhb* by high frequencies, whereas *Cga* (the gene encoding the common α subunit) is stimulated by GnRH regardless of pulse frequency. Moreover, GnRH induces an increase in intracellular calcium that promotes gonadotropin secretion (Stamatiades & Kaiser, 2018).

From the gonadotropes, the gonadotropins are transported to the circulation and their target cells in the testes and ovaries where FSH and LH bind to their respective G-protein coupled receptors, FSHR and LHR. Activation of the receptors induces signaling pathways (primarily the AC-cAMP-PKA-CREB) which ultimately translate into the effects of the gonadotropins (Arey & López, 2011). In boys, FSH activates testicular growth and spermatogenesis in Sertoli cells, as well as promotes the conversion of testosterone to estradiol, whereas LH stimulates testosterone production from Leydig cells (Corradi et al., 2016). In girls, the increased pulsatile GnRH secretion finally triggers the first ovulation and menstrual cycle via FSH and LH. FSH regulates estradiol production from granulosa cells and promotes follicular growth until the preovulatory stage, whereas LH surge triggers ovulation. After ovulation, LH promotes follicular luteinization, as well as ovarian production of progesterone and androgens, a part of the latter being converted into estradiol under the promotion of FSH (Reed & Carr, 2018; Richards & Pangas, 2010). The sex hormones further induce secondary sexual characteristics in both sexes, and especially estrogen supports the pubertal growth spurt (Riggs et al., 2002). Finally, GnRH and gonadotropin secretion is regulated by the sex hormones: Testosterone, especially when converted to estrogen, negatively regulates GnRH and gonadotropin release (Corradi et al., 2016; Smith et al., 2005), whereas estradiol and progesterone exert both positive and negative feedback on the hypothalamus and pituitary. During most of the menstrual cycle, their feedback is negative, yet around ovulation, estradiol induces the LH surge and progesterone the mid-cycle FSH surge (Reed & Carr, 2018; Herbison, 2016). In addition, inhibin B, a local autocrine/paracrine factor secreted from the ovarian follicles and Sertoli cells in response to FSH, exerts negative feedback on the pituitary FSH secretion (Corradi et al., 2016; Reed & Carr, 2018; Namwanje & Brown, 2016).

2.3.2 The role of miRNAs in pubertal development

MicroRNAs (miRNAs) are small non-coding RNAs, which regulate gene expression in a wide variety of organisms. Classically, miRNAs are considered gene suppressors, which inhibit translation or degrade their target gene mRNAs, although they might additionally activate or silence genes at the transcriptional level. The microRNA-encoding genes can locate in the introns or exons of noncoding RNAs, or in the introns of protein-coding genes. Furthermore, miRNAs can be derived from tRNAs and snoRNAs (Saliminejad et al., 2019; Stavast & Erkeland, 2019). The miRNAs undergo complex biogenesis pathways to reach their mature forms: Most miRNAs, following the so-called canonical mode of biogenesis (and gene regulation), are transcribed by RNA polymerase II as long primary microRNA molecules containing at least one hairpin structure. The hairpin structures are further processed into precursor-miRNAs by DROSHA and DGCR8, and then exported to the cytoplasm. In the cytoplasm, the precursory miRNAs are cleaved near the loop by DICER and TRBP into double-stranded RNAs, miRNA duplexes, which are separated (Stavast & Erkeland, 2019). One of the separated strands is degraded and the other is loaded to the RNA-induced silencing complex (RISC). In the RISC, the mature miRNAs base pair to the seed regions in the 3'UTRs of their target mRNAs, thus guiding the RISC complex to suppress the target (Stavast & Erkeland, 2019). A single miRNA can regulate multiple genes, and conversely, one gene can be regulated by several miRNAs – in fact, most mammalian genes are miRNA targets (Friedman et al., 2009).

The miRNAs are implicated in various complex traits and developmental processes, including pubertal onset and development (Rao & Pak, 2016; Cao et al., 2018). Papers demonstrating miRNA regulatory networks in the hypothalamic-pituitary-gonadal axis of laboratory animals have been mushrooming, especially those addressing regulation at the hypothalamic level (Sangiao-Alvarellos et al., 2013; Garaffo et al., 2015; Messina et al., 2016; Heras et al., 2019; Li et al., 2020; Li et al., 2019; Ju et al., 2019). Sangiao-Alvarellos et al. (2013) first demonstrated "dramatic changes" in the expression levels of the *Lin28/let-7* miRNA regulatory network in the rat hypothalamus. In the system, *Lin28a* and *Lin28b* repress the synthesis of *let-7* miRNAs, which in turn post-transcriptionally repress the expression of both *Lin28* genes. Sangiao-Alvarellos et al. (2013) showed that *Lin28a* and *Lin28b,* as well as the *let-7* family members *let-7a* and *let-7b,* were expressed in the hypothalamus, and the hypothalamic expression levels of *Lin28a* and *Lin28b* declined in male and female rats from the neonatal period towards the onset of puberty and remained low in adulthood, whereas *let-7a* and *let-7b* levels increased and stayed high. Furthermore, neonatal administration of sex steroids as well as photoperiod manipulation delayed pubertal development and affected the *Lin28/let-7* expression ratios at the expected time of puberty. The paper suggested that the *Lin28/let-7* system might play a role in the permission of the pubertal onset (Sangiao-Alvarellos et al., 2013).

Later studies have shed more light on how miRNAs link to GnRH neuron development or GnRH production, either directly or via known modulators of GnRH (Garaffo et al., 2015; Messina et al., 2016; Heras et al., 2019; Li et al., 2020; Li et al., 2019; Ju et al., 2019). Simultaneously, new potential mechanisms underlying the disorders of puberty have been highlighted. In 2015, Garaffo et al. demonstrated the importance of two miRNA families in GnRH neuron development in zebrafish. Depletion of the miR-9 and miR-200 families, likely exerting their functions via the transcription factor *foxg1*, resulted in altered GnRH neuron genesis and migration from the nasal primordium to the forebrain, as well as in delayed olfactory neuron differentiation, axon extension, and connectivity. In other words, the miRNA-depleted zebrafish recapitulated the human Kallmann syndrome phenotype (Garaffo et al., 2015).

In 2016, the study by Messina et al. indicated that the miR-200/429 family, as well as miR-155, are vital components of a complex developmental switch that controls *GnRH* promoter activity in infancy. Expression levels of both these miRNAs were upregulated in GnRH neurons in the mouse "minipubertal period", and they were shown to target several genes whose activation or repression is necessary for the minipubertal GnRH increase. Of importance, the miR-200/429 family and miR-155 affected *Gnrh1* expression via *Zeb1*, a transcriptional repressor of *Gnrh1* activators as well as *Gnrh1* itself, and *Cebpb*, a nitric oxide-mediated repressor of *Gnrh1* that acted both directly and indirectly via *Zeb1* (Messina et al., 2016). Furthermore, both miR-200/429 and miR-155 influenced *Gnrh1* expression by repressing the kisspeptin receptor. The drastic phenotype of mice with inactivated *Dicer* in their GnRH neurons further demonstrated the role of miRNAs in postnatal GnRH expression: the mutant mice exhibited GnRH deficiency, lacked the minipubertal GnRH increase, and had hypogonadotropic hypogonadism with infertility (Messina et al., 2016).

The paper by Heras et al. in 2019 integrated another miRNA regulatory axis to the multilayered control of pubertal onset: The authors showed that miR-30b, a member of the miR-30 family, targeted

the 3'UTR of *Mkrn3*, and that the two were co-expressed in the rodent arcuate nucleus Kiss1 neurons. Similarly to the *Lin28/let-7* system, the hypothalamic levels of miR-30b increased from the neonatal period towards the onset of puberty, whereas the levels of Mkrn3 decreased simultaneously (Heras et al., 2019). Neonatal estrogen administration or miR-30b target site blocking in the juvenile phase delayed pubertal development and changed hypothalamic levels of Mkrn3 and miR-30b at the expected time of puberty in female rats, indicating that the miR-30b/Mkrn3 system participates in the hypothalamic programming of pubertal onset during discrete maturational windows (Heras et al., 2019). In 2019 and 2020, more miRNAs have been added to the hypothalamic network controlling GnRH in rodents, including the miR-199 family, which regulates *Kiss1* via p38 MAPK pathway; the miR-29 family which represses the *Gnrh1* transcriptional activator *Tbx21,* and miR-664-2, which might participate in the pathogenesis of precocious puberty via hypothalamic NMDAR1 signaling (Li et al., 2020; Li et al., 2019; Ju et al., 2019).

In contrast to the reviewed papers so far, which concentrated on the hypothalamus, Ahmed et al. (2017) showed the importance of miRNAs in the hypothalamic-pituitary-gonadal axis at the level of the pituitary. Namely, homozygous miR-7a2, a member of the miR-7 family, systemic knockout mice presented with reduced pituitary weights, hypogonadotropic hypogonadism, and infertility. In the knockout mouse pituitaries, expression levels of *Lhb, Fshb,* and *Cga* were drastically decreased, and so were plasma concentrations of gonadotropins. MiR-7a2 seemed to regulate the expression and secretion of pituitary gonadotropins via BMP and prostaglandin signaling by targeting *Glg1* and *Ptgfrn* (Ahmed et al., 2017).

In humans, studies on miRNAs in the control of puberty are scarcer. Genome-wide as well as epigenome-wide association studies have linked some miRNA genes to the onset of puberty, especially age at menarche (Hollis et al., 2020; Day et al., 2017; Huan et al., 2020). In addition, several papers have investigated, with negative results, potentially pathogenic variants in *LIN28B/A* in patients with pubertal disorders (Tommiska et al., 2010; Tommiska et al., 2011; Silveira-Neto et al., 2012). In 2021, Varimo & Wang contributed to the field by showing that miR-30b levels increase during puberty in boys (Varimo & Wang, 2021). Although evidence from animal models might be relevant for human puberty and its disorders, much remains to be investigated in our species.

2.4 Central precocious puberty (CPP)

2.4.1 Clinical manifestation of CPP

Precocious puberty is defined by the appearance of the first signs of puberty before the age of 8 years in girls and before the age of 9 years in boys. These estimates may vary depending on the ethnic background: African American and Hispanic American girls tend to exhibit an earlier onset of puberty than Caucasian girls, and thus, in these two ethnic groups puberty is defined early if it commences before the age of 7.5 years (Maione et al., 2021; Aguirre & Eugster, 2018).

Central precocious puberty (CPP) refers to the onset of puberty due to premature activation of GnRH secretion, contrary to peripheral precocious puberty, in which excessive sex hormone secretion occurs independently of GnRH. In approximately 80% of cases, precocious puberty is central (Maione et al., 2021; Cheuiche et al., 2021). The incidence rates of CPP are over 10 times higher among girls than

boys. In Europe, for instance, a Danish study calculated the mean annual incidence of CPP as 9.2 in 10 000 for girls and 0.9 in 10 000 for boys (Bräuner et al., 2020). An earlier study from Spain reported the mean incidence of CPP as 0.217 in 10 000 for girls and 0.023 in 10 000 for boys (Soriano-Guillén et al., 2010), whereas a study from France reported the mean incidences as 2.6 in 10 000 for girls and 0.24 in 10 000 for boys (Le Moal et al., 2018). Furthermore, Bräuner et al. (2020) detected an increase in the annual incidence of CPP between 1998 and 2017.

Known causes of CPP include hypothalamic tumors, such as hamartomas or gliomas; other central nervous system abnormalities, such as hydrocephalus, encephalopathies, brain injuries, irradiation, and infections; environmental factors, such as nutritional excess, endocrine disruptors, and adoption, as well as gene defects (Maione et al., 2021; Mucaria et al., 2021). CPP is idiopathic in approximately 90% of girls and 25-60% of boys (Aguirre & Eugster, 2018). The genetic forms of CPP can be a manifestation of a syndrome, such as McCune-Albright syndrome or neurofibromatosis type 1, or occur without syndromic features (Maione et al., 2021; Mucaria et al., 2021). In most cases, CPP is sporadic, and rarely familial: de Vries et al. (2004) estimated that 27.5% of idiopathic CPP cases have a familial component and suggested that the mode of inheritance in CPP is autosomal dominant with sex-dependent penetrance. The genetic basis of CPP is poorly comprehended, but chromosomal abnormalities, epimutations, or imprinting defects at least on chromosomes 14, 15, 7, 1, 9, and X are known to underlie certain syndromic forms, and pathogenic variants in *MKRN3, DLK1*, *KISS1*, and *KISS1R* have been reported as monogenic causes of CPP (Canton et al., 2019).

Children with CPP typically present with progressive pubertal development, an advanced bone age, and accelerated growth velocity. Boys may exhibit transient pubertal gynecomastia. In syndromic forms of CPP, café-au-lait spots, neurofibromas, or skin freckling may be present (Mucaria et al., 2021). Brain imaging with MRI is vital to determine whether CPP is due to central nervous system lesions, and it is recommended for all boys with CPP. An additional imaging option for girls is pelvic ultrasound, which can detect increased ovarian and uterine volume (Cheuiche et al., 2021). The circulating levels of sex steroids and gonadotropins may be elevated, the latter either at the basal level or in response to GnRH stimulation. The follow-up of pubertal development facilitates distinguishing CPP from benign variants of precocious puberty, such as isolated premature breast development without other findings indicative of puberty, or premature adrenarche with consequent pubic and axillary hair, apocrine body odor, and acne (Cheuiche et al., 2021). CPP may have numerous (and severe) health implications, such as shortened adult height, altered body proportions, psychosocial distress, risk-taking behavior, and an increased risk for obesity, cardiovascular diseases, type 2 diabetes, and breast cancer (Mucaria et al., 2021; Maione et al., 2021). CPP is commonly treated with GnRH analogs, which desensitize GnRH receptors, suppress gonadotropin secretion, and reduce gonadal steroids to prepubertal levels. Successful treatment should stabilize pubertal progression, reduce growth velocity and reserve adult height, hinder the advancement of the bone age, and alleviate psychosocial distress (Mucaria et al., 2021; Cheuiche et al., 2021).

2.4.2 Molecular genetics of CPP

At the time of writing, defects in four genes, *KISS1, KISS1R, MKRN3,* and *DLK1,* are regarded as potential monogenic causes of CPP. Genetic testing is typically considered if the patient has family

history of precocious puberty or features suggestive of a syndrome, and genetic causes are identified in a minority of patients. Genetic investigations by high-throughput sequencing methods with ever-lowering costs, such as whole genome and whole exome sequencing, are expected to reveal new gene defects in a growing number of idiopathic CPP cases (Canton et al., 2019; Roberts & Kaiser, 2020; Maione et al., 2021).

KISS1* and *KISS1R

The first identified monogenic defect in idiopathic CPP was a heterozygous activating variant in *KISS1R* (previous name *GPR54*), encoding kisspeptin receptor (Teles et al., 2008). This missense variant was detected in an adopted girl with progressive premature breast development since birth, which was followed by accelerated growth, skeletal maturation, and accelerated development of secondary sexual characteristics since the age of 7. The variant led to prolonged activation of intracellular signaling pathways in response to kisspeptin *in vitro* (Teles et al., 2008). Later elaboration of the variant function revealed that it prolonged responsiveness to kisspeptin by reducing *KISS1R* degradation so that internalized variant receptors were recycled back to the plasma membrane instead of being normally degraded (Bianco et al., 2011). In addition, numerous *KISS1R* polymorphisms or variants of unknown significance have had potential associations with sporadic and familial CPP (Pagani et al., 2020; Ghaemi et al., 2020; Oh et al., 2017; Luan et al., 2007), but the variant identified by Teles and colleagues in 2008 seems to be the only potentially causative one.

Silveira et al. (2010) identified two novel missense variants in *KISS1*, encoding kisspeptin, in three unrelated children with idiopathic CPP. One variant was found in heterozygous state in a boy with sporadic CPP, which had started at 1 year of age. His mother and grandmother were asymptomatic carriers. The other variant was found in a homozygous state in two girls with sporadic CPP. *In vitro* experiments indicated that only the variant harbored by the boy made the kisspeptin protein more resistant to degradation, suggesting an activating and potentially causative role, whereas the variant harbored by the girls changed no kisspeptin bioavailability. In addition, the variant detected in the girls had been previously found in a few control individuals (Silveira et al., 2010). As in the case of *KISS1R*, other investigators have found polymorphisms or variants of unknown significance in *KISS1* with potential association with CPP but without evidence of causality (Rhie et al., 2014; Ko et al., 2010; Luan et al., 2007; Mazaheri et al., 2015; Li et al., 2020).

All in all, activating variants in *KISS1* and *KISS1R* seem to be infrequent causes of CPP. Despite the rarity of potentially causative variants identified so far, the fact that inactivating variants in these genes underlie congenital hypogonadotropic hypogonadism supports the roles of *KISS1* and *KISS1R* as strong candidate genes for CPP (Maione et al., 2018; Trevisan et al., 2018; Ke et al., 2019).

DLK1

DLK1 encodes delta-like non-canonical Notch ligand 1, also known as preadipocyte factor 1 (Pref-1), a transmembrane glycoprotein. *DLK1* has been implicated in tissue development and regeneration, but its role as an inhibitor of adipocyte differentiation and a regulator of glucose homeostasis seems to be best established (Traustadóttir et all., 2019). The potential interaction partners of DLK1 are numerous, including at least NOTCH and fibronectin, and DLK1 is proposed to prevent adipocyte

differentiation via the latter (Traustadóttir et all., 2019). The *DLK1* gene lies on chromosome 14q32.2 in an imprinted locus (Prasasya et al., 2020). Maternal uniparental disomy of chromosome 14, and epimutations or deletions in the imprinted region on the paternal chromosome associate with Temple syndrome, which is characterized by prenatal and postnatal growth failure, developmental delay, facial dysmorphism, relative macrocephaly, central precocious puberty, obesity, short stature, type 2 diabetes, and hypercholesterolemia. *Dlk1* knockout mice have recapitulated the growth delay, adiposity, hypercholesterolemia, hyperlipidemia, and fatty liver disease (Prasasya et al., 2020).

Some of the patients with CPP and paternally inherited *DLK1* loss-of-function variants have presented with Temple syndrome-like metabolic features (Dauber et al., 2017; Gomes et al., 2019; Montenegro et al., 2020). In 2017, Dauber et al. detected a complex defect in *DLK1* in five individuals with non-syndromic CPP from a large multigenerational family: in four girls with CPP, and their paternal grandmother with a presumed CPP based on her early age at menarche. All five individuals had an increased body fat percentage (Dauber et al., 2017). The girls, their grandmother, and unaffected fathers carried a heterozygous ~14 kb deletion on 14q32.2 encompassing the first exon of *DLK1.* The deletion was absent in control genomes and included no other genes. Furthermore, a duplicated 269 bp segment from *DLK1* intron 3 had been inserted between the ends of the deletion. Serum DLK1 levels were undetectable in the affected individuals, suggesting a lack of DLK1 production due to the chromosomal defect (Dauber et al., 2017).

Gomes et al. (2019) identified three different heterozygous, segregating frameshift variants in three families with CPP. In addition to CPP, the affected variant carriers presented with variable metabolic abnormalities, including overweight or obesity, type 2 diabetes, hyperlipidemia, hypercholesterolemia, or insulin resistance or several of these, as well as short stature. Additionally, two sisters had hepatic steatosis, polycystic ovarian syndrome, and infertility. Serum DLK1 levels were undetectable in selected variant carriers compared to healthy controls, indicating loss of function. The metabolic alterations, short stature, and polycystic ovarian syndrome were more prevalent in the *DLK1* variant carriers than non-carriers. Given the high prevalence of metabolic alterations among patients and the phenotypic similarity to *Dlk1* knockout mice, the authors suggested that *DLK1* might be a link between reproduction and metabolism (Gomes et al., 2019). The more recent paper by Montenegro and colleagues (2020) reported a rare heterozygous *de novo* splice site deletion in *DLK1* a girl with idiopathic CPP, normal weight and metabolic profile, and undetectable DLK1 serum levels. Three other rare variants were detected in two girls with CPP, but their DLK1 serum levels were normal. One of these two girls presented with obesity and hyperandrogenism (Montenegro et al., 2020). Taken together, *DLK1* variants potentially inflict metabolic consequences and seem to be a relatively rare cause of CPP (Lee et al., 2020; Chen et al., 2019; Montenegro et al., 2020).

Paternally inherited variants at the *DLK1* locus are associated with age at menarche (Day et al., 2017; Perry et al., 2014). Furthermore, *Dlk1* is expressed in mouse hypothalamic arcuate nucleus as well as in arcuate- and anteroventral periventricular nucleus-derived kisspeptin neuron cell lines (Dauber et al., 2017; Villanueva et al., 2012), and its murine hypothalamic expression levels increase between birth and adulthood correlating with *Kiss1* expression, suggesting that *DLK1* has a neuroendocrine

function (Dauber et al., 2017; Villanueva et al., 2012). However, the connection between *DLK1* function and pubertal timing remains unknown.

MKRN3

The maternally imprinted gene *MKRN3* encodes makorin ring finger protein 3 and lies on chromosome 15q11-13, in the Prader-Willi syndrome critical region. Prader-Willi syndrome is caused by maternal uniparental disomy of chromosome 15, paternal deletion of 15q11-13, or imprinting defects. Common features of the syndrome include intellectual disability, behavioral problems, increased appetite, dysmorphic features, and endocrinological abnormalities, including hypogonadism, infertility, and rarely central precocious puberty (Tauber & Hoybye, 2021). Some scholars have considered *MKRN3* as a candidate gene for pubertal aberrations in this syndrome, although its role in the overall syndrome development is unclear (Costa et al., 2019; Abreu et al., 2015; Meader et al., 2020). Of note, additional dysmorphisms in *MKRN3*-related isolated CPP are rare, and methylation defects remain undetected (Valadares et al., 2019; Maione et al., 2020; Meader et al., 2020).

Abreu et al. (2013) first reported variants in *MKRN3* to underlie CPP in five families. Three of the variants were frameshift and one missense, and all of them were heterozygous and paternally inherited. Since 2013, a plethora of inactivating *MKRN3* variants, including missense, nonsense, regulatory region, frameshift, and copy number variants, have been identified in diverse populations in sporadic and familial cases (Maione et al., 2020; Meader et al., 2020). Some genotype-phenotype correlation has been characterized: patients harboring missense variants tend to be older at diagnosis, have less bone age advancement, and lower basal LH levels than those harboring other types of variants (Valadares et al., 2019; Seraphim et al., 2021). A debatable subject has been whether some boys with *MKRN3* variants could be even asymptomatic (Dimitrova-Mladenova et al., 2016; Brito & Latronico, 2017). Overall, defects in *MKRN3* are the most frequent genetic cause of CPP, especially in Western countries, boys, and familial cases. The estimated overall prevalence of *MKRN3* variants among patients with idiopathic CPP is 9% (Valadares et al., 2019; Bessa et al., 2017).

As previously mentioned, (see **Factors regulating the onset of puberty**) *MKRN3* has been suggested to function as a pubertal brake. The initial suggestion originated from Abreu et al. (2013) based on the findings that *Mknr3* expression levels in the murine arcuate nucleus decreased postnatally until the onset of puberty, simultaneously with an increase of *Kiss1* and *Tac2* levels, and that the variants they identified were predicted to be inactivating. Subsequent studies have been in line with the pubertal brake hypothesis. In addition to mice, *Mkrn3* expression is high in the hypothalamus of rats and non-human primates early in life, and then decreases towards the onset of puberty (Abreu et al., 2020; Heras et al., 2019). In humans, circulating MKRN3 levels decline before the onset and through puberty in healthy boys and girls, and the levels are lower, even undetectable, in early maturing girls than healthy controls (Hagen et al., 2015; Busch et al., 2017). In girls with CPP without *MKRN3* variants, MKRN3 levels are alike lower in comparison to healthy controls (Grandone et al. 2018; Ge et al., 2020).

The mechanism of how *MKRN3* regulates pubertal timing remains elusive but unraveling the functional network of the gene has begun. MKRN3 harbors three canonical zinc finger domains, one makorin-type Cys-His domain, and one zinc RING finger domain, the latter being characteristic of RING-class E3 ubiquitin ligases (Maione et al., 2020). MKRN3 inhibits *KISS1* and *TAC3* promoter activity, for which the RING finger domain is essential. This might imply that the promoter repression involves ubiquitination of certain *KISS1* and *TAC3* transcriptional regulators (Abreu et al., 2020). Furthermore, MKRN3 seems to have E3 ubiquitin ligase activity for MBD3, a transcriptional activator of *GNRH1* via demethylation, and several Poly(A)-binding proteins (PABPs), which regulate *GNRH1* mRNA stability and formation of the translation initiation complex (Li et al., 2020; Li et al., 2021). MKRN3 is predicted to have up to 81 protein-protein interaction partners, including proteins implicated in puberty timing, hypogonadotropic hypogonadism, insulin signaling, RNA-metabolism, as well as other zinc finger proteins (Yellapragada et al., 2019). In turn, *Mkrn3* itself is a target of miR-30b at least in rodents (Heras et al., 2019).

Other candidate genes

LIN28A and ***LIN28B*** encode the homologs A and B of the heterochronic *Caenorhabditis elegans lin-28* gene. In *C. elegans*, inactivating variants in *lin-28* cause precocious development, whereas activating variants lead to a developmental delay (Moss et al., 1997). In mice, *Lin28a* and *Lin28b* abnormal expression can alter growth and pubertal timing in a sex-dependent manner (Corre et al., 2016), and central administration of Lin28b to prepubertal rats induces dynorphin synthesis (Srivastava et al., 2015). The hypothalamic expression levels of *Lin28a* and *Lin28b* markedly decrease from the neonatal period towards puberty in rats (see section **2.3.2**), and a decline in the hypothalamic expression levels of *Lin28a* or *Lin28b* has been observed in mice, monkeys, goats, sheep, and chickens during the juvenile-to-pubertal transition (Cao et al., 2020; Xing et al., 2019; Sangiao-Alvarellos et al., 2013). The animal models suggest that *LIN28A* or *LIN28B* or both may play an inhibitory role in the pubertal onset, making them potential candidate genes for pubertal disorders. Furthermore, GWAS studies have shown that variation at the *LIN28B* locus is associated with pubertal timing and adult height (Day et al., 2017; Ong et al., 2009; Hollis et al., 2020; Perry et al., 2014). In humans, however, no conclusive variants in *LIN28A* or *-B* have been found in patients with CPP (Tommiska et al., 2011; Silveira-Neto et al., 2012; Cao et al., 2020).

Based on their roles in the regulation of GnRH release, ***TAC3*** and ***TACR3***, encoding neurokinin B and its receptor, respectively, as well as ***GABRA1*** and ***NPY1R***, have been proposed as CPP candidate genes (Teles et al., 2011). *GABRA1* encodes gamma-aminobutyric acid type A receptor subunit alpha1, which mediates the inhibitory effects of GABA on GnRH neurons (Terasawa & Fernandez, 2001), whereas *NPY1R* encodes neuropeptide Y receptor Y1, implicated in the inhibitory effects of neuropeptide Y on GnRH secretion (Teles et al., 2011; Hessler et al., 2020; Plant & Barker-Gibb, 2004). Variants in *TAC3, TACR3, GABRA1*, and *NPY1R* have been tentatively investigated in CPP patients, but conclusive variants have remained undetected (Brito et al., 2006; Freitas et al., 2007; Barker-Gibb et al., 2004; Tusset et al., 2012; Krstevska-Konstantinova et al., 2014; Leka-Emiri et al., 2014; Ortiz-Cabrera et al., 2017; Xin et al., 2015).

Another player in the regulation of GnRH release is ***PROKR2,*** encoding prokineticin receptor 2, a G protein-coupled receptor implicated in the GnRH neuron migration and olfactory bulb development (Dodé & Rondard, 2013). Variants in *PROKR2*, often causing functional impairment, are known causes of congenital hypogonadotropic hypogonadism with or without anosmia as well as hypopituitarism (Dodé & Rondard, 2013; Maione et al., 2018). In 2017, Fukami et al. detected a novel heterozygous frameshift variant in *PROKR2* in a 3.5-year-old girl with idiopathic CPP without family history of the condition. The variant produced an mRNA resistant to nonsense-mediated decay, and it seemed to have a gain-of-function effect by enhancing the activity of the wild-type receptor (Fukami et al., 2017). Later, Aiello et al. (2021) reported that one polymorphism in *PROKR2* had a significantly higher frequency among a cohort of girls with idiopathic CPP than among controls, but no potentially pathogenic variants were detected (Aiello et al., 2021). All in all, causative variants in *TAC3, TACR3, GABRA1*, *NPY1R*, or *PROKR2*, if they exist, seem to be exceptional in CPP patients.

Single studies have identified associations between potential candidate gene polymorphisms and CPP: in 2020, Li et al. reported that a single-nucleotide polymorphism in ***PLCB1****,* a gene in the KISS1/KISS1R pathway, was associated with a risk of CPP in Chinese Han girls. Once KISS1 binds to KISS1R, *PLCB1,* encoding phospholipase C beta 1, activates and produces second messengers mediating kisspeptin downstream signaling (Li et al., 2020). Similarly, polymorphisms in ***FSHB, LHB***, and ***CYP19A1*** (encoding cytochrome P450 family 19 subfamily A member 1, which is involved in the aromatization of androgens to estrogen) have been associated with CPP (Zhao et al., 2010; Lee et al., 2014). However, these associations await to be confirmed.

In 2020, two new candidate genes of CPP were uncovered, when Lee et al., (2020) identified a rare, potentially inactivating, missense variant in both ***NOTCH2*** and ***HERC2*** in two sisters with CPP. Their parents without a known history of CPP had a missense variant in either *NOTCH2* or *HERC2*, which suggested digenic inheritance (Lee et al., 2020). Inactivating variants in *NOTCH2,* encoding notch receptor 2, have been previously associated with Alagille syndrome, a multisystem disorder affecting the liver, kidneys, lens, vertebra, vasculature, and craniofacial development, whereas inactivating variants in *HERC2,* encoding HECT and RLD domain containing E3 ubiquitin protein ligase 2, have been associated with developmental delay, autism spectrum disorder, and facial dysmorphism (Yamamoto, 2020; Siebel & Lendahl, 2017; García-Cano et al., 2019; Puffenberger et al., 2012). Possible syndromic features in the two siblings were unreported (Lee et al., 2020).

NOTCH2 and *HERC2* are both associated with NOTCH signaling. NOTCH2, a component of NOTCH signaling, participates in human neural progenitor cell differentiation and proliferation, and, intriguingly, is a target of miR-9, which has been implicated in GnRH neuron development in zebrafish (Roese-Koerner et al., 2017; Garaffo et al., 2015). In mice, activated *Notch2* is sufficient to delay gonadotrope differentiation, and Rbpj-κ-dependent Notch signaling is required for the arcuate and anteroventral periventricular nucleus kisspeptin neuron development (Raetzman et al., 2006; Biehl & Raetzman, 2015). HERC2, in turn, interacts with the Notch ligand Dll1/Dl as a part of a protein complex, slowing down its turnover. This interaction negatively regulates Notch signaling and promotes neural cell differentiation during mouse development (Imai et al., 2015). However, Lee

et al., (2020) performed no functional studies, and so far, the mechanism of how *NOTCH2* and *HERC2* inactivating variants could lead to CPP remains unknown.

2.5 Manifestations of delayed puberty: congenital hypogonadotropic hypogonadism (CHH) and Kallmann syndrome (KS)

2.5.1 Clinical manifestation of CHH

Traditionally, delayed puberty is defined as the absence of testicular enlargement by the age of 14 years or breast development by the age of 13 years. Delayed puberty can occur due to numerous reasons, and in most cases, it represents the normal spectrum of pubertal development, called constitutional delay of growth and puberty (CDGP) (Palmert & Dunkel, 2012; Sedlmeyer & Palmert, 2002; Varimo et al., 2017). The pathological causes of delayed puberty are classified into three categories: functional hypogonadotropic hypogonadism, hypergonadotropic hypogonadism, and permanent hypogonadotropic hypogonadism. Permanent hypogonadotropic hypogonadism explains approximately 8% of cases with delayed puberty in boys and 15% in girls, congenital hypogonadotropic hypogonadism being the most frequent cause in this category (Raivio & Miettinen, 2019).

In adolescents with CDGP, once puberty starts eventually, it progresses normally and should be completed by the age of 18 years. In contrast to CDGP, CHH is a rare genetic condition that is caused by deficient synthesis, secretion, or action of GnRH (Boehm et al., 2015; Young et al., 2019; Raivio & Miettinen, 2019). CHH manifests as absent or incomplete puberty, infertility, and lack of growth spurt. In addition, patients typically have low serum levels of testosterone or estradiol, and low or normal serum gonadotropin levels as well as otherwise normal pituitary function. CHH is commonly diagnosed in late adolescence or early adulthood when puberty has failed to occur (Boehm et al., 2015). Before the age of 18 years, differential diagnosis of CHH from CDGP is sometimes impossible, although severe CHH can manifest as cryptorchidism or micropenis in infancy in boys. Infant girls lack these signs, but altered levels of reproductive hormones during minipuberty can give a cue of CHH in both sexes if known family history exists or if CHH is suspected based on genital abnormalities (Boehm et al., 2015; Raivio & Miettinen, 2019). An important diagnostic step is to separate CHH from functional hypogonadotropic hypogonadism, which can be caused by, for instance, other diseases, malnutrition, excessive exercise, or stress. Structural lesions in the hypothalamus or pituitary should be ruled out with brain MRI (which can also reveal abnormal olfactory bulbs) (Seminara et al., 1998). Genetic testing can help with diagnosis, prognosis, and genetic counseling, although the genetic architecture of CHH is complex and defects in known disease genes are found in only a half of patients (Boehm et al., 2015; Maione et al., 2018; Young et al., 2019).

Although CHH is usually a permanent condition, some patients can undergo a spontaneous, sometimes only temporary, recovery of the reproductive axis following hormonal treatment. The patients undergoing this reversal have achieved a sex steroid milieu for months and have usually received sex steroid treatment. It has been suggested that sex steroids could enhance the plasticity of the GnRH neuronal network and induce its maturation via regulatory genes that are responsive to sex hormones (Dwyer et al., 2016; Young et al., 2019; Sidhoum et al., 2014; Raivio et al., 2007).

Sometimes, CHH is detected later in adulthood. Adult patients typically present with undeveloped genitals, low libido, infertility, and occasionally bone loss and fractures (Young et al., 2019). Some of the male patients may show poor masculinization but are fertile with an increased testicular volume and enfeebled GnRH secretion that is sufficient for spermatogenesis and local testosterone production, but insufficient for the achievement of testosterone levels required for full virilization. In turn, other patients may have experienced normal pubertal development, but later develop irreversible GnRH deficiency called adult-onset hypogonadotropic hypogonadism (Seminara et al., 1998; Dwyer et al., 2010).

CHH can be isolated, associated with panhypopituitarism, or syndromic with various non-reproductive abnormalities. Approximately 50% of patients have partly or fully lacking sense of smell (named hyposmia and anosmia, respectively), and often hypoplastic or absent olfactory bulbs (Boehm et al., 2015). CHH with a defective sense of smell is called Kallmann syndrome (KS), which was first described by the Spanish physician Aureliano Maestre de San Juan in 1856, and later named after the German-American psychiatrist and geneticist Franz Josef Kallmann, who proposed a genetic basis for the condition in 1944 (Dodé & Hardelin, 2010; Pow & Stahnisch, 2016). To make a distinction between Kallmann syndrome and the form of CHH with a normal sense of smell, the latter is named normosmic CHH. In addition to the defective sense of smell, other possible manifestations of CHH include hearing loss, cleft lip or palate, renal agenesis, pigmentation defects, missing teeth, and mirror movements. These additional manifestations stem from the biological function of the causative gene. Sometimes, CHH can be a manifestation of a syndrome, such as combined pituitary hormone deficiency, septo-optic dysplasia, and CHARGE-, Hartsfield-, and Waardenburg syndromes, although the line between syndromic CHH and CHH as a part of a syndrome may be thin (Cangiano et al., 2021).

The estimated overall incidence of CHH is 1 to 10 in 100 000 births, and it is up to 5 times more common in men than women (Bianco & Kaiser, 2009; Good, 2021). In Finland, the estimated minimal incidence of Kallmann syndrome is 1 in 48 000 births, 1 in 30 000 among boys, and 1 in 125 000 among girls (Laitinen et al., 2011). The male predominance might be consequent to ascertainment bias, as females might be more likely managed in the primary health care than at tertiary centers. Additionally, partial CHH might be misdiagnosed as functional hypothalamic amenorrhea, or mild, non-syndromic forms of CHH might be treated with contraceptives or hormonal therapy so that an accurate diagnosis is unreceived (Cangiano et al., 2021; Young et al., 2019).

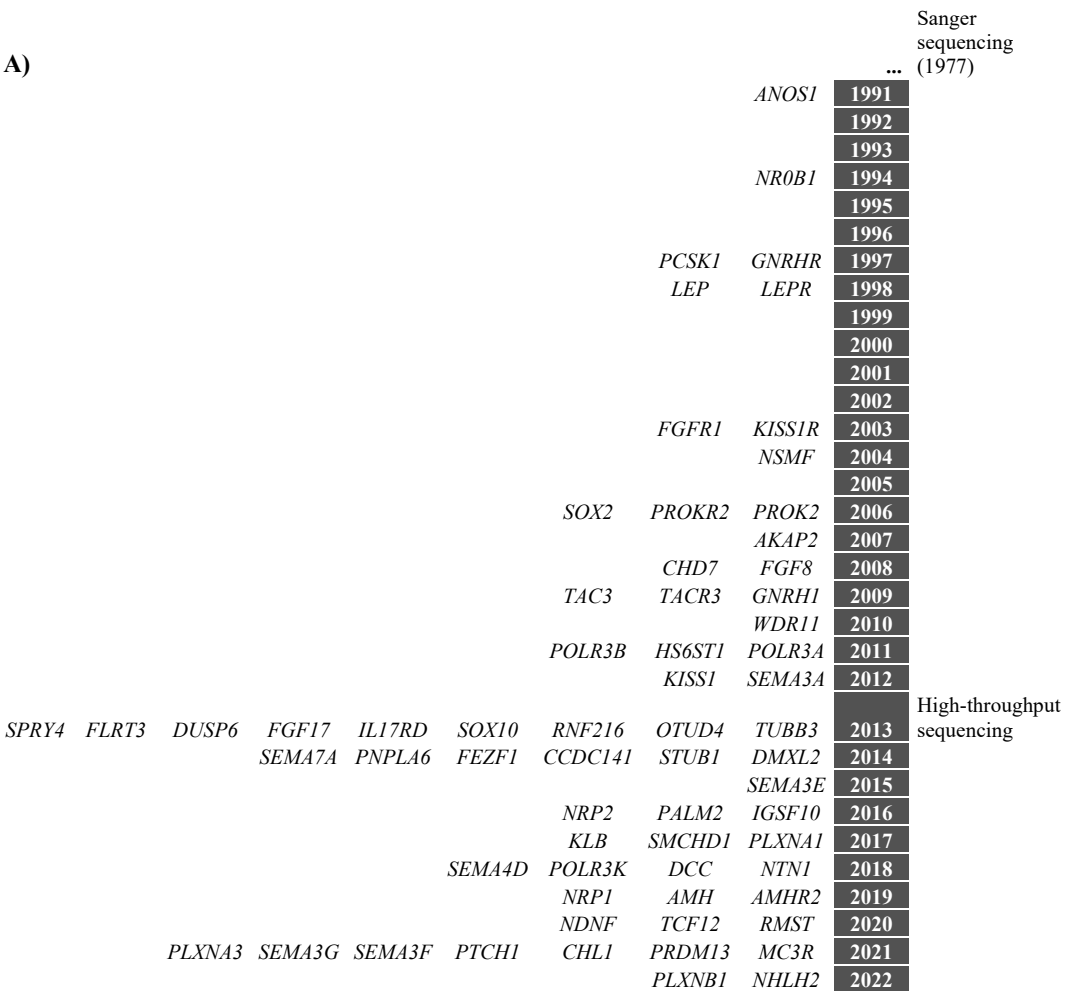
2.5.2 Overview of the molecular genetics in CHH

CHH is found in both sporadic and familial cases, and it exhibits great genetic complexity with X-linked recessive, autosomal dominant, and autosomal recessive modes of inheritance (Cangiano et al., 2021; Maione et al., 2018). The Mendelian view of CHH as a strictly monogenic disorder has been updated after the identification of oligogenic forms of CHH, and it has been estimated that 2.5-15% of cases might be explained by oligogenic inheritance (Cassatella et al., 2018; Sykiotis et al., 2010; Boehm et al., 2015). In addition, defects in certain genes can show variable expressivity and incomplete penetrance that might be explained by oligogenicity or environmental factors (Cangiano et al., 2021). In the era of next-generation sequencing, on the one hand, detection of multiple rare

variants in one patient is expected to become increasingly common. On the other hand, evaluation of each variant's pathogenicity and synergistic effects between variants brings a new challenge (Maione et al., 2018; Young et al., 2019). Therefore, new methods, such as variant classification criteria and mutant-wild type interaction assays, are being implemented to distinguish relevant variants (Cox et al., 2018; Richards et al., 2015). The increasingly complex genetic architecture of CHH has made reliable genetic counseling challenging (Maione et al., 2018).

By the time of writing, defects in over 60 genes (at least *LEP, LEPR, GNRH1, GNRHR, KISS1R, KISS1, TAC3, TACR3, ANOS1 (KAL1), HS6ST1, PROK2, PROKR2, SEMA3A, PLXNA1, SEMA7A, SEMA3E, NSMF (NELF), CCDC141, FEZF1, DCC, NTN1, AMH, AMHR2, NDNF, SOX10, TUBB3, FGFR1, IL17RD, FGF17, FGF8, DUSP6, FLRT3, SPRY4, KLB, WDR11, IGSF10, NR0B1 (DAX1), CHD7, SOX2, LHX4, HESX1, LHX3, SOX3, OTX2, PROP1, GLI2, PITX2, GATA2, PCSK1, LHB, FSHB, DMXL2, SMCHD1, TBX3, OTUD4, RNF216, PNPLA6, STUB1, POLR3A, TCF12, PTCH1, SRA1, RMST, PLXNA3, NRP1, NRP2, SPRY2,* and *POLR3B*) (previous names in brackets) have been implicated in CHH (Cangiano et al., 2021; Davis et al., 2020; Barraud et al., 2021; Stamou et al., 2020; Neocleous et al., 2020; Oleari et al., 2019; Marcos et al., 2017; Miraoui et al., 2013). The reported gene defects, in addition to single-nucleotide and insertion-deletion variants, include genomic rearrangements, as well as mosaic and copy number variants encompassing CHH genes with or without additional genetic material (Acierno et al., 2020; Izumi et al., 2014; Young et al., 2012; Sato et al., 2006; Xu et al., 2015; Choucair et al., 2015; Salaria et al., 2012; Stamou et al., 2020; Telvi et al., 1996). Normosmic CHH and KS are mostly caused by defects in different genes, although some overlap exists. Normosmic CHH is principally caused by defects in genes affecting GnRH neuron activation, GnRH secretion or GnRH action at the gonadotroph level, and KS by defects in genes that affect GnRH neuron migration, GnRH neuron fate specification, or differentiation (Cangiano et al., 2021). Notably, CHH and combined pituitary hormone deficiency also share some disease genes (Gregory & Dattani, 2020). Despite the vast number of associated genes, as already mentioned, defects in them are found in only a half of patients (Boehm et al., 2015; Maione et al., 2018; Young et al., 2019), indicating that new disease genes remain to be discovered. Because of the large number of associated genes, this work is not all-inclusive. The next section (2.5.3), for the most part, reviews the KS-associated genes and briefly summarizes the genes associated with normosmic CHH. It also highlights the genes in the 9q31.2 locus and their potential associations with CHH as well as recent findings in the field of CHH genetics (**Figure 2**).

A)


Sanger sequencing (1977)
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ANOS1
1991
1992
1993
NR0B1
1994
1995
1996
PCSK1
GNRHR
1997
LEP
LEPR
1998
1999
2000
2001
2002
FGFR1
KISS1R
2003
NSMF
2004
2005
SOX2
PROKR2
PROK2
2006
AKAP2
2007
CHD7
FGF8
2008
TAC3
TACR3
GNRH1
2009
WDR11
2010
POLR3B
HS6ST1
POLR3A
2011
KISS1
SEMA3A
2012
High-throughput sequencing
SPRY4
FLRT3
DUSP6
FGF17
IL17RD
SOX10
RNF216
OTUD4
TUBB3
2013
SEMA7A
PNPLA6
FEZF1
CCDC141
STUB1
DMXL2
2014
SEMA3E
2015
NRP2
PALM2
IGSF10
2016
KLB
SMCHD1
PLXNA1
2017
SEMA4D
POLR3K
DCC
NTN1
2018
NRP1
AMH
AMHR2
2019
NDNF
TCF12
RMST
2020
PLXNA3
SEMA3G
SEMA3F
PTCH1
CHL1
PRDM13
MC3R
2021
PLXNB1
NHLH2
2022

B)

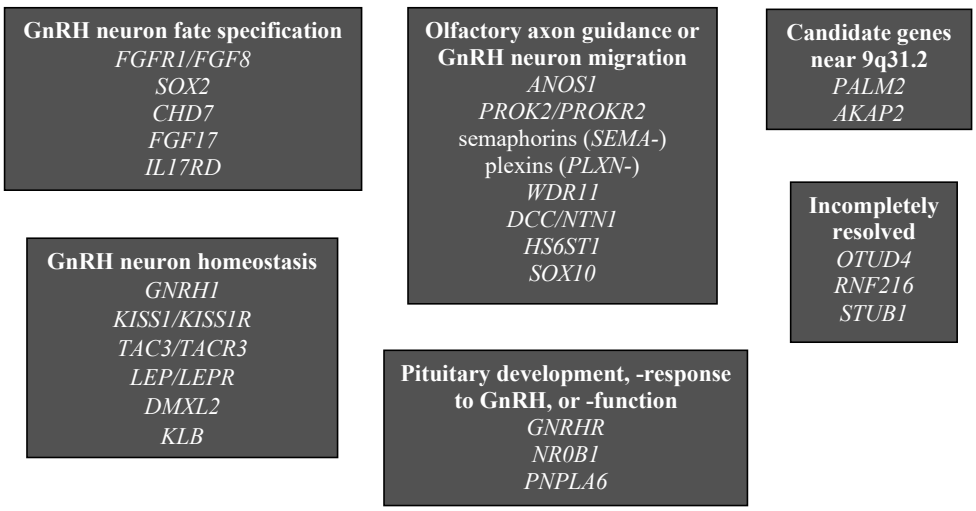
GnRH neuron fate specification
FGFR1/FGF8
SOX2
CHD7
FGF17
IL17RD
Olfactory axon guidance or GnRH neuron migration
ANOS1
PROK2/PROKR2
semaphorins (SEMA-)
plexins (PLXN-)
WDR11
DCC/NTN1
HS6ST1
SOX10
Candidate genes near 9q31.2
PALM2
AKAP2
Incompletely resolved
OTUD4
RNF216
STUB1
GnRH neuron homeostasis
GNRH1
KISS1/KISS1R
TAC3/TACR3
LEP/LEPR
DMXL2
KLB
Pituitary development, -response to GnRH, or -function
GNRHR
NR0B1
PNPLA6

Figure 2. Genes implicated in isolated or syndromic CHH, limited to those described in this review of literature.
A) Timeline of gene discovery. **B)** Biological processes in which the CHH genes can be involved with example genes in each box, along with two KS candidate genes near the 9q31.2 locus. The figure is modified from Young et al. (2019).

2.5.3 Genes implicated in KS and normosmic CHH

Genes regulating GnRH neuron fate specification

FGFR1 and *FGF8*

FGFR1 encodes fibroblast growth factor receptor 1, whereas *FGF8* encodes fibroblast growth factor 8. Fibroblast growth factors (FGFs) comprise a large family of structurally related proteins, which play a role in embryonic morphogenesis and act as ligands for the FGF receptors (FGFRs) (Moosa & Wollnik, 2016). FGF-FGFR signaling is crucial for especially cranial, skeletal, and limb development (Moosa & Wollnik, 2016), and Fgf8 signaling via Fgfr1 is needed for the emergence of GnRH neurons from the olfactory placode during embryonic development. Mice with reduced expression of *Fgf8* or *Fgfr1* or both exhibit absent or a reduced number of GnRH neurons, which can be due to enfeebled survival or fate specification of the GnRH progenitor cells (Chung et al., 2016; Chung et al., 2008; Chung et al., 2010). Additionally, Fgfr1 and Fgf8 are implicated in the olfactory bulb morphogenesis, and FGFR1 in the migration of GnRH neurons (Miraoui et al., 2011; Hébert et al., 2003; Hu et al., 2013). FGF signaling requires heparan sulphate proteoglycans, with which anosmin-1 also interacts, and can enhance FGF signaling (also see next section) (González-Martínez et al., 2004).

Defects in *FGFR1* in CHH were first reported in 2003, and in *FGF8* in 2008 (Dodé et al., 2003; Falardeau et al., 2008). Inactivating variants in *FGFR1* and *FGF8* have been identified in both KS and normosmic CHH patients. The prevalence of *FGFR1* variants among CHH patients is approximately 9%, whereas the prevalence of *FGF8* variants is 1% (Cangiano et al., 2021). Autosomal dominant mode of inheritance with incomplete penetrance is possible for defects in both *FGFR1* and *FGF8* (Dodé et al., 2003; Falardeau et al., 2008; Trarbach et al., 2010), although biallelic and oligogenic inheritance modes have also been suggested (Pitteloud et al., 2007; Maione et al., 2018; Dodé et al., 2003; Villanueva et al., 2015; Falardeau et al., 2008; Quaynor et al., 2011). Rare *FGFR1* and *FGF8* variants can manifest as variable phenotypes even between family members, ranging from unaffected individuals, isolated anosmia, delayed (or partial, in the case of *FGFR1*) puberty to severe CHH with non-reproductive anomalies (Dodé et al., 2003; Pitteloud et al., 2006; Falardeau et al., 2008; Trarbach et al., 2010). Some patients with *FGFR1* variants can undergo a reversal of CHH (Laitinen et al., 2012; Raivio et al., 2007; Pitteloud et al., 2006), and an *FGF8* variant has been found in at least one patient with adult-onset hypogonadotropic hypogonadism (Falardeau et al., 2008). Non-reproductive abnormalities detected in patients with defects in *FGFR1* include dental agenesis, cleft lip or palate, mirror movements, ear, skeletal, and digit anomalies, and split hand/foot malformation (Costa-Barbosa et al., 2013; Miraoui et al., 2011; Villanueva et al., 2015). In addition, defects in *FGFR1* can underlie a multitude of skeletal and craniofacial disorders, such as Pfeiffer-, and Hartsfield syndromes and osteoglophonic dysplasia (Moosa & Wollnik, 2016). In turn, *FGF8*-associated non-reproductive features include cleft lip or -palate, hearing loss, osteoporosis, hyperlaxity of the digits, and camptodactyly (Falardeau et al., 2008; Trarbach et al., 2010). *FGF8*

defects have also been associated with holoprosencephaly and septo-optic dysplasia (McCabe et al., 2011; Gregory & Dattani, 2020).

***FGF8* synexpression group**

IL17RD, DUSP6, FLRT3, SPRY4, and *SPRY2* belong to the *FGF8* synexpression group, that is, they show similar developmental expression patterns to *FGF8* and are able to modulate the FGF8 signaling via FGFR1 (Miraoui et al., 2013). *FGF17* and *FGF18*, in turn, belong to the *FGF8* subfamily, are highly homologous to *FGF8,* and can signal via FGFR1. Additionally, *Fgf17* and *Fgf18* are coexpressed with *Fgf8* in the olfactory placode (Miraoui et al., 2013). Rare variants in *IL17RD, FGF17, DUSP6, FLRT3,* and *SPRY4* have been identified in normosmic CHH and KS patients *(*Miraoui et al., 2013; Amato et al., 2019; Men et al., 2020; Men et al., 2021), and their estimated prevalences range from 1% to 3% among CHH patients (Cangiano et al., 2021). Miraoui et al. (2013) investigated the presence of potentially pathogenic variants in the *FGF8* synexpression group/subfamily genes in a cohort of CHH patients and detected oligogenicity (variants in more than one of the *FGF8* synexpression group/subfamily gene or other known CHH genes) in 19%. In most of the oligogenic cases, at least one FGF8 signaling-associated gene was involved, suggesting that this group of genes could contribute to oligogenic CHH (Miraoui et al., 2013). Men et al. (2021), who investigated variants in *DUSP6, IL17RD, SPRY2,* and *SPRY4* in a Chinese cohort, suggested that heterozygous variants in *DUSP6* could be sufficient to cause CHH, but heterozygous variants in *IL17RD* or *SPRY4* would be insufficient, requiring contribution of alleles in the same or different genes (Men et al., 2021). KS and hearing loss have been frequent findings in carriers of rare *IL17RD* variants (Miraoui et al., 2013), whereas cryptorchidism, dental agenesis, syndactyly, and blue color blindness have been common in rare *DUSP6* variant carriers (Men et al., 2021). In addition, a rare variant in *SPRY4* has been found in a patient with anosmia and adult-onset HH (Indirli et al., 2019).

CHD7

CHD7 encodes chromodomain helicase DNA binding protein 7, a member of the CHD family, which functions as an ATP-dependent chromatin-remodeling protein (Balasubramanian & Crowley, 2017). During the embryonic development of the human and mouse, *CHD7* is ubiquitously expressed in tissues, including the olfactory bulb and -nerves, hypothalamus, and pituitary (Sanlaville et al., 2006; Bergman et al., 2010). *Chd7*-insufficient mice show olfactory bulb hypoplasia, reduced numbers of olfactory sensory- and GnRH neurons, and abnormalities in the reproductive organs. Consequently, these mice have impaired olfaction, delayed puberty, and reproductive dysfunction (Bergman et al., 2010; Layman et al., 2009; Layman et al., 2011). In the mouse model, Chd7 seems to be vital for GnRH neurogenesis and neural stem cell proliferation in the olfactory epithelium during development (Layman et al., 2009; Layman et al., 2011). Furthermore, CHD7 is required for the formation of multipotent migratory neural crest cells, and in mice, a subpopulation of GnRH neurons may arise from the neural crest (Bajpai et al., 2010; Forni et al., 2011). Thus, the reduced number of GnRH neurons may be, at least in part, consequent to neural crest dysregulation.

Defects in *CHD7* were originally associated with CHARGE (coloboma, heart defects, choanal atresia, retardation of growth and development, gonadal/genital defects, and ear/hearing abnormalities) syndrome, and most CHARGE patients harbor inactivating heterozygous variants in *CHD7*

(Balasubramanian & Crowley, 2017). The gonadal/genital defects, which can manifest as cryptorchidism, micropenis, or delayed puberty or all, are secondary to CHH. CHH, often with anosmia, is present in the majority of patients with CHARGE syndrome (Bergman et al., 2011). In 2008, Kim et al. reported that *CHD7* defects can underlie normosmic CHH or KS without the full CHARGE phenotype, indicating that CHH can be a milder representation of the syndrome (Kim et al., 2008). Truncating variants in *CHD7* are mainly associated with the full CHARGE phenotype, whereas pathogenic missense variants are enriched in patients with CHH (Balasubramanian & Crowley, 2017). A reversal of the reproductive phenotype in a patient with KS and a truncating variant in *CHD7* have been reported (Laitinen et al., 2012). CHH-associated *CHD7* variants can be inherited in an autosomal dominant fashion with incomplete penetrance, but also cases with variants in *CHD7* and other CHH genes exist (Balasubramanian & Crowley, 2017). The prevalence of rare *CHD7* variants among CHH patients is approximately 8% (Cangiano et al., 2021).

SOX2

SOX2 encodes SRY-box transcription factor 2, which is required for maintaining pluripotency and directing neural differentiation of pluripotent stem cells (Zhang & Cui, 2014). *Sox2* expression is vital for the genesis of GnRH neurons from the olfactory epithelium (Tucker et al., 2010), and the long non-coding RNA *RMST* regulates neurogenesis with SOX2 through downstream target genes (Ng et al., 2013). Pathogenic variants in or deletions of the entire *SOX2* gene are implicated in an autosomal dominant phenotypic spectrum that can include, among other features, anophthalmia or microphthalmia, normosmic CHH with genital abnormalities, developmental delay, pituitary hypoplasia, brain malformations, seizures, learning difficulties, short stature, hearing loss, dystonia, and esophageal atresia (Williamson et al., 2020; Kelberman et al., 2006). CHH has been included in the spectrum since 2006 (Kelberman et al., 2006). In 2020, a translocation was reported to disrupt *RMST* (rhabdomyosarcoma 2 associated transcript) leading to defective neural crest cell differentiation in a KS patient with skeletal dysplasia and delayed mental development (Stamou et al., 2020).

Genes regulating olfactory axon guidance or GnRH neuron migration

ANOS1* and *HS6ST1

ANOS1 (formerly known as *KAL1*) encodes anosmin 1, an extracellular matrix protein, which binds to the cell membrane with the help of heparan sulfate proteoglycans (Soussi-Yanicostas et al., 1996). In turn, *HS6ST1* encodes heparan sulfate 6-O-sulfotransferase 1, an enzyme that can introduce a sulfate to the 6-*O*-position of heparan sulfates, which are integral components of heparan sulphate proteoglycans (Tornberg et al., 2011; Bülow et al., 2002). In *C. elegans*, the homologous *ANOS1* gene is involved in neural branching in a heparan sulfate proteoglycan-dependent manner, requiring 6-*O*-sulfate modifications in heparan sulfates to function (Tornberg et al., 2011; Bülow et al., 2002). Furthermore, in human fetuses with a deleted or truncated *ANOS1*, GnRH neurons and olfactory axons leave the olfactory placode but fail to reach the brain and accumulate in the cribriform plate (Schwanzel-Fukuda et al., 1989; Teixeira et al., 2010), indicating that anosmin 1 plays a role in olfactory axon guidance and GnRH neuron migration (Cariboni et al., 2004). Furthermore, anosmin 1 has been implicated in the development of cranial neural crest via regulation of FGF8, BMP5, and WNT3a signaling in the chick embryo (de Castro et al., 2014).

In 1991, *ANOS1* was identified as the first gene in which defects can underlie KS (Franco et al., 1991; Legouis et al., 1991). In turn, rare variants in *HS6ST1* were detected in CHH patients in 2011 (Tornberg et al., 2011). Inactivating variants in *ANOS1* and *HS6ST1* have been found in both normosmic CHH and KS patients, although more frequently in KS patients, especially in the case of *ANOS1* (Danda et al., 2021; Sato et al., 2004; Tornberg et al., 2011; Cangiano et al., 2021). Carriers of inactivating *ANOS1* variants may also manifest with mirror movements, cryptorchidism, renal agenesis, and hearing loss, whereas reversal of CHH and cleft lip or palate have been found in those with inactivating *HS6ST1* variants (Costa-Barbosa et al., 2013). *ANOS1* variants are inherited in an X-linked recessive manner with high penetrance, whereas variants in *HS6ST1* are suggested to have an oligogenic mode of inheritance with variable expressivity (Tornberg et al., 2011; Maione et al., 2018). The requirement of more than one allele in the same or different genes for the inheritance of *HS6ST1*-related CHH is supported by the report from Howard et al. (2018), in which a heterozygous, potentially pathogenic *HS6ST1* variant was linked to self-limited delayed puberty (Howard et al., 2018). The estimated prevalence of rare *ANOS1* variants among CHH patients is 5% and that of *HS6ST1* variants 2% (Cangiano et al., 2021; Tornberg et al., 2011).

PROK2* and *PROKR2

PROK2 encodes prokineticin 2 and *PROKR2* its G protein-coupled receptor. In addition to reproduction, PROK2 participates in various biological processes, such as nociception, circadian rhythms, angiogenesis, food intake, and control of the energy balance (Magnan & Migrenne-Li, 2021). Homozygous (but not heterozygous) *Prok2*- and *Prokr2* knockout mice have a phenotype resembling Kallmann syndrome with hypoplastic olfactory bulbs, infertility, and reduced numbers of GnRH neurons (Ng et al., 2005; Pitteloud et al., 2007; Matsumoto et al., 2006; Prosser et al., 2007). Moreover, olfactory bulb neuronal progenitor cells start their migration from the subventricular zone, but instead of reaching their destination in the olfactory bulb, they accumulate in the olfactory ventricle and rostral migratory stream in both types of the knockout mice (Ng et al., 2005; Prosser et al., 2007). The findings in the mice imply that *PROK2/PROKR2*-associated CHH is caused by defective development and migration of GnRH- and olfactory neurons; however, *Prok2* and *Prokr2* are also expressed in the gonads, and it is possible that the genes can contribute to the phenotype by directly affecting the gonadal development or function (Martin et al., 2011).

Potentially pathogenic variants in *PROK2* and *PROKR2* were reported in KS patients for the first time in 2006 (Dodé et al., 2006). Since 2006, also normosmic *PROK2* or *PROKR2* rare variant carriers have been reported. The variants, in turn, have occurred in heterozygous and biallelic forms with or without rare variants in other known CHH genes, and most of the variants have been missense, heterozygous, and inactivating (Martin et al., 2011; Dodé & Rondard, 2013; Mkaouar et al., 2021; Méndez et al., 2015). Patients with biallelic genotypes have exhibited more severe phenotypes, whereas variable expressivity and incomplete penetrance have been associated with heterozygous variants (Martin et al., 2011). In fact, some of the heterozygous *PROKR2* variants originally considered pathogenic were reclassified as benign or of unknown significance by Cox et al. in 2018. Moreover, rare *PROKR2* variants have been detected in patients with adult-onset- and reversal of CHH (Cangiano et al., 2019; Sinisi et al., 2008). On the one hand, the dominant negative function has been indicated for a few *PROKR2* heterozygous variants (Cox et al., 2018; Abreu et al., 2012). On

the other hand, it has been suggested that most *PROK2/PROKR2* heterozygous (and even some homozygous!) variants could require oligogenicity to cause a phenotype (Cangiano et al., 2021; Mkaouar et al., 2021; Cox et al., 2018). No additional abnormalities are significantly enriched in CHH patients with rare variants *PROK2* or *PROKR2*, although some papers have detected hearing loss, synkinesia, epilepsy, sleep disorders, and obesity (Dodé & Rondard, 2013; Costa-Barbosa et al., 2013; Cole et al., 2008). Rare variants in *PROK2* and *PROKR2* are present in approximately 2% and 6% of CHH patients, respectively (Cangiano et al., 2021).

NTN1* and *DCC

NTN1 encodes netrin 1, and *DCC* its receptor. Netrin 1 acts as a secreted guidance cue for commissural axons crossing the midline of the neural tube during brain development. DCC, in turn, is expressed in the commissural axons (and, of note, in a subpopulation of GnRH neurons) and required for netrin-induced attractive axon guidance (Marsh et al., 2018; Meijers et al., 2020, Low et al., 2012). Heterozygous pathogenic variants in *NTN1* and *DCC* are implicated in congenital mirror movements. Additionally, heterozygous *DCC* variants are associated with corpus callosum agenesis, and biallelic variants with developmental split-brain syndrome (Méneret et al., 2020; Marsh et al., 2018). *NTN1* and *DCC* are expressed along the human and murine GnRH migratory pathway (Bouilly et al., 2018; Deiner & Stretavan, 1999), and *Ntn1*- and *Dcc*-deficient mice show the migration of GnRH neurons to inappropriate positions in the cerebral cortex and accessory olfactory bulb, as well as reduced GnRH axon projections to the median eminence (Deiner & Stretavan, 1999; Schwarting et al., 2004). Netrin 1 acts as a chemoattractant for migrating GnRH neurons in the forebrain and stimulates neurite outgrowth from *DCC*-expressing GnRH neurons towards the median eminence in a non-chemoattractive fashion (Low et al., 2012; Murakami et al., 2010). Moreover, netrin 1-DCC signaling is required for correct olfactory sensory axon projections (Lakhina et al., 2012).

Schwarting et al. (2004) stated: “It is tempting to speculate whether a variant form of Kallmann syndrome might occur in people hemizygous for either the *Ntn1* or *Dcc* genes. These individuals would be hypogonadal (--) but would not necessarily be anosmic (--).” Fourteen years later, Bouilly et al. (2018) indeed described the first rare heterozygous, inactivating *NTN1* and *DCC* variants in CHH patients (Bouilly et al., 2018). In their report, 5/7 patients manifested with KS and 2/7 with normosmic CHH. All KS patients harbored one variant in *DCC* with or without additional variants in *NTN1* or other known CHH genes. The variants in *NTN1* were always present with an additional variant in *DCC* or another CHH gene, implicating oligogenic inheritance (Bouilly et al., 2018). The CHH was severe in all, and some of the patients exhibited mirror movements, obesity, hearing loss, mild facial asymmetry, or mild facial malformation. In the cohort, the estimated prevalence of *NTN1/DCC* rare variants was 5.2% (Bouilly et al., 2018). By 2021, other *NTN1* or *DCC* variants with potentially pathogenic effects in CHH have remained scarce (Zhang et al., 2021).

TUBB3

TUBB3, encoding tubulin beta 3 class III, is a neuron-specific microtubule component that interacts with DCC and is required for netrin 1-induced axon outgrowth and -guidance (Qu et al., 2013). A single missense variant in *TUBB3*, p.E410K, has been associated with autosomal dominant KS, congenital fibrosis of the extraocular muscles, developmental delay, facial weakness, midface

hypoplasia, and progressive peripheral neuropathy, a constellation named "TUBB3 E410K syndrome". In some cases, the syndrome may also manifest as cyclic vomiting, tracheomalacia, vocal cord paralysis, hypoplasia of the pituitary, and normal reproductive function (Chew et al., 2013; Balasubramanian et al., 2015; Nakamura et al., 2017; Dentici et al., 2020). It has been proposed that the p.E410K variant disrupts axon guidance via altered kinesin–microtubule interactions (Chew et al., 2013).

Genes encoding semaphorins, plexins, and neuropilins

Semaphorins are a group of guidance proteins with diverse developmental functions, including the regulation of the GnRH neuron migration, plasticity, and survival; guidance of the olfactory axons, as well as the plasticity of the median eminence (Oleari et al., 2019). Semaphorins predominantly signal via plexin receptors; additionally, SEMA3 family members bind to neuropilins (NRP1 and NRP2), which act as coreceptors for type A plexins (PLXNAs) (although SEMA3E can directly bind to its receptor, PLXND1) (Oleari et al., 2019). Rare variants in the semaphorins *SEMA3A, SEMA3E*, *SEMA3F*, *SEMA3G*, *SEMA4D,* and *SEMA7A***,** in the plexins *PLXNA1* and *PLXNA3*, as well as in the neuropilins *NRP1* and *NRP2* have been identified in CHH patients (Kotan et al., 2021; Dai et al., 2020; Känsäkoski et al., 2014; Hanchate et al., 2012; Young et al., 2012; Kotan et al., 2019; Marcos et al., 2017; Quaynor et al., 2016; Chen et al., 2020; Cariboni et al., 2015; Mkaouar et al., 2020; Zhao et al., 2020; Oleari et al., 2021; Zhou et al., 2018). Most of the CHH-associated variants in these genes have been heterozygous and present in KS patients (which might partly reflect the inclusion of solely KS patients in the studied cohorts). Co-occurrence with additional variants in other CHH genes has not been uncommon (Marcos et al., 2017; Kotan et al., 2019; Chen et al., 2020; Cariboni et al., 2015; Känsäkoski et al., 2014; Mkaouar et al., 2020; Dai et al., 2020; Hanchate et al., 2012; Kotan et al., 2021). The mode of inheritance for variants in *SEMA3A* was originally thought to be autosomal dominant, but because some variants in *SEMA3A* have been present in controls or co-occurred with variants in other CHH genes, the view has turned towards oligogenicity and a modifying role of the variants in the phenotype (Dai et al., 2020; Känsäkoski et al., 2014; Hanchate et al., 2012; Young et al., 2012). Oligogenic inheritance also seems possible for variants in *PLXNA1, SEMA3E, SEMA7A, SEMA3F*, and *PLXNA3* in CHH, whereas autosomal recessive is the prevailing mode of inheritance for *SEMA3G* and *SEMA4D* variants so far (Marcos et al., 2017; Oleari et al., 2021; Cariboni et al., 2015; Känsäkoski et al., 2014; Mkaouar et al., 2020; Zhou et al., 2018; Kotan et al., 2021). Of note, *in vitro* or *in vivo* functional validation is lacking for variants in *NRP1, NRP2, SEMA4D*, and *SEMA7A*, but they remain credible CHH candidate genes based on the existing mouse models (Messina et al., 2011; Oleari et al., 2019).

WDR11

WDR11, encoding WD repeat domain 11 protein, has been implicated in GnRH neuron migration through ciliogenesis, and induction of GnRH production via Hedgehog signaling (Kim et al., 2018). *WDR11* was detected as a gene underlying CHH upon breakpoint definition of a balanced translocation on 10q26 in a patient with KS. *WDR11* was in the vicinity of the breakpoint, and likely inactivating missense variants in the gene were subsequently identified in unrelated CHH patients (Kim et al., 2010). Since 2010, findings of rare, potentially pathogenic heterozygous variants in *WDR11* in both KS and normosmic CHH patients have been published, but the number of confirmed

cases remains relatively small by 2021 (Neocleous et al., 2020; Quaynor et al., 2011; Sutani et al., 2020; Izumi et al., 2014; Choucair et al., 2015; Ayers et al., 2017). In mice, only homozygote knockouts show the CHH phenotype, but in humans, *WDR11* variants in CHH seem to be inherited in an autosomal dominant fashion with incomplete penetrance. However, potential oligogenicity cannot fully be excluded (Quaynor et al., 2011; Neocleous et al., 2020; Kim et al., 2010; Kim et al., 2018). The recent findings by Haag et al. (2021) have brought (at least the monogenic) role of *WDR11* in CHH under debate: biallelic carriers of *WDR11* variants presented no signs of CHH (but microcephaly, short stature, and intellectual disability instead), and their heterozygote relatives were unaffected.

NSMF

NSMF (previous name *NELF,* "nasal embryonic LHRH factor") encodes NMDA receptor synaptonuclear signaling and neuronal migration factor, also known as Jacob. Jacob has been implicated in the guidance of olfactory axons and migration of GnRH neurons, and some female *Nsmf* knockout mice have exhibited delayed puberty and subfertility (Spilker et al., 2016). The first rare, possibly CHH-associated *NSMF* variant was reported in a patient with normosmic CHH in 2004 (Miura et al., 2004). Later, several rare *NSMF* variants, often heterozygous and in combination with variants in other CHH genes, have been detected in normosmic CHH and KS patients, suggesting oligogenicity (Zhang et al., 2021; Xu et al., 2011; Pitteloud et al., 2007; Tornberg et al., 2011). A recent finding in another murine *Nsmf* knockout model suggests that the Jacob protein plays no major role in the migration of GnRH neurons (these mice exhibited hippocampal dysplasia but no clear CHH phenotype in either sex), questioning the causation of CHH at least by monogenic *NSMF* variants (Spilker et al., 2016).

FEZF1* and *CCDC141

FEZF1 encodes a zinc-finger protein, which functions as a transcriptional repressor. In mice, *Fezf1* enables the olfactory axons and GnRH neurons to penetrate the central nervous system basal lamina and enter the brain (Watanabe et al., 2009; Hirata et al., 2006). Consistently, *Fezf1* knockout mice recapitulate the human KS phenotype with hypoplastic olfactory bulbs and stalled migration of GnRH neurons (Watanabe et al., 2009; Hirata et al., 2006). The first inactivating *FEZF1* variants in KS, inherited in an autosomal recessive manner, were detected in two consanguineous families in 2014 (Kotan et al., 2014). In one of the families, an additional homozygous nonsense variant in *CCDC141* was detected, potentially suggesting an additional contribution to the phenotype (Kotan et al., 2014; Hutchins et al., 2016). In mice, knockdown of *Ccdc141* reduces GnRH neuron migration but not olfactory axon outgrowth or GnRH neuron adhesion in murine nasal explants, implying that defects in cell motility could underlie the abnormal migration (Hutchins et al., 2016; Turan et al., 2017). Turan et al. (2017) later identified an association between rare *CCDC141* variants and normosmic CHH (with incomplete penetrance and possible reversal) rather than KS. In three out of four families, rare variants in other CHH genes were present (Turan et al., 2017). Similarly, most other reported variants in *CCDC141* have co-occurred with different CHH gene variants, which suggests that their contribution to the CHH phenotype, if any, is minor (Turan et al., 2017; Correa-Silva et al., 2018; Mkaouar et al., 2021; Kotan et al., 2019; Hou et al., 2020). After 2014, only Zhang et al. (2020) have

reported a *FEZF1* variant in a CHH patient; however, this variant was of uncertain significance and its contribution to the phenotype remains unknown.

SOX10

SOX10 is a transcription factor that participates in the early development of neural crest cells, including the olfactory ensheathing cells (Pingault et al., 2021; Barraud et al., 2013). *Sox10* null mice exhibit a massive loss of the olfactory ensheathing cells (which are crucial for olfactory axon targeting and GnRH neuron migration) supporting the role of *SOX10* in the human KS phenotype (Barraud et al., 2013; Pingault et al., 2013). Pathogenic variants in *SOX10* have been classically associated with Waardenburg syndrome, which has phenotypic overlap, (especially olfactory bulb agenesis) with KS (Pingault et al., 2021). Most KS patients with pathogenic *SOX10* variants exhibit hearing loss, and sometimes additional features of or full Waardenburg syndrome (Chen et al., 2020; Wakabayashi et al., 2021; Hamada et al., 2020; Rojas et al., 2021; Maione et al., 2016; Wang et al., 2018; Vaaralahti et al., 2014, Suzuki et al., 2015). It has been proposed that these two syndromes, in fact, are representations of a *SOX10*-related phenotypic continuum (Rojas et al., 2021). Additionally, normosmic CHH patients with *SOX10* variants have been reported, but the variants in them have lacked functional validation (Pingault et al., 2021; Gach et al., 2020; Amato et al., 2019; Rojas et al., 2021). CHH-associated *SOX10* variants are usually missense and inherited in an autosomal dominant manner. The variants show variable expressivity and penetrance, which might be explained by the influence of the genetic or environmental background (Pingault et al., 2013; Pingault et al., 2021; Xu et al., 2020; Rojas et al., 2021).

IGSF10

Howard et al., (2016) identified rare and heterozygous, potentially pathogenic variants in *IGSF10* (immunoglobulin superfamily member 10) in individuals with self-limited delayed puberty, CHH, and functional HH. The authors also showed that *Igsf10* was expressed in the nasal mesenchyme during the migration of GnRH neurons in mice and humans, and that knockdown of the gene reduced migration of immature GnRH neurons *in vitro,* as well as perturbed migration and axonal outgrowth of GnRH3 neurons in zebrafish (Howard et al., 2016). In later studies, rare heterozygous variants in *IGSF10* have been detected in individuals with CDGP, CHH with or without hyposmia or reversal, and KS-like phenotype with normal gonadotropin levels (Amato et al., 2019; Barroso et al., 2020; Kotan et al., 2019; Zhang et al., 2021; Jolly et al., 2019). However, some of these variants were present with variants in other genes, and their functional significance remained unclear (Amato et al., 2019; Barroso et al., 2020; Kotan et al., 2019; Zhang et al., 2021; Jolly et al., 2019). It has been proposed that *IGSF10* variants may predispose to delayed puberty via reduction of GnRH neuron number in the hypothalamus or an impaired GnRH neuronal network due to abnormally timed arrival of these neurons to the hypothalamus (Howard et al., 2016). Furthermore, it might be that *IGSF10* defects represent a common basis for different forms of GnRH deficiency, so that additional defects in other genes are required for the manifestation of the full CHH phenotype (Amato et al., 2019; Howard et al., 2016).

SMCHD1

SMCHD1 encodes an epigenetic gene silencer, structural maintenance of chromosomes flexible hinge domain containing 1, the molecular function of which is largely unknown (Gurzau et al., 2020). Heterozygous missense variants in the *SMCHD1* ATPase domain or its vicinity are implicated in autosomal dominant, variably expressed Bosma arhinia microphthalmia syndrome (BAMS) with KS (Gordon et al., 2017; Shaw et al., 2017). In addition, an *SMCHD1* variant close to the ATPase domain has been identified in a patient exhibiting combined pituitary hormone deficiency with (apparently) normosmic CHH and septo-optic dysplasia but absent arhinia or microphthalmia (Kinjo et al., 2020). *Smchd1* is expressed in the murine immature olfactory sensory neurons (Gordon et al., 2017), and the ATPase domain-ablated zebrafish show reduced projection length of the terminal nerve where GnRH3 neurons reside (Shaw et al., 2017). In neural crest cells, *SMCHD1* variants in BAMS are associated with changes in the expression of genes involved in neural crest cell migration and differentiation as well as axon guidance (Laberthonnière et al., 2021), supporting the hypothesis that the CHH in BAMS patients might be due to impaired GnRH neuron projection (Gordon et al., 2017; Shaw et al., 2017). In turn, the CPHD phenotype might reflect an aberrant expression of *SMCHD1* target genes implicated in central nervous system development (Kinjo et al., 2020; Massah et al., 2020).

AMH* and *AMHR2

AMH encodes anti-Mullerian hormone and *AMHR2* a receptor of AMH. AMH/AMHR2 signaling has been implicated in GnRH neuron migration and secretion in mice and *in vitro* (Cimino et al., 2016; Malone et al., 2019; Cannarella et al., 2021). Malone et al. (2019) detected heterozygous, *in vitro* validated inactivating variants in *AMH and AMHR2* in patients with KS or normosmic CHH exhibiting partial or absent puberty. Oligogenic contribution of *AMH* or *AMHR2* variants to CHH seems most likely (Malone et al., 2019; Brunello & Rey, 2021). However, the roles of *AMH* and *AMHR2* in CHH remain to be confirmed in other large patient series.

TCF12

Heterozygous variants in *TCF12*, encoding the basic helix-loop-helix transcription factor 12, have previously been implicated in craniosynostosis (Wilkie et al., 2017). The phenotypic spectrum of *TCF12* variants was expanded, when Davis et al. (2020) reported inactivating variants in the gene in KS patients. The phenotype associated with *TCF12* variants was however highly variable, ranging from KS with craniosynostosis to sole anosmia or a normal phenotype (Davis et al., 2020). Loss of *tcf12* resulted in reduced olfactory bulb size and disturbed GnRH neuron patterning in zebrafish, which was rescued by *STUB1* mRNA. Interestingly, most *TCF12* variants seemed to be autosomal dominant, while one was autosomal recessive. In addition, some of the variants in KS patients had been previously detected in individuals with craniosynostosis and no apparent signs of CHH, suggesting that other *cis* or *trans* acting factors likely modulate the phenotypic spectrum (Davis et al., 2020).

PTCH1

Barraud et al. (2021) showed that a novel nonsense variant in *PTCH1* (patched 1), a component of the Hedgehog signaling pathway, segregated with Gorlin-Goltz syndrome (predominantly basal cell

carcinoma and odontogenic keratocysts) and defective sense of smell in one family, the proband of which manifested with KS (Barraud et al., 2021). In addition, three *PTCH1* variants that were predicted to be deleterious were detected in additional KS patients without Gorlin-Goltz manifestations. Based not only on their pedigree but also previous *Ptch1*-deficient rodent models and *in vitro*-demonstrated interactions between Hedgehog- and other signaling pathways regulating olfactory bulb morphogenesis and GnRH neuron migration, the authors suggested that *PTCH1*-associated Gorlin-Goltz- and Kallmann syndromes share a common molecular basis (Barraud et al., 2021). However, the predicted deleteriousness of the identified *PTCH1* variants was not confirmed in functional experiments (Barraud et al., 2021).

NDNF

NDNF (neuron-derived neurotrophic factor) is known to promote the migration and neurite outgrowth of cultured mouse brain neurons, and the protein contains the fibronectin-3 domain (Kuang et al., 2010). Messina et al. (2020) probed the role of fibronectin-3 domain-containing proteins in CHH by employing a strategy that included WES of 240 CHH patients and prioritization of the best candidate genes among the fibronectin-3 domain superfamily by bioinformatic tools. As a result, the authors identified three protein-truncating variants and a missense variant in *NDNF* in four KS patients (Messina et al., 2020). Autosomal dominant inheritance with variable expressivity and incomplete penetrance seemed most likely for the variants, all of which compromised NDNF function *in vitro*. NDNF was further shown to be expressed along the embryonic GnRH neuron migratory route in humans and mice, and deficiency of the gene impaired GnRH neuron migration and olfactory axon innervation *in vivo* (Messina et al., 2020). Tamaoka et al. (2021) later attempted to identify pathogenic *NDNF* variants in 61 CHH patients but failed to detect any. Currently, defects in *NDNF* seem to be rare causes of CHH.

Genes regulating GnRH neuron homeostasis

GNRH1

GNRH1 encodes a pre-prohormone that is cleaved into the active GnRH decapeptide (Bouligand et al., 2010). *GNHR1* was a speculated human CHH gene for over two decades until 2009, when biallelic inactivating variants in *GNRH1* were discovered in patients with non-syndromic normosmic CHH (Bouligand et al., 2010; Bouligand et al., 2009; Mason et al., 1986; Weiss et al., 1991). Some studies have detected CHH in carriers of heterozygous *GNRH1* variants, but the roles of the heterozygous variants as sole causes of CHH remain a matter of controversy (Patil et al., 2021). *GNRH1* variants are rare findings among CHH patients, their estimated prevalence ranging from 0.3% to 4% in cohorts (Francou et al., 2016; Patil et al., 2021).

DMXL2

DMXL2 encodes Dmx like 2, or rabconnectin 3, a vesicular protein. *Dmxl2* is required, likely through afferent neurons or glial cells, for the morphological maturation of GnRH neuron dendrites during puberty as well as GnRH neuron responsiveness to kisspeptin (Tata et al., 2017). In addition, Dmx like 2 is involved in the regulated secretion of insulin (Tata et al., 2014). Homozygous intragenic deletions in *DMXL2* have been identified in three brothers showing normosmic CHH with central

hypothyroidism, intellectual disability, abnormal glucose regulation, and peripheral neuropathy (Tata et al., 2014). Other patients with similar genotype-phenotype association remain to be reported.

PCSK1

PCSK1 encodes proprotein convertase subtilisin/kexin type 1, which participates in the cleavage of proneuropeptides and prohormones into bioactive forms (Stijnen et al., 2016). Compound heterozygous or homozygous pathogenic variants in *PCSK1* have been reported to underlie rare cases of normosmic CHH with obesity and malabsorptive diarrhea. In addition, patients may variably exhibit deficiency of several neuroendocrine hormones, hypocortisolism with elevated pro-opiomelanocortin levels, and reactive hypoglycemia with elevated levels of proinsulin as consequences of PCSK1 deficiency and defective proprotein processing (Stijnen et al., 2016; Pépin et al., 2019; Jackson et al., 1997; O'Rahilly et al., 1995). Recently, a digenic combination of *PCSK1* and *CHD7* variants was speculated to underlie hypogonadism in a proband with CHH and maturity-onset diabetes (Cho et al., 2020).

LEP* and *LEPR

Leptin signaling regulates food intake and energy homeostasis and is thought to contribute to puberty onset by mediating information on sufficient nutritional status to GnRH neurons via ventral premammillary nucleus signaling to kisspeptin neurons (Childs et al., 2021). Pathogenic variants in leptin gene *LEP* or leptin receptor *LEPR* are implicated in autosomal recessive normosmic CHH with severe early-onset obesity due to relentless appetite. In addition, decreased GH and thyroid hormone or immune dysfunction might be associated with pathogenic *LEP* and *LEPR* variants (Nunziata et al., 2018; Fischer-Posovszky et al., 2010; Ozata et al., 1999; Clément et al., 1998). *LEP/LEPR*-associated CHH was described for the first time in 1998 (Strobel et al., 1998; Clément et al., 1998).

TAC3* and *TACR3

Signaling of neurokinin B and its receptor, encoded by *TAC3* and *TACR3*, is thought to initiate or accelerate KNDy neuronal activity and contribute to GnRH pulse generation via kisspeptin release (Uenoyama et al., 2021). Pathogenic variants in *TAC3* and *TACR3* were implicated in autosomal recessive, normosmic CHH for the first time in 2009 (Topaloglu et al., 2009). In addition, heterozygous *TACR3* variants have been detected in a few CHH patients (Gianetti et al., 2010). However, heterozygous family members have repeatedly been unaffected, indicating that pathogenic variant(s) in other genes must be present to produce CHH in patients with heterozygous variants in *TACR3* (Quaynor et al., 2011; Young et al., 2010; Topaloglu et al., 2009; Francou et al., 2011). Null *Tac2* (the murine homolog of *Tac3*) and *Tacr3* female mice phenotypically resemble reversal seen in patients with *TAC3/TACR3* variants, whereas null males have normal reproductive capacity (True et al., 2015; Yang et al., 2012). In contrast, an adult male cat with a homozygous, likely pathogenic *TAC3* variant showed absent puberty and low testosterone levels (Hug et al., 2019).

KLB

KLB encodes klotho beta, a single-pass transmembrane glycoprotein, which binds to FGF21 and is required for FGF21 interaction with FGFR1 isoform c (Phan et al., 2021). Xu et al. (2017) identified heterozygous pathogenic variants in *KLB* in 4% of investigated CHH patients, in whom at least some

degree of oligogenicity modifying the phenotypes was present. The variant carriers had either normal or absent smell, and most of them exhibited metabolic defects, such as obesity and dyslipidemia. In the same study, experiments on Klb-deficient mice showing delayed puberty and subfertility implicated a role for KLB in FGF21-mediated postnatal GnRH release (Xu et al., 2017). As FGF21/KLB/FGFR1 signaling plays a role in cell metabolism, this signaling pathway might connect metabolic and reproductive functions (Phan et al., 2021; Xu et al., 2017).

KISS1* and *KISS1R

The first *KISS1R* variants underlying CHH were reported in 2003 (de Roux et al., 2003; Seminara et al., 2003) and *KISS1* variants in 2012 (Topaloglu et al., 2012). Inactivating variants in these genes resulted in normosmic, autosomal recessive CHH (de Roux et al., 2003; Seminara et al., 2003; Topaloglu et al., 2012). By the time of writing, multiple *KISS1R* variants have been implicated in CHH with an estimated rare variant prevalence of 1.6% among patients, whereas *KISS1* variants with confirmed pathogenicity in CHH are extremely rare (Abbara et al., 2021; Trevisan et al., 2018; Cangiano et al., 2021; Ke et al., 2019). Both *Kiss1r* and *Kiss1* knockout mice recapitulate the normosmic human CHH phenotype (d'Anglemont de Tassigny et al., 2007; Funes et al., 2003; Seminara et al., 2003; Ikegami et al., 2020).

Genes regulating the pituitary development, response to GnRH, or function

GNRHR

GNRHR encodes the GnRH receptor, a G protein-coupled receptor which, upon GnRH binding, couples with and signals via the $G\alpha_{q/11}$ and $G\alpha_{s}$ proteins to stimulate gonadotropin secretion from the pituitary (Stamatiades et al., 2022). *GNRHR* was the first gene in which a defect was associated with normosmic, non-syndromic CHH (de Roux et al., 1997; Louden et al., 2021; Cioppi et al., 2019). Not only autosomal recessive but also oligogenic inheritance might be considered possible for *GNRHR* variants implicated in CHH (Louden et al., 2021). Phenotypic consequences of *GNRHR* variants are highly variable and can range from complete CHH to mild or moderately severe disease and reversal, depending on the variant severity and -combination (Cioppi et al., 2019; Laitinen et al., 2012; Raivio et al., 2007; Tommiska et al., 2013). Heterozygous *GNRHR* variants possibly contribute to mild forms of GnRH deficiency (Vaaralahti et al., 2011; Gianetti et al., 2012; Tommiska et al., 2016). *GNRHR* variants are frequent in familial CHH, their prevalence reaching up to 40% among affected families (Nair et al., 2016).

NR0B1

NR0B1 (previously named *DAX1*) encodes an orphan nuclear receptor, a regulator of transcription. Little is known about the exact function of NR0B1, but it has been implicated in the development of adrenal glands and organs constituting the hypothalamic-pituitary-gonadal axis (Achermann et al., 2018). Pathogenic variants in *NR0B1* associate with X-linked adrenal hypoplasia congenita and co-occurring normosmic CHH, which was first discovered in 1994 (Muscatelli et al., 1994). CHH in the *NR0B1*-linked condition may manifest as partial or absent puberty or adult-onset HH (with adult-onset adrenal insufficiency), often with defective spermatogenesis in males (Kyriakakis et al., 2017; Suntharalingham et al., 2015; Hasegawa et al., 2020). Female heterozygotes may occasionally present with adrenal insufficiency, HH, or extreme pubertal delay; a case of a female homozygote with

isolated CHH is also known (Seminara et al., 1999). So far, a consensus seems to prevail that the *NR0B1*-related CHH is due to a combined hypothalamic and pituitary defect leading to impaired gonadotropin secretion (Achermann et al., 2018; Suntharalingham et al., 2015).

POLR3A, POLR3B,* and *POLR3K

POLR3A, *POLR3B,* and *POLR3K* encode A, B, and K subunits of RNA polymerase III. Biallelic pathogenic variants in these genes are associated with variably severe 4H leukodystrophy, classical features of which include hypomyelination, hypodontia, and hypogonadotropic hypogonadism (Lata et al., 2021; Dorboz et al., 2018; Tétreault et al., 2011; Bernard et al., 2011). In addition, potentially pathogenic recessive variants in *POLR3B* have been identified in four patients with normosmic CHH but without neurological signs or dental anomalies. One of these patients presented with resistance to GnRH treatment, indicating CHH of pituitary origin (Richards et al., 2017). Recently, a normosmic CHH patient with mild leukodystrophy and a putatively pathogenic combination of *POLR3A* and *FGFR1* variants was added to the list of *POLR3*-associated cases of CHH (Neocleous et al., 2020).

PNPLA6

PNPLA6 encodes patatin like phospholipase domain containing 6 (neuropathy target esterase), which is required for the biosynthesis of the neurotransmitter acetylcholine, and which can regulate membrane permeability and vesicle trafficking via its lysophospholipase activity (Wortmann et al., 2015; Pamies et al., 2014). Biallelic variants in *PNPLA6* were initially implicated in CHH as a part of neurodegenerative disease spectrum including Gordon-Holmes and Boucher-Neuhäuser syndromes (Synofzik et al., 2014). In addition, hypopituitarism in the *PNPLA6*-associated Oliver-McFarlane and Lawrence-Moon syndromes can include CHH (Synofzik et al., 2021). Apart from CHH, the four syndromes are characterized by combinations of ataxia, chorioretinal dystrophy, trichomegaly, and brisk reflexes/spasticity (Synofzik et al., 2021). Defects in *PNPLA6* disrupt gonadotropin secretion in response to GnRH, although a hypothalamic defect cannot be excluded (Topaloglu et al., 2014).

Genes with incompletely resolved roles in the GnRH neuronal system

RNF216, OTUD4,* and *STUB1

Pathogenic variants in *RNF216* (ring finger protein 216), *OTUD4* (OTU deubiquitinase 4), and *STUB1* (STIP1 homology and U-box containing protein 1) are implicated in Gordon-Holmes -like syndrome with or without dementia (Hayer et al., 2017; Margolin et al., 2013; Shi et al., 2014; Alqwaifly & Bohlega, 2016; Santens et al., 2015). Variants in *RNF216* seem to require at least one additional pathogenic variant in the same or different gene to cause the syndrome with CHH, whereas syndrome-associated biallelic variants in *OTUD4* have only been identified in combination with biallelic variants in *RNF216* (Margolin et al., 2013; Santens et al., 2015). *STUB1*-associated CHH is typically a feature of an autosomal recessive disease, and rarely a manifestation of an autosomal dominant condition caused by heterozygous variants in *STUB1* (Shi et al., 2014; Heimdal et al., 2014; Lieto et al., 2020). Proteins encoded by *RNF216*, *OTUD4*, and *STUB1* are involved in the ubiquitination process (Shi et al., 2014; Zhao et al., 2018; Seenivasan et al., 2019). Additionally, RNF216 regulates GnRH neuron migration by suppressing Beclin-mediated autophagy (Li et al., 2019), and is vital for spermatogenesis and male fertility (Melnick et al., 2019). STUB1 alike

regulates an autophagy pathway, as well as cAMP-induced cilia resorption (Sha et al., 2017; Porpora et al., 2018), whereas OTUD4 enhances TGFβ signaling, implicated in the migration of immature GnRH neurons and GnRH expression (Jaynes et al., 2020; Bouret et al., 2004; Larco et al., 2018). The *RFN216*-, *OTUD4*-, and *STUB1*-associated normosmic CHH likely originates from both hypothalamic and pituitary defects, but the exact disease mechanism requires further elucidation (Margolin et al., 2013; Li et al., 2019; Melnick et al., 2019; Shi et al., 2014).

Genes in the 9q31.2 locus

The 9q31.2 locus is associated with age at menarche (Perry et al., 2009; He et al., 2009; Elks et al., 2010), and an SNP in the locus with the sense of smell (Dong et al., 2017). In fact, in the study by Perry et al. (2009), the strongest association signal to the age at menarche came from an intergenic SNP in 9q31.2, the nearest protein-coding genes being *TMEM38B, FSD1L, FKTN*, *SLC44A1, TAL2*, and *ZNF462* (Perry et al., 2009). According to the UCSC Genome Browser (GRCh37), the locus also includes *RAD23B* and *KLF4*. Of these, *TMEM38B* is associated with the timing of puberty in men and women (Day et al., 2017; Hollis et al., 2020). Microdeletions and inactivating variants in *ZNF462* (zinc finger protein 462) have been associated with Weiss-Kruszka syndrome, characterized by ptosis, metopic ridging, down-slanting palpebral fissures, craniosynostosis, hearing loss, dysgenesis of the corpus callosum, autism, and developmental delay (Weiss et al., 2017; Cosemans et al., 2018; Kruszka et al., 2019; González-Tarancón et al., 2020). In addition, *ZNF462* harbors potential links to the reproductive system: Fgf8 can induce *Znf462* expression in the chick preplacodal region, and hypothalamic expression of *ZNF462* decreases at puberty in monkeys (Hintze et al., 2017; Lomniczi et al., 2015). *Klf4*, in turn, is coexpressed with *Sox10* in the chick neural crest (Lignell et al., 2017). Additionally, KLF4 interacts with β-catenin and inhibits Wnt-signaling (Zhang et al., 2006) by preventing β-catenin binding to TCF7L2 (Sellak et al., 2012), a transcription factor implicated in GnRH signaling and development of the pituitary (Chen et al., 2014; Brinkmeier et al., 2007).

Patients with deletions in the 9q31.2 chromosomal region have been described since at least the 1970s (for instance, by Turleau et al., 1978; Farrell et al., 1991; Kulharya et al., 2008; and Dugan et al., 2018). One of the patients with a 9q31.2 deletion has manifested with CHH, one with olfactory bulb hypoplasia, and a few with cleft lip or palate (Xu et al., 2013; Dugan et al., 2018; Farrell et al., 1991; Chien et al., 2010; Cao et al., 2015). In addition, one family with delayed puberty and a segregating 9q31.2 deletion has been reported (Ramineni et al., 2019). The ***PALM2AKAP2*** gene, encoding **PALM2 and AKAP2** fusion, lies ~1.2 Mb downstream of the 9q31.2 locus (UCSC Genome Browser, GRCh37). *PALM2,* a member of the paralemmin protein family implicated in plasma membrane dynamics, and *AKAP2*, a protein kinase A-anchoring protein, can form fusion transcripts via transcriptional readthrough and alternative splicing (Hultqvist et al., 2012; Panza et al., 2007; Hu et al., 2001). In 2007, Panza et al. reported a balanced translocation disrupting *AKAP2* in a boy with KS and bone abnormalities and showed that *Akap2* is expressed in the murine olfactory bulb and -epithelium. In 2016, Quaynor et al. detected a potentially pathogenic, heterozygous missense variant in *PALM2* in a female with KS and hyperthyroidism. The putative roles of *PALM2* or *AKAP2* in KS are lacking further confirmation.

Recent findings (2021-2022)

Chen et al. (2021) identified a rare heterozygous variant ***CHL1*** (cell adhesion molecule L1 like) in two KS patients and their mother with anosmia and normal fertility (Chen et al., 2021). *Chl1* null mutant mice have previously exhibited aberrant olfactory axon projections (Heyden et al., 2008). In functional experiments, Chen et al. (2021) demonstrated that *CHL1* regulated immature GnRH cell migration and death. The rare variant possibly impaired migration through reduced ERK1/2 activation, and increased cell death through activated calcium loading and transcription of *RIPK3* and *MLKL,* two cell death -related genes.

The publication by Whittaker et al. (2021) demonstrated that a variant in ***PRDM13*** (PR/SET domain 13), previously known as a transcriptional regulator and a determinant of GABAergic cell fate in the developing spinal cord and retina, was associated with a recessive syndrome presenting with intellectual disability, ataxia with cerebellar hypoplasia, scoliosis, and normosmic CHH in three patients. Mice homozygous for a *Prdm13* mutant allele displayed cerebellar hypoplasia, a reduced number of the arcuate nucleus Kiss1 neurons, and delayed puberty with normal gonads, suggesting a vital role for PRDM13 in the regulation of cerebellar GABAergic cell fate and kisspeptin neuron development (Whittaker et al., 2021).

Nhlh2 has been implicated in kisspeptin synthesis from KNDy neurons in response to leptin, and in the regulation of puberty onset in male mice (Leon et al., 2021). Topaloglu et al. (2022) reported that rare ***NHLH2*** (nescient helix-loop-helix 2) variants underlay normosmic CHH in three male patients, two of whom also showed late-onset obesity. One of the detected variants was homozygous (p.R79C) while the two others (p.A9L and p.V31M) were heterozygous. Moreover, the p.V31M variant was present with variants in other CHH genes, leaving the inheritance of the *NHLH2*-related phenotype ambiguous (Topaloglu et al., 2022). All variants showed impaired transactivation of the human *KISS1* promoter, and p.R79C reduced Nhlh2 binding to the promoter of *Mc4R* (melanocortin 4 receptor), a gene in the central leptin-POMC-melanocortin pathway regulating satiety and energy expenditure (Fatima et al., 2021). Although these findings support the role of *NHLH2* as a link between the metabolic circuitry and GnRH pulse generator in humans, the causality of the p.A9L and p.V31M variants in CHH with obesity needs further confirmation (Topaloglu et al., 2022).

Like MC4R, **MC3R** (melanocortin 3 receptor) is activated by the POMC-derived α-MSH in the central leptin-POMC-melanocortin pathway (Fatima et al., 2021). Lam et al. (2021) reported that individuals with inactivating *MC3R* variants had a later onset of puberty as well as reduced linear growth, lean mass, and circulating levels of IGF-1. Both sexes of *Mc3r*-deficient mice displayed delayed onset of puberty, and the estrous cycle length was not affected by acute energy deficit in females (Lam et al., 2021). *Mc3r* expression was enriched in the arcuate nucleus KNDy and Ghrh neurons, and its expression in AVPV increased from the juvenile to pubertal stage of development, indicating a potential role in the regulation of puberty onset. Lam et al. (2021) suggested that while signaling via MC4R controls the acquisition and retention of calories, signaling through MC3R principally regulates the disposition of calories into growth, lean mass, and puberty timing. It is currently unclear whether *MC3R* has implications in CHH.

PLXNB1 (plexin B1), a receptor of SEMA4D, has been implicated in the migration of murine GnRH neurons independent of the olfactory axonal pathway (Giacobini et al., 2008). Rare *PLXNB1* variants have been detected in CHH patients, but no functional confirmation on their roles in the phenotype has been performed (Zhou et al., 2018; Chen et al., 2021; Chen et al., 2020) until the study by Welch et al. (2022). Welch and colleagues (2022) detected six rare *PLXNB1* variants in six patients with normosmic CHH and two with CDGP; it was concluded that the CDGP was likely due to a *CCDC141* variant that these two individuals also carried. Autosomal dominant inheritance with variable expressivity seemed most likely for the *PLXNB1* variants, all of which were predicted to be deleterious in *in silico* mutagenesis modeling (Welch et al., 2022). The variant shared by the CHH and CDGP individuals was further shown to attenuate PLXNB1 membranous expression in immortalized GnRH cells and reduce their migration. The authors concluded that *PLXNB1* variants are implicated in the etiology of CHH (Welch et al., 2022), but it remains to be seen whether they could also be implicated in CDGP in the absence of additional genetic defects.

3. AIMS OF THE STUDY

The main aim was to discover defects in specific genes and evaluate their roles in disorders where the normal growth or timing of pubertal development is disrupted due to aberrant pituitary hormone secretion. Specific aims were to discover the:

1) presence of *KCNQ1* or *KCNE2* gene variants in patients with acromegaly (I)
2) presence of variants in the microRNA genes *MIR7-3, MIR141, MIR429, MIR200A, MIR200B*, or *MIR200C* in congenital hypogonadotropic hypogonadism or Kallmann syndrome patients without genetic diagnoses (II)
3) the genetic features in two families with central precocious puberty (III)
4) genetic basis of Kallmann syndrome in a patient with a deletion on chromosome 9q31.2 (IV)

4. SUBJECTS AND METHODS

4.1 Subjects and DNA/RNA extraction

4.1.1 Subjects with somatotropinomas

In study I, 44 sporadic and one familial (patient 852, who had an affected sister) patients with somatotropinomas (23 men, 22 women) were investigated. Moreover, previous whole-genome sequencing (WGS) data of eight somatotropinoma patients (four men, four women) were exploited. The tumors of these eight patients were Gsp mutation negative, and their germline (blood-derived) WGS data revealed no mutations in the known genes associated with inherited forms of pituitary adenoma (Välimäki et al., 2015). All patients had been previously sequenced negative for *AIP* and *CDKN1B* variants as well as the *GPR101* variant p.Glu308Asp (Georgitsi et al., 2007). No genetic testing for *MEN1* and *PRKAR1A* variants had been performed as the patients' family histories and clinical phenotypes were negative for them.

The patient age at diagnosis ranged from 14 to 56 years with the mean age being 39 years. One of the studied cases presented with gigantism (ST10) and the rest had typical acromegalic phenotypes. Forty-three cases had somatotropinomas and two had adenomas that secreted both prolactin and growth hormone. Forty-nine cases represented the Finnish population and four had non-Finnish origins: these patients were from Estonia (patient ST7), Spain (patient ST12), Italy (patient 852), and Tunis (patient ST6). All patients were operated in Finland.

4.1.2 Subjects with KS or normosmic CHH

In study IV, the proband was the second child of healthy Finnish nonconsanguineous parents who had a healthy daughter before him. He was born at gestation week H42+1 after an uneventful pregnancy. After birth, he was noted to have a muscular ventricular septal defect that closed spontaneously. His testes were normally descended. Before starting primary school, he was diagnosed with attention deficit disorder and mild developmental delay. He had distinctive facial features including ptosis, flat nasal tip, and low set ears. In addition, he presented with mild bilateral sensorineural hearing loss with normal semicircular canals in MRI.

At the age of 13.5 years, he was noted to have delayed puberty and a hypoplastic scrotum and testes. He had self-reported anosmia and absent olfactory bulbs in MRI. He had no synkinesia, hand or foot deformities, pigmentation defects, missing teeth, or problems with balance. He had two kidneys in abdominal ultrasound, and low reproductive hormone and inhibin B levels (LH 0.1 IU/L, FSH 0.2 IU/L, testosterone 0.7 nM, and inhibin B 14 ng/l). At the age of 14 years and 11 months, he remained prepubertal (Tanner stage G1 and pubic hair stage P3), and his reproductive hormone and inhibin B levels remained low as well. He had remarkably small testes (volume <1 ml). Based on these clinical evaluations (small testicular size, low reproductive hormone and inhibin B levels, anosmia, and the absence of the olfactory bulbs in the MRI), he was diagnosed with Kallmann syndrome according to the criteria described in Varimo et al. (2017), and low-dose testosterone treatment was commenced to induce his puberty. During the treatment, his testis size remained extremely small (assessed at the age of 16 years and 9 months) in the setting of low gonadotropin and inhibin B levels (23 ng/l at the age of 15 years and 9 months; 29 ng/l at 16 years 9 months; and 28 ng/l at 17 years 7 months), which supported the KS diagnosis.

As a part of the clinical evaluation, he had undergone two genetic assays: a comparative genetic hybridization microarray (Agilent 44K; Agilent Technologies, Santa Clara, CA, USA), in which a 2.38 Mb *de novo* deletion on chromosome 9q31.2 was detected. The precise deletion breakpoints, however, remained undetermined in this assay. The other genetic assay was a BluePrint Genetics® Kallmann Syndrome Panel Plus (version 3), which covered the coding regions of the following genes: *ANOS1, CHD7, FGF8, FGFR1, GNRHR, KISS1R, PROK2, PROKR2,* and *TACR3* up to 20 bp of intronic sequence with single nucleotide changes, small indels up to 220 bp and copy number variations defined as single exon or larger deletions and duplications.

Other KS patients in study IV, and the KS and normosmic CHH patients in study II had been enrolled from the five university hospitals in Finland. They had been previously diagnosed with CHH, as described in Laitinen et al. (2011) and Laitinen et al. (2012), based on: i) missed or partial puberty by the age of 18 in adult patients or other evident signs of CHH in patients under 18 years old, ii) excluded organic cause for their condition, iii) low sex steroid levels in the setting of subnormal or normal gonadotropin levels, iv) otherwise normal function of the anterior pituitary, and in case of KS, v) defective sense of smell discovered by formal testing, anamnesis, or absent/rudimentary olfactory bulbs in magnetic resonance imaging. The KS patients had been screened for variants in *ANOS1, FGFR1, FGF8, PROK2, PROKR2, CHD7,* and *WDR11* (Laitinen et al., 2011), and the normosmic CHH patients for variants in *FGFR1, FGF8, PROK2, PROKR2, CHD7, WDR11, GNRHR, GNRH1, KISS1R, KISS1, LHB, TAC3,* and *TACR3* (Laitinen et al., 2012). Conclusively pathogenic variants had not been found in these genes.

4.1.3 Subjects with CPP

In study III, the Finnish index patient (a girl) with CPP was evaluated in the New Children's Hospital at the Helsinki University Hospital in Finland, and the Polish siblings (brother and sister) at the Karol Jonscher's Clinical Hospital in Poznan, Poland. All patients in study III were evaluated for serum LH and FSH levels, bone ages, height SD scores, and age-adjusted body mass index (ISO-BMI) values. All patients underwent GnRH stimulation tests and brain magnetic resonance imaging (MRI). Additionally, estradiol levels were measured in the Polish girl and testosterone levels in the Polish boy. The Finnish index patient was genetically evaluated with Blueprint Genetics MKRN3 single gene test Plus.

The Finnish index patient in study III was born at term. She was appropriate for gestational age, and her early developmental milestones were normal. At the age of 6 years, she presented with breast development and was referred to the Pediatric Endocrine Outpatient Clinic at the age of 6.4 years. In a physical examination, the index patient had Tanner breast stage 2 and pubic hair stage 1. She displayed accelerated growth (growth velocity 7.8 cm/year) and advanced bone age (6.8 years) at the age of 6.4. Her baseline serum LH level was 0.3 IU/l and FSH level 5.2 IU/l. The GnRH stimulation test revealed a clearly pubertal LH response (max. 10.4 IU/l), and her brain MRI showed a normal pituitary region. She was started on a GnRH analog (Leuprorelin 3.75 mg every 4 weeks) at the age of 6.5 years. At the age of 7.9 years, she had Tanner breast stage 1 and pubic hair stage 1. Her weight was normal before and after the initiation of the treatment.

The Finnish patient's mother had menarche at the age of 13, and she was 171 cm tall (+0.5 SDS). The father had experienced precocious puberty; his pubertal growth had ended at the age of 12, and his adult height was 160 cm (−3.4 SDS). The father had another daughter (half-sister to the index patient) who also had been diagnosed with CPP at the age of 5.5 years, and she had received treatment thereafter.

The Polish family in study III included a boy and a girl with CPP. They had no family history of CPP: their mother's menarche age had been 13 years and her adult height was 170 cm (+0.7 SDS), and the father was 186 cm tall (+1.2 SDS) and he had experienced normal pubertal timing. The son was diagnosed with CPP at the age of 9 years and 3 months when he presented with adult-sized testes (20 ml), advanced bone age (11.2 years), and tall stature without growth acceleration. His testosterone level was 3.6 nmol/l. In the GnRH-stimulation test, his maximal LH and FSH responses were 8.9 IU/l and 1.7 IU/l, respectively, and his brain MRI showed an incidental small pineal cyst. He had presented with pubarche at the age of 7 and axillarche at the age of 9. He showed the first signs of a pubertal spurt at the age of 9 years and 9 months, and he was followed up until the age of 15.4 years, when he had reached his adult height, 180.5 cm (mid-parental target 184.5 cm; +1.0 SDS). He never received treatment for CPP. Based on the ISO-BMI values, he was overweight during the follow-up.

The younger sister of the Polish boy was diagnosed with CPP at the age of 4.5 years, when she had presented with thelarche, advanced bone age (5 years), and tall stature without growth acceleration. Her serum estradiol level was 91.8 pmol/l. In the GnRH stimulation test, her maximal LH response was 12.6 IU/l, and her brain MRI scan was unremarkable. She was started on a GnRH analog (Triptorelin 3.75 mg every 4 weeks), and at the age of 5.3 years, she had Tanner breast stage 1 showing regression of the breast tissue. She was overweight prior to the initiation of the treatment, and thereafter her ISO-BMI values were within the normal limits.

4.1.4 Control databases

Controls used in studies I-IV were the Finnish (studies I, II, and IV), European (study IV), and total (studies I and III) populations in the gnomAD 2.1.1 database (https://gnomad.broadinstitute.org/) (Karczewski et al., 2020), from which the presence and frequency of the variants identified in Sanger sequencing were checked. In study III, the SISu database (Sequencing Initiative Suomi project, http://www.sisuproject.fi/, [SISu v4.1, accessed 2/2021]) was additionally used for checking the presence of the *MKRN3* variant, which was found in the Finnish family. In study IV, the dbSNP (https://www.ncbi.nlm.nih.gov/snp/) (Sherry et al., 2001), database was utilized for checking the frequency of the two variants in *AKAP2* (rs879255366 and rs373159646) which were absent in gnomAD.

In study IV, the gnomAD (Karczewski et al., 2020), ExAC (data integrated into gnomAD) (Karczewski et al., 2017), 1000 Genomes (https://www.internationalgenome.org/) (1000 Genomes Project Consortium, 2015), and Exome Variant Server (Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), https://evs.gs.washington.edu/EVS/, [accessed 1/2021]) databases (including all their subpopulations), which were provided in the ANNOVAR (https://annovar.openbioinformatics.org/en/latest/#reference) (Wang et al., 2010) annotation, as well

as dbSNP (Sherry et al., 2001), were used as control databases for checking the presence and frequency of potentially causative variants in other but known KS genes in the proband's whole exome sequencing data.

4.1.5 DNA/RNA extraction

In all studies, genomic DNA samples had been previously obtained from the patients, as well as from the Polish (study III) and the proband's (study IV) family members, by extraction from peripheral blood leukocytes. The Finnish parents' DNAs in study III were collected with Oragene DNA saliva kit and extracted by using the prepIT-L2P kit (both kits by DNA Genotek, Ottawa, ON, Canada) according to the manufacturer's instructions.

In study IV, the proband's and his parents' RNAs were isolated with the QIAamp RNA Blood Mini Kit (QIAGEN, Hilden, Germany) from peripheral blood leukocytes.

4.1.6 Ethical consideration

Written informed consents were obtained from all participants, and in the case of a minor/children, a parent or guardian gave the consent. All studies (I-IV) were approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa and conducted in accordance with the Declaration of Helsinki. All studies adhered to the approved guidelines.

4.2 Genetic and bioinformatic methods

4.2.1 Whole exome sequencing

The whole exome sequencing (WES) of the proband and his family in study IV was performed with Illumina NovaseqS2 PE100 technology (Illumina Inc., San Diego, CA, USA) at the Institute for Molecular Medicine Finland (FIMM) according to their established pipeline (Sulonen et al., 2011): First, the adapter was trimmed from the reads, as well as any low-quality nucleotides from the 5' or 3' ends of the read, removing pairs with less than 36 bp. The reads were aligned to the GRCh37 (hg19) reference genome with the Burrows-Wheeler Aligner (BWA) (Li & Durbin, 2010). Non-unique read pairs and non-unique single reads were removed and Genome Analysis Toolkit (GATK) Base Recalibrator (DePristo et al., 2011) was used to clean the alignment. Any potential PCR duplicates were removed using GATK Picard MarkDuplicates, and GATK IndelRealigner was used for indel sites. The mpileup from the SAMtools package (Li et al., 2009) was used for variant calling. The sequencing yielded a mean target coverage of 222X and 98% of 20X coverage.

4.2.2 Whole genome linked-read sequencing

The whole genome linked-read sequencing of the proband in study IV was performed at FIMM according to 10X Genomics Chromium library preparation (Chromium Genome Reagent Kits v2 Rev B; 10X Genomics, Pleasanton, CA, USA). The sample was sequenced with Illumina NovaSeq 6000 system S4 flow cell and XP workflow. Read length for the paired-end run was 2x151. The reads were aligned to the GRCh37 (hg19) reference genome. The data were analyzed with Long Ranger v.2.2.2 WGS pipeline (10X Genomics) with default parameters and GATK (DePristo et al., 2011) was used for variant calling. The genome was covered with a mean depth of 34.7X and a mean molecule length of 26.8 kb.

4.2.3 RNA sequencing and data alignment

The RNA sequencing of the proband and his parents in study IV was performed at the Functional Genomics Unit (FUGU) of the University of Helsinki, Finland. The quality and integrity of the blood-derived RNA were assessed with high sensitivity D5000 Screen Tape® System (Agilent Technologies). After the quality control, RNA sequencing libraries were prepared with the NEBNext Ultra II Directional RNA Library Prep Kit (New England Biolabs Inc., Ipswich, MA, USA). RNA sequencing was carried out as a paired-end run with Illumina NextSeq 500 Mid Output System.

The raw sequencing data in the adaptor-trimmed FASTQ format were subjected to quality assessment with the FASTQC tool (Simon Andrews, Babraham Bioinformatics, Cambridge, the United Kingdom), and based on the overall quality of the data and the individual reads, further trimming was unnecessary. Binary Alignment Map (BAM) files were produced by aligning the FASTQ files to the human genome GRCh37 version with the Spliced Transcripts Alignment to a Reference (STAR) package (Dobin et al., 2013).

4.2.4 RT-qPCR

The RNA extracted from the proband and his parents in study IV was converted into cDNA using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's instructions in a regular thermocycler with 1 µg of total RNA. The synthesized cDNAs were used as templates (25 ng of first-strand cDNA and 0.5 µM forward and reverse primers) to assess the *PALM2AKAP2* mRNA expression with HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis Biodyne, Tartu, Estonia) in a quantitative PCR machine (Roche LightCycler® 480 II). The PCR program included 45-50 cycles of 95°C for 15 s; 60°C for 20 s, and 72°C for 20 s. The expression levels of *PALM2AKAP2* were normalized to *GAPDH.* The relative expression levels were standardized against a healthy unrelated control sample, which was given an arbitrary value of 1.0, by using the ΔCT method.

4.2.5 Sanger sequencing

The Sanger sequencing method was applied to screen *KCNQ1* and *KCNE2* in study I; the selected miRNA genes in study II; *MKRN3* in study III; *PALM2* and *AKAP2* in study IV; and to verify the potentially causative variants in *RIMBP3C* and *SARS1* in study IV (**Table 2**).

Table 2. Details of the Sanger-sequenced genes and investigated subjects in each study.

Study	Subjects (n)	Gene			Assembly
I	somatotropinoma patients (45)	*KCNQ1*	ENSG00000053918	ENST00000155840.9	GRCh38
		KCNE2	ENSG00000159197	ENST00000290310.3	GRCh38
II	KS patients (19)	*MIR141*	ENSG00000207708	ENST00000384975.1	GRCh38
		MIR429	ENSG00000198976	ENST00000362106.1	GRCh38
		MIR200A	ENSG00000207607	ENST00000384875.3	GRCh38
		MIR200B	ENSG00000207730	ENST00000384997.3	GRCh38
		MIR200C	ENSG00000207713	ENST00000384980.3	GRCh38
	normosmic CHH patients (5)	*MIR7-3*	ENSG00000207630	ENST00000384898.1	GRCh38
III	Finnish parents & the Polish family (6)	*MKRN3*	ENSG00000179455	ENST000314520.5	GRCh38
IV	KS patients (16)	*PALM2*	ENSG00000243444	ENST00000314527.4	GRCh37
		AKAP2	ENSG00000241978	ENST00000434623.2	GRCh37
	the proband and his family (4)	*RIMBP3C*	ENSG00000183246	ENST00000433039.1	GRCh37
		SARS1	ENSG00000031698	ENST00000234677.2	GRCh37

The coding exons and exon-intron boundaries of the genes (**Table 2)**, or in the case of the *RIMBP3C* and *SARS1* variants, the nearby sequences surrounding the variants of interest, were PCR-amplified from the genomic DNA samples. The PCR products were purified with ExoProStar (GE Healthcare Life Sciences, IL, USA) treatment. The purified products of *KCNQ1* and *KCNE2* were sequenced from the forward direction and the purified products of all other genes from both directions with the ABI BigDyeTerminator Cycle Sequencing Kit (v3.1) and ABI Prism 3730xl DNA Analyzer automated sequencer (Applied Biosystems, Foster City, CA, USA). The DNA sequences were aligned and read by using Sequencher 5.4 software (Gene Codes Corporation, Ann Arbor, MI, USA).

***AKAP2* RT-PCR analysis**

In study IV, RNAs of the KS patient, who carried the intronic *AKAP2* variant c.2570-13C>T, and of an unrelated KS patient, who did not carry the variant, were converted into complementary DNA (cDNA) by using the SuperScript® III First-Strand Synthesis System for RT-PCR Kit (Invitrogen by Life Technologies, Carlsbad, CA, USA) with random hexamer primers (50 ng/µl) according to the manufacturer's instructions. 2 µl of the synthesized *AKAP2* cDNA was PCR-amplified with cDNA-specific primers surrounding the variant of interest, and the PCR products were visualized on a 1.5% agarose gel. The bands of expected sizes from the patient and control samples were extracted from the gel with NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany) and Sanger sequenced from both directions as described above.

4.2.6 Bioinformatic prediction tools

Web-based tools

Effects of the detected variants in *KCNQ1, KCNE2* (study I), *MKRN3* (study III), and *AKAP2* (study IV) on transcripts were manually predicted with the *in silico* tools in **Table 3**. The ClinVar database and Intervar tool were additionally utilized to evaluate the clinical significance of the variant in *KCNE2* (study I) and in *AKAP2* (study IV), respectively (**Table 3**).

Table 3. *In silico* tools and databases applied in predicting the effects and clinical significance of the detected variants in the protein-coding genes. All analyses were performed with default settings.

Variants	Study	Tools or databases
KCNQ1 synonymous or intronic	I	MutationTaster[a], Human Splicing Finder[b]
KCNE2 p.(Thr8Ala)	I	MutationTaster[a], PolyPhen-2[c], SIFT[d], ClinVar[e]
MKRN3 p.(Ile313Met) & p.(Gly413Thrfs*63)	III	MutationTaster[a], PolyPhen-2[c], SIFT[d]
AKAP2 c.2570-13C>T	IV	MutationTaster[a], Human Splicing Finder[b], InterVar[f], NetGene2[g], BDGP NNSPLICE[h]

[a]Schwarz et al. (2014) (http://www.mutationtaster.org/)
[b]Desmet et al. (2009) (https://www.genomnis.com/access-hsf)
[c]Adzhubei et al. (2010) (http://genetics.bwh.harvard.edu/pph2/)
[d]Kumar et al. (2009) (https://sift.bii.a-star.edu.sg/)
[e]Landrum et al. (2018) (https://www.ncbi.nlm.nih.gov/clinvar/)
[f]Li & Wang (2017) (http://wintervar.wglab.org/). Used for clinical interpretation according to the 2015 ACMG/AMP guidelines ((Richards et al., 2015)
[g]Hebsgaard et al. (1996) (http://www.cbs.dtu.dk/services/NetGene2/)
[h]Reese et al. (1997) (https://www.fruitfly.org/seq_tools/splice.html)

In study II, the mouse gene *Mir7-2* (ENSMUSG00000065609, ENSMUST00000083675.1, GRCm38, reference strain CL57BL6) was aligned against the human reference genome (GRCh38) with the web-based BLASTN (Altschul et al., 1990) and BLAT (Kent, 2002) in Ensembl database (http://www.ensembl.org/index.html) (Zerbino et al., 2018) to find the closest equivalent human gene. The BLAT search was run with 'Genomic sequence' and the BLASTN search with 'Ensembl Non-coding RNA genes' as DNA databases. In both alignment types, other settings were default. In study II, miRWalk 3.0 (http://mirwalk.umm.uni-heidelberg.de/) (Sticht et al., 2018) with default settings was applied to search the predicted target genes of hsa-miR-7-5p, the mature human miR-7 annotated in miRBase (https://www.mirbase.org/index.shtml) (Kozomara & Griffiths-Jones, 2014).

ANNOVAR

The proband's (study IV) VCF file, included in the WES data, was annotated with the functional annotation tool ANNOVAR (Wang et al., 2010) to find potentially causative variants in other but known KS genes in the proband. The annotation was performed at FIMM. Based on the annotation, the following criteria were applied to filter the potentially causative variants: the variant should be 1) nonsynonymous and 2) exonic or located in the consensus splice site according to the Ensembl database, and 3) the variant should not be classified as benign by any of the ten applied *in silico* tools that were included in the annotation (SIFT (Kumar et al., 2009); LRT (http://www.genetics.wustl.edu/jflab/lrt_query.html) (Chun & Fay, 2009); MutationTaster (Schwarz et al., 2014); MutationAssessor (http://mutationassessor.org/r3/) (Reva et al., 2011); FATHMM (http://fathmm.biocompute.org.uk/) (Shihab et al., 2013); PROVEAN (http://provean.jcvi.org/index.php) (Choi & Chan, 2015); MetaSVM (https://sites.google.com/site/jpopgen/dbNSFP) (Dong et al., 2015); MetaLR (https://sites.google.com/site/jpopgen/dbNSFP) (Dong et al., 2015); M-CAP

(http://bejerano.stanford.edu/mcap/) (Jagadeesh et al., 2016), and FATHMM-MKL (http://fathmm.biocompute.org.uk/) (Shihab et al., 2015)).

Additionally, 4) the potentially causative variant(s) should fit the putative modes of inheritance and fulfil the frequency criteria that were selected for each inheritance mode. The frequency criteria should be fulfilled in all ethnic subpopulations of the databases included in the annotation (gnomAD (Karczewski et al., 2020); ExAC (data integrated into gnomAD (Karczewski et al., 2017); 1000 Genomes (1000 Genomes Project Consortium, 2015), and Exome Variant Server (Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA, https://evs.gs.washington.edu/EVS/). The putative modes of inheritance and frequency criteria were: a) *de novo* so that the variant would occur only in the proband, and such a variant should have a minor allele frequency (MAF)<0.1%; b) autosomal recessive so that the variant would be biallelic in the proband (homozygous or compound heterozygous), MAF<2%; c) autosomal dominant so that the variant would be monoallelic and inherited from one parent and be absent in the sister, MAF<0.1% or d) X-linked recessive so that the variant would be monoallelic and inherited in the X chromosome, MAF<2%.

4.2.7 Bioinformatic visualization tools

Loupe

In study IV, Loupe 2.1.1 interactive visualization tool (10X Genomics) was employed to visualize the whole genome linked-read sequencing data, define the exact 9q31.2 deletion breakpoints, and verify the absence of genomic structural variants of over 40 bp in size in the coding regions of 32 KS genes (*ANOS1, FGFR1, FGF8, FGF17, PROK2, PROKR2, CHD7, NSMF, HS6ST1, WDR11, SEMA3A, SEMA7A, PLXNA1, SOX10, IL17RD, FEZF1, NDNF, TCF12, TUBB3, DCC, SMCHD1, KLB, NTN1, SPRY4, PTCH1, FLRT3, AMH, DUSP6, PLXNA3, NRP1, SPRY2,* and *NRP2*) in the proband.

BasePlayer

In study I, BasePlayer analysis and visualization tool (Katainen et al., 2018) was used to evaluate the allelic imbalance in the chromosomal region at the c.22A>G, p.(Thr8Ala) variant in the acromegaly patient ST6's blood- and tumor tissue-derived whole genome sequencing data (Välimäki et al., 2015). In study IV, BasePlayer was used to verify that the coding exons of the 32 KS genes (*ANOS1, FGFR1, FGF8, FGF17, PROK2, PROKR2, CHD7, NSMF, HS6ST1, WDR11, SEMA3A, SEMA7A, PLXNA1, SOX10, IL17RD, FEZF1, NDNF, TCF12, TUBB3, DCC, SMCHD1, KLB, NTN1, SPRY4, PTCH1, FLRT3, AMH, DUSP6, PLXNA3, NRP1, SPRY2,* and *NRP2*) were covered in the WES data of the proband.

Integrative Genomics Viewer (IGV)

In study IV, the Sashimi plot utility of the Mixture-of-Isoforms (MISO) framework (Katz et al., 2015) in the Integrative Genomics Viewer (IGV) browser (http://software.broadinstitute.org/software/igv/) (Broad Institute, Cambridge, MA, USA) (Thorvaldsdóttir et al., 2013) was used to visualize the splicing events of the 13 known KS genes which were expressed in peripheral blood leukocytes,

FGFR1, PROK2, CHD7, NSMF, HS6ST1, WDR11, SEMA3A, SEMA7A, PLXNA1, TCF12, SMCHD1, PTCH1, and *DUSP6*, in the RNA sequencing data (BAM files) of the proband and his parents.

4.3 Statistics

In study I, the significance of the *KCNE2* variant c.22A>G, p.(Thr8Ala) allele frequency differences between the acromegaly patient set and the gnomAD database controls was calculated with Fisher's exact test. $P < 0.05$ was accepted to indicate significance. The proband's (study IV) and his parents' relative expression values of *PALM2AKAP2* in peripheral blood leukocytes were compared with one-way ANOVA with four replicates of *PALM2AKAP2* expression from each person. The replicates were measured by qPCR, normalized by the housekeeping gene (*GAPDH*), and standardized against an unrelated control sample. ANOVA was followed by Tukey's HSD post-hoc test. $P<0.05$ was accepted to indicate significance.

4.3.1 Probability estimate for the occurrence of 9q31.2 microdeletions

To evaluate the role of the 9q31.2 deletion in the proband in study IV, the CNV data obtained from the UK Biobank (Aguirre et al., 2019; McInnes et al., 2019) was utilized. The CNV data contained 4 deletions of 1 Mb or larger, which overlapped the 9q31.2 area in a set of 472,734 people (Global Biobank Engine, Stanford, CA (URL: http://gbe.stanford.edu), [3rd March, 2021 accessed]). The probability to randomly pick a Finnish male with KS, without a causal mutation in known KS genes, and with a microdeletion larger than 1 Mb in the 9q31.2 area, was calculated as follows: 1:30,000 x 1:2 x 4:472,734 = 1.41e-10, as the frequency of KS in males in Finland is approximately 1:30,000 (Laitinen et al., 2011), and that approximately 50% of the CHH patients can be given a molecular genetic diagnosis (Young et al., 2019). The respective probability for a male KS patient, who would be selected based on his phenotype, was calculated as 1:2 x 4:472,734 = 4.23e-06.

5. RESULTS

5.1 *KCNQ1* and *KCNE2* variants in patients with somatotropinoma

5.1.1 Genomic sequencing of *KCNQ1* and *KCNE2*

Sanger sequencing of the coding exons and exon-intron boundaries in *KCNQ1* revealed two synonymous coding variants: ENST00000155840.9:c.1638G>A, p.(Ser546=) (rs1057128) in 11 patients, and ENST00000155840.9:c.1986C>T, p.(Tyr662=) (rs11601907) in 18 patients. Moreover, three patients' previous WGS data contained both the c.1638G>A, p.(Ser546=) and c.1986C>T, p.(Tyr662=) variants. The MAF of the c.1638G>A, p.(Ser546=) variant was 0.2013 in the gnomAD total control population, and five patients carried it in a homozygous form. In turn, the MAF of the c.1986C>T, p.(Tyr662=) variant was 0.1748 in gnomAD, and it was heterozygous in all carriers among the patients. One of its carriers manifested with gigantism. Additionally, screening of *KCNQ1* revealed several deep (13>bp from exon borders) intronic variants in all 45 Sanger-sequenced patients.

Sanger sequencing of *KCNE2* revealed one missense variant, ENST00000290310.3:c.22A>G, p.(Thr8Ala) (rs2234916), in two patients, and it was found in one patient's previous WGS data. The variant was heterozygous in all carriers. It had a MAF of 0.0038 in the Finnish and total control populations in gnomAD, and an allele frequency of 0.028 in the patient set. In gnomAD, eight individuals carried the variant in a homozygous state.

Moreover, *KCNQ1* and *KCNE2* were screened for somatic variants in the eight previously whole-genome sequenced patient samples. No somatic hits appeared.

5.1.2 *In silico* analyses and visual evaluation of allelic imbalance at the *KCNE2* variant

Effects of the coding and deep intronic *KCNQ1* variants on transcript were predicted with MutationTaster and Human Splicing Finder v.3.0. According to the prediction tools, the identified variants had no likely effects on splicing. Moreover, the c.1638G>A, p.(Ser546=) and c.1986C>T, p.(Tyr662=) variants were silent and induced no changes to the amino acid sequence of KCNQ1.

Three *in silico* tools were utilized to predict the effects of the *KCNE2* missense variant, c.22A>G, p.(Thr8Ala) on protein. MutationTaster predicted this variant to be "disease-causing", SIFT "deleterious" (score 0), and PolyPhen-2 "probably damaging" (score 0.991). Moreover, allelic imbalance in the chromosomal region of the variant was evaluated by using patient ST6's WGS data acquired from both peripheral blood-derived and tumor tissue DNAs (Välimäki et al., 2015). Visual evaluation of the data with BasePlayer revealed no allele loss or gain in the chromosomal region (**Figure 3**).

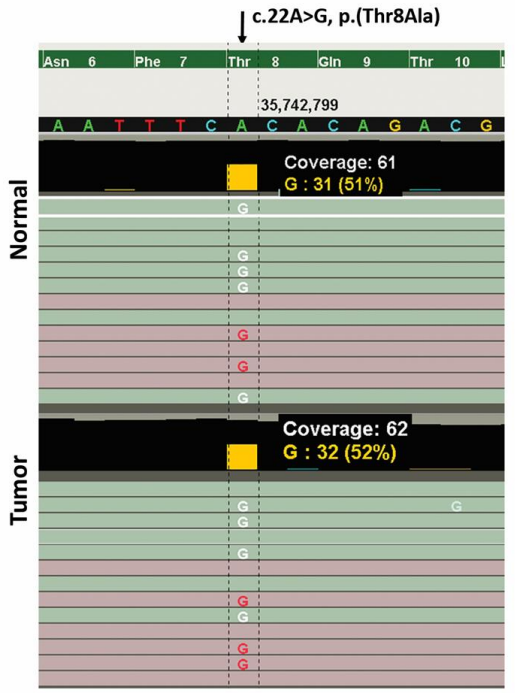

Figure 3. Visual evaluation of allelic imbalance at the c.22A>G, p.(Thr8Ala) locus.
Patient ST6's whole genome sequence reads display a heterozygous c.22A>G *KCNE2* variant in peripheral blood leukocyte ("normal") and tumor tissue ("tumor") DNAs. Both tissues display approximately 50–50% allelic fraction. The figure is cropped to a representative section of the reads, which were visualized with BasePlayer. Coverage: number of total reads covering the position, G: number and percentage (in brackets) of reads with the G allele. Red and green colors mark the + and – direction of the reads, respectively. Appearing originally in Iivonen et al. (2018). Copyright: the authors.

5.1.3 *KCNE2* variant frequencies between the patient set and control population

The significance of the *KCNE2* variant c.22A>G, p.(Thr8Ala) allele frequency differences between the acromegaly patient set and the gnomAD controls was calculated with Fisher's exact test, which yielded a significant difference (OR, 7.73: 95% CI, 1.56–23.32: P=0.008).

Table 4a summarizes the coding variants identified in *KCNQ1* and *KCNE2*, as well as the patients' clinical characteristics, and **Table 4b** the intronic *KCNQ1* variants. According to MutationTaster, all intronic variants were polymorphisms, and according to Human Splicing Finder, none of them affected splicing.

Table 4a. The coding *KCNQ1* and *KCNE2* variants detected in the acromegaly patients (n=54) and the patients' clinical characteristics. The variants are marked according to transcripts ENST00000155840.9 for *KCNQ1* and ENST00000290310.3 for *KCNE2*. Appearing originally in Iivonen et al. (2018). Copyright: the authors.

Patient	Sex	Age at Dg	Age at Op	Clinical Dg	*KCNQ1*		MAF[c]	*KCNE2*		MAF[c]
331	M	39		GH	-			-		
332	F		39	GH	c.1986C>T, p.(Tyr662=)	rs11601907	0.1748	-		
335	M	42	42	GH	-			-		
336	F	25		GH	c.1986C>T, p.(Tyr662=)	rs11601907	0.1748	-		
337	F	43	53	GH	c.1638G>A[b], p.(Ser546=)	rs1057128	0.2013	-		
338	M	38		GH	-			-		
340	M	38	60	GH	c.1986C>T, p.(Tyr662=)	rs11601907	0.1748	-		
341	F	39		GH	-			-		
343	M	42	42	GH	c.1986C>T, p.(Tyr662=)	rs11601907	0.1748	-		
344	M	36		GH	c.1986C>T, p.(Tyr662=)	rs11601907	0.1748	-		
345	M	38		GH	c.1986C>T, p.(Tyr662=)	rs11601907	0.1748	-		
346	M	29		GH	-			c.22A>G, p.(Thr8Ala)	rs2234916	0.0038
347	M	30	34	GH	c.1986C>T, p.(Tyr662=)	rs11601907	0.1748	-		
348	F	42	42	GH	-			-		
349	M	38	40	GH	c.1986C>T, p.(Tyr662=)	rs11601907	0.1748	-		
350	F	36	36	GH	-			-		
351	M	40	40	GH	-			-		
352	M	33	33	GH	c.1986C>T, p.(Tyr662=)	rs11601907	0.1748	-		
353	F	38	39	GH	c.1986C>T, p.(Tyr662=)	rs11601907	0.1748	-		
415	F	39	45	GH	c.1638G>A, p.(Ser546=)	rs1057128	0.2013	-		
416	F	40	40	GH	c.1638G>A[b], p.(Ser546=)	rs1057128	0.2013	-		
417	F	42	43	GH	c.1986C>T, p.(Tyr662=)	rs11601907	0.1748	-		
418	F	44	44	GH	c.1986C>T, p.(Tyr662=)	rs11601907	0.1748	-		
419	F	22	38	GH	c.1986C>T, p.(Tyr662=)	rs11601907	0.1748	-		
420	F	33	33	GH	-			-		
421	M	35		GH	-			-		
422	F	32	47	GH	c.1986C>T, p.(Tyr662=)	rs11601907	0.1748	-		
					c.1638G>A[b], p.(Ser546=)	rs1057128	0.2013	-		

Patient	Sex	Age at Dg	Age at Op	Clinical Dg	*KCNQ1*		MAF[c]	*KCNE2*		MAF[c]
423	M	34	46	GH	c.1638G>A, p.(Ser546=)	rs1057128	0.2013	-		
445	F	39	39	GH	c.1986C>T, p.(Tyr662=)	rs11601907	0.1748	-		
720	F	36		GH	-			-		
721	M	43		GH	-			-		
725	M	40		GH	c.1638G>A[b], p.(Ser546=)	rs1057128	0.2013	-		
813	F		43	GH	c.1638G>A, p.(Ser546=)	rs1057128	0.2013	-		
860	F		31	GH	-			-		
861	M	47	47	GH	-			c.22A>G, p.(Thr8Ala)	rs2234916	0.0038
862	F	53		GH/PRL	c.1986C>T, p.(Tyr662=)	rs11601907	0.1748	-		
833	M		44	GH	c.1638G>A[b], p.(Ser546=)	rs1057128	0.2013	-		
842	M		60	GH	c.1986C>T, p.(Tyr662=)	rs11601907	0.1748	-		
847	F	26		GH	c.1638G>A, p.(Ser546=)	rs1057128	0.2013	-		
848	F	53		GH	c.1986C>T, p.(Tyr662=)	rs11601907	0.1748	-		
849	M	44	44	GH	-			-		
852[d](I)	M	37	37	GH	-			-		
856	M	25		GH	-			-		
857	M	41		GH/PRL	c.1638G>A, p.(Ser546=)	rs1057128	0.2013	-		
858	F	33		GH	c.1638G>A, p.(Ser546=)	rs1057128	0.2013	-		
ST5[a]	M	56		GH	c.1986C>T, p.(Tyr662=)	rs11601907	0.1748	-		
ST6[a](T)	M	40		GH	-			c.22A>G, p.(Thr8Ala)	rs2234916	0.0038
ST7[a](E)	F	40		GH	c.1638G>A, p.(Ser546=)	rs1057128	0.2013	-		
ST8[a]	F	55		GH	-			-		
ST9[a]	F	38		GH	c.1638G>A, p.(Ser546=)	rs1057128	0.2013	-		
ST10[a,e]	M	14		GH	c.1986C>T, p.(Tyr662=)	rs11601907	0.1748	-		
ST11[a]	F	24		GH	c.1986C>T, p.(Tyr662=)	rs11601907	0.1748	-		
ST12[a](S)	M	37		GH	c.1638G>A, p.(Ser546=)	rs1057128	0.2013	-		

[a]Whole genome sequencing data from Välimäki et al. (2015); [b]Homozygous variant; [c]Minor allele frequency (MAF) in gnomAD total population; [d]Familial background; [e]Gigantism. Dg: diagnosis; E: Estonian; GH: growth hormone; I: Italian; Op: operation; PRL: prolactin; S: Spanish; T: Tunisian

Table 4b. Intronic variants in *KCNQ1* and their *in silico* predictions. The variants are marked according to transcript ENSG00000053918 (ENST00000155840.9). Modified from Iivonen et al. (2018). Copyright: the authors.

Variant	Number of patients carrying the variant (hom/het)[a]	MAF[b]	
c.387-51T>C	0/8 (0.0755)	0.0077	rs192393524
c.477+86_88delGG	13/28 (0.5094)	0.6125[c]	rs566926544
c.478-24G>A	0/1 (0.0094)	0.00056	rs375027164
c.781-117A>G	1/1 (0.0283)	0.0201	rs41282926
c.781-99A>G	0/15 (0.1415)	0.3572	rs4930127
c.1394-14C>T	0/1 (0.0094)	0.0108	rs28730758
c.1394-39T>G	0/11 (0.1038)	0.1224	rs739502
c.1514+50C>G	0/1 (0.0094)	0.0015	rs199948521
c.1514+46A>G	6/23 (0.3302)	0.4495	rs760419
c.1514+18C>T	0/1 (0.0094)	0.0090	rs12577654
c.1590+14T>C	0/13 (0.1226)	0.08532	rs11024034
c.1685+36A>G	23/9 (0.5189)	0.6685	rs163150
c.1732+43T>C	4/19 (0.2547)	0.2249	rs81204
c.1794+32G>T	0/4 (0.0377)	0.0354	rs41282928

[a]homozygous (hom) and heterozygous(het) form; variant frequencies among the patients are in brackets
[b]Minor allele frequency (MAF) in gnomAD total population
[c]later merged into rs145292450, MAF of which is marked

5.2 *MIR7-3*, *MIR141*, *MIR429* and *MIR200A-C* in normosmic CHH and KS

5.2.1 Identified miRNA gene variants

Screening of *MIR141, MIR429*, *MIR200A, MIR200B,* and *MIR200C* in 19 Finnish KS patients and *MIR7-3* in 5 Finnish normosmic CHH patients revealed one *MIR200A* variant, ENST00000384875.3:c.42C>T, rs202051309, in one KS patient. This variant had a frequency of 0.01996 in the Finnish population in gnomAD. No variants were identified in other genes.

5.3 Two novel *MKRN3* variants in a Finnish and a Polish family

5.3.1 The identified *MKRN3* variants

In the Finnish index patient, *MKRN3* was analyzed with a commercial gene panel, in which a heterozygous missense variant, ENST00000314520.5:c.939C>G, p.(Ile313Met), was identified. The variant was predicted to be deleterious according to the applied *in silico* tools (SIFT: score 0.01, "deleterious"; PolyPhen-2: score 1.000, "probably damaging" and MutationTaster: score 29, "disease-causing"), and it was absent in the gnomAD and SISu databases. Sanger sequencing of her parents revealed that the variant was present in her father and absent in her mother. Genotypes of the index patient's half-sister and cousin, who had early puberty as well, were unavailable (**Figure 4**).

In the Polish family, *MKRN3* was Sanger sequenced in all family members, and a heterozygous deletion, ENST00000314520.5:c.1237_1252del, p.(Gly413Thrfs*63), was found in the father and both children (**Figure 4**). The variant was predicted to lead to a frameshift and a premature stop codon, and it was absent in gnomAD.

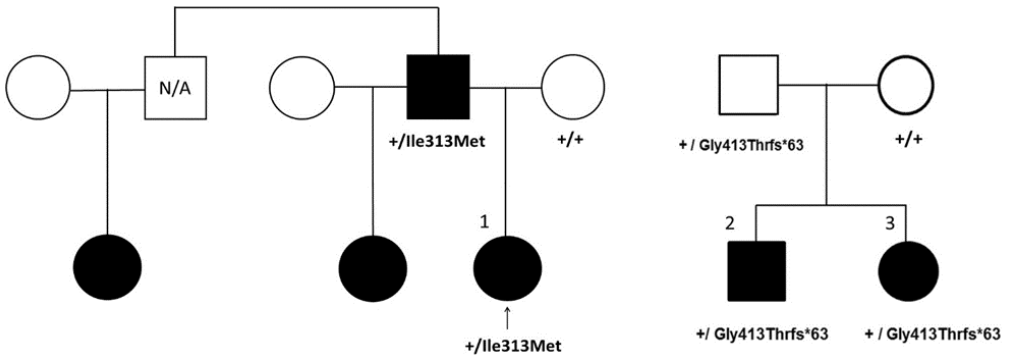

Figure 4. Pedigrees of the Finnish (left) and Polish (right) families showing the segregation of the *MKRN3* variants and available genotypes of the family members.
Filled symbols: central precocious puberty; arrow: index patient; N/A: phenotype not available. Modified from Varimo & Iivonen et al. (2021).

5.4 Deletion on 9q31.2 and *PALM2AKAP2* in Kallmann syndrome

5.4.1 Determination of the 9q31.2 deletion breakpoints and *PALM2AKAP2* expression

The proband's (in study IV) DNA was whole genome linked-read sequenced to determine the exact breakpoints of the 2.38 Mb deletion on chromosome 9q31.2, which had been previously detected in a comparative genetic hybridization microarray. Visualization of his whole genome linked-read sequencing data with Loupe revealed that the deletion lied in 9:108,331,353-110,707,332 (GRCh37) (**Figure 5**). The deletion laid approximately 1.8 Mb upstream of the *PALM2AKAP2* locus in 9:112,542,589-112,934,792 (GRCh37). Instead of *PALM2AKAP2*, the deletion disrupted *FKTN*, which contains 10 exons, at 9:108,331,353 in the first intron, and no other known gene at 9:110,707,332. In total, the deletion encompassed six protein-coding genes (*FKTN, TAL2, TMEM38B, ZNF462, RAD23B,* and *KLF4*). Investigation of the *PALM2AKAP2* expression in the proband's and his parents' blood leukocyte-derived DNA with RT-qPCR revealed that the proband's *PALM2AKAP2* transcript was expressed at a higher level than in the father (**Figure 6**), indicating that the deletion, despite the relative vicinity, did not suppress *PALM2AKAP2* expression.

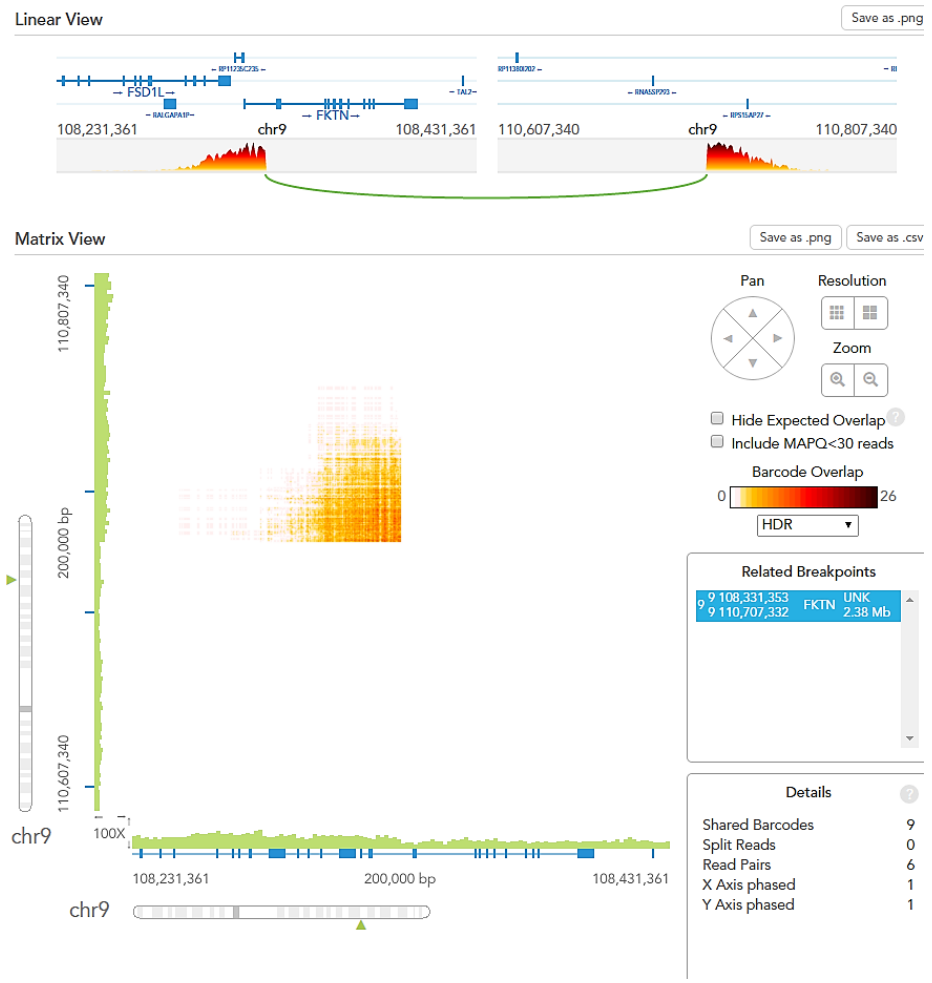

Figure 5. Visualization of the proband's whole genome linked-read sequencing data with Loupe.
In the Linear View, the deletion is drawn as a green arc between the breakpoints. The genes close to each breakpoint are shown in blue; at the 5' end, the deletion breaks *FKTN* in the first intron, and it disrupts no other gene at the 3' end. Additionally, the Linear View draws a linearized view of the barcode overlap matrix below. In the Matrix View, the corresponding breakpoints are displayed in the matrix in the center. Each position of the matrix corresponds to a pair of loci from the two axes. The intensity of the color shows the number of barcodes that were observed in reads from both loci, that is the number of distinct common barcodes shared between the two loci. Outside the matrix plot, the vertical green bars show the average coverage per base, and the scale is shown in the lower left (100X). The Related Breakpoints box shows the log-likelihood-based quality score for the deletion, 9, which, being positive, indicates that the observed barcode overlap between the two breakpoints can better be explained by the presence of a structural variant than by the absence of it. In addition, the Related Breakpoints box shows the breakpoint genomic coordinates (9:108,331,353 - 9:110,707,332, GRCh37) and the distance between them (2.38 Mb). **(**Iivonen & Kärkinen et al. (2021); unpublished results).

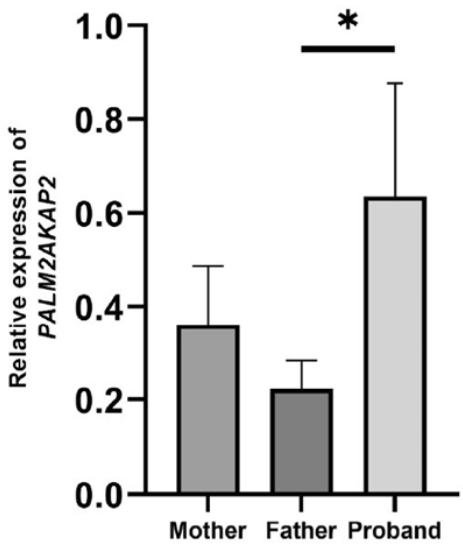

Figure 6. Relative expression of *PALM2AKAP2* in the peripheral blood leukocytes of the proband and his parents.
The bars represent four replicates of *PALM2AKAP2* expression that were measured by qPCR, normalized to the housekeeping gene (*GAPDH*) and standardized against an unrelated healthy control sample. *, P<0.05 (one-way ANOVA followed by Tukey's HSD post-hoc test). Appearing originally in Iivonen & Kärkinen et al. (2021). Copyright: the authors.

5.4.2 Sanger sequencing of *PALM2* and *AKAP2*

To further probe the putative role of *PALM2AKAP2* in KS, the exons, and exon-intron boundaries of *PALM2* and *AKAP2* were Sanger sequenced in 16 Finnish KS patients (one woman, 15 men) without conclusive variants in *ANOS1, FGFR1, FGF8, PROK2, PROKR2, CHD7,* or *WDR11*. In intron 2 of *AKAP2,* one rare heterozygous variant, ENSG00000241978 (ENST00000434623.2):c.2570-13C>T (rs777796314), was detected. The frequency of this variant was 0.00004267 in the gnomAD Finnish population, and its highest frequency among all gnomAD subpopulations was 0.0001702. According to the 2015 ACMG/AMP guidelines, this variant was classified (by using InterVar) as likely benign. The carrier was diagnosed with KS at the age of 14, and he had presented with sensorineural hearing loss in the right ear, micropenis, normal MRI, and alopecia at the age of five. He had no family history of delayed puberty. Four *in silico* tools (MutationTaster, NetGene2, Human Splicing Finder v.3.0 and BDGP NNSPLICE v.0.9) were applied to predict the effects of the variant, and according to two of them (MutationTaster: "disease-causing – splice site changes"; NetGene2: alteration of acceptor sites), the variant might affect splicing. To investigate the putative effect of the variant on splicing *in vitro*, the carrier's and a control KS patient's (who did not carry the variant) RNAs were converted into cDNAs, Sanger sequenced and compared, which revealed that the splicing was normal in the carrier. All other encountered *PALM2* and *AKAP2* variants had MAFs above 1% in the gnomAD Finnish or European, or in cases where the variant was absent in gnomAD, in dbSNP European control population.

5.4.3 Variants in KS-implicated genes

The commercial KS gene panel, which the proband had previously undergone and which had covered the coding regions of *ANOS1, CHD7, FGF8, FGFR1, GNRHR, KISS1R, PROK2, PROKR2,* and *TACR3* up to 20 bp of intronic sequence as well as structural variants, had been negative for likely pathogenic variants. In addition, as his deletion on chromosome 9q31.2 *per se* or the adjacent candidate gene *PALM2AKAP2* gave no equivocal answer to whether the 9q31.2 deletion underlies his KS, his whole family was whole exome sequenced. First, it was visually verified with BasePlayer that the 32 genes implicated in KS (*IL17RD, SPRY2, DUSP6, CHD7, FGFR1, SOX10, ANOS1, FGF8, FGF17, PROK2, PROKR2, NSMF, HS6ST1, WDR11, SEMA3A, SEMA7A, PLXNA1, FEZF1, NDNF, TCF12, TUBB3, DCC, SMCHD1, KLB, NTN1*, *SPRY4, PTCH1, FLRT3, AMH, PLXNA3, NRP1,* or *NRP2*) were indeed covered the in proband's WES data. All these genes had been covered, and no rare sequence variants were found in them. In addition, the proband's whole genome linked-read sequencing data were visually investigated for genomic structural variants of over 40 bp in the coding regions of the 32 KS genes. None of the genes were disrupted by structural variants. Finally, as WES may not detect non-coding variants that affect splicing, the splicing events of the KS genes in the proband and his parents were investigated with RNA sequencing of blood-derived samples and subsequent visualization of the sequencing data. Overall, 15 KS genes, *FGFR1, PROK2, CHD7, NSMF, HS6ST1, WDR11, SEMA3A, SEMA7A, PLXNA1, TCF12, SMCHD1, PTCH1,* and *DUSP6*, were expressed in peripheral blood leukocytes, and none of them displayed aberrant splicing events in the visual data evaluation with Sashimi plots.

5.4.4 Potentially causative variants from WES

Lastly, the proband's annotated WES data were investigated for variants in new potential KS candidate genes, and two rare heterozygous, potentially causative variants emerged: ENST00000433039.1:c.1189_1191del, p.(Glu397del) (rs1555881978) in *RIMBP3C,* and ENST00000234677.2:c.950G>A, p.(Arg317Gln) (rs1412278011) in *SARS1*. The *SARS1* and *RIMBP3C* variants were also present in the mother. The *RIMBP3C* variant was absent in all applied databases (ExaC, gnomAD, 1000 Genomes, dbSNP and Exome Variant Server), whereas the *SARS1* variant was present in dbSNP with a MAF of 0.000008. Both variants were absent in the sister and the father. As the associations of the *SARS1* and *RIMBP3C* genes to the proband's phenotype are uncertain, the variants that occurred in them were classified as variants of uncertain significance following the 2015 ACMG/AMP guidelines (Richards et al., 2015).

6. DISCUSSION

Disorders of growth and puberty may cause remarkable psychosocial stress for the affected individual and can have long-term health outcomes. For instance, anxiety, depression, and poor self-image are frequent among patients with acromegaly, and those with precocious puberty and CHH (Dimopoulou et al., 2017; Temelturk et al., 2021; Dwyer et al., 2019). Identifying the genetic defects underlying these disorders increases understanding of the genetic regulation of growth and puberty, and is valuable for the diagnosis, treatment, and genetic counseling of the patient and their family. Knowing the genetic cause of their condition might also be a relief for the patient. Although numerous genes have been implicated in disorders of growth and puberty, the genetic cause in known disease genes is identified in only a half of the patients with somatotropinoma or CHH (Rostomyan et al., 2015; Boehm et al., 2015; Maione et al., 2018; Young et al., 2019), indicating that novel disease genes remain to be identified.

The aim of this study was to discover defects in specific genes and evaluate their roles in disorders of growth and puberty, which stem from the aberrant secretion of pituitary hormones. In a broader perspective, the selected disorders are due to abnormal activation or inactivation of two hypothalamic-pituitary-target organ axes: the hypothalamic-pituitary-IGF-1 axis and the hypothalamic-pituitary gonadal axis, which are interconnected to regulate the maturation of the body into its adult form and to maintain vital functions (Tenuta et al., 2020; Dees et al., 2021; Cannarella et al., 2021). The hypothalamic-pituitary-IGF-1 axis was examined for activating genetic factors after puberty. *KCNQ1*, a voltage-gated potassium channel gene implicated in GH deficiency, and its auxiliary subunit *KCNE2*, were screened for germline variants potentially predisposing to somatototropinoma formation in a cohort of somatotropinoma patients of Finnish, European, and African origin operated in Finland. A rare variant of unknown significance in *KCNE2* was found to have a significantly higher frequency among somatotropinoma patients compared to controls.

In turn, the hypothalamic-pituitary gonadal axis was examined for both activating and inactivating genetic factors before puberty, as the genetic defects underlying precocious and absent puberty were investigated in Finnish and Polish families. Segregating, paternally inherited novel missense and frameshift variants in *MKRN3*, a gene suggested to function as a pubertal brake, were detected in a Finnish and a Polish family, supporting the remarkable role of defects in this gene in precocious puberty. In addition, a Finnish patient with KS, Weiss-Kruszka syndrome, and a *de novo* 2.38 Mb deletion in 9q31.2, a region associated with pubertal timing and the sense of smell, was described. Potentially pathogenic variants in the miRNA genes *MIR141, MIR429, MIR200A, MIR200B, MIR200C,* and *MIR7-3* as well as in *PALM2AKAP2*, promising CHH candidate genes, were found to be rare in Finnish CHH patients. These results are further discussed in the following subchapters.

6.1 Current knowledge on the roles of *KCNQ1* and *KCNE2* in aberrant GH secretion

In *KCNQ1*, two synonymous coding variants, p.(Ser546=) and p.(Tyr662=) were detected in hetero- or homozygous states in fourteen and twenty-one patients, respectively. The minor allele frequencies of both variants in gnomAD total population were over 5%, which, according to the ACMG/AMP 2015 criteria, is sufficient to indicate a benign variant (Richards et al., 2015). In addition, the deep intronic variants c.387-51T>C, c.477+86_88delGG, c.781-99A>G, c.1394-39T>G, c.1514+46A>G,

c.1590+14T>C, c.1685+36A>G, and c.1732+43T>C, were found in hetero- or homozygous forms in eight or more somatotropinoma patients, whereas c.781-117A>G was found in two, c.1794+32G>T in four, and c.478-24G>A, c.1394-14C>T, c.1514+50C>G, and c.1514+18C>T each in one patient. The minor allele frequencies of the deep intronic variants ranged from 0.00056 to 0.6685 in the gnomAD total population, and none of the variants were predicted to affect splicing according to Human Splicing Finder or MutationTaster. The variant c.477+86_88delGG, which was not identified in gnomAD or dbSNP at the time of publication, has been merged into rs145292450, MAF of which is 0.6125 in gnomAD. Similarly, the MAFs of all the deep intronic variants except c.478-24G>A, c.781-117A>G, c.1394-14C>T, c.1514+50C>G, and c.1514+18C>T were over 5% in at least one subpopulation in gnomAD, dbSNP, or SISu.

The rare (MAF under 5%) deep intronic variants c.781-117A>G, c.1394-14C>T, and c.1514+18C>T have been classified (based on the ACMG/AMP 2015 criteria) as benign; and c.478-24G>A and c.1514+50C>G variants of unknown significance in VarSome (Kopanos et al., 2019). Moreover, the variants classified as VUS have no associated publications in PubMed, ClinVar, dbSNP, VarSome or LitVar. Although it cannot be excluded that the rare deep intronic variants could have a pathogenic role, based on the observations in the current study and the public data, the variants identified in *KCNQ1* seem to be unlikely associated with somatotropinoma formation.

Nevertheless, *KCNQ1* is an intriguing candidate gene for somatotropinoma formation. First, ion channels are essential for the regulation of GH secretion. Somatotrope cell membranes express potassium, sodium, and calcium channels, which contribute to GH secretion through electrical signaling and in response to GHRH and SST (Eigler & Ben-Shlomo, 2014; Fletcher et al., 2018). In addition to GH secretion, ion channels participate in setting the resting membrane potential (Fletcher et al., 2018). K_V-type voltage-gated potassium channels like *KCNQ1* play a role in reducing excitation in pituitary cells (Stojilkovic et al., 2010).

Second, potassium channel genes have also been implicated in tumorigenic processes, such as angiogenesis as well as tumor resistance to apoptosis, invasion, and growth (Rapetti-Mauss et al., 2020; Tommiska et al., 2017). KCNQ1 has been recently connected to Wnt/β-catenin signaling, which is implicated in pituitary development, formation of craniopharyngiomas (epithelial tumors arising from the Rathke's pouch) (Gaston-Massuet et al., 2011), and several cancers (Rapetti-Mauss et al., 2020). KCNQ1 can suppress Wnt/β-catenin signaling and hold β-catenin in the plasma membrane with E-cadherin by forming a complex with β-catenin and E-cadherin; it has been suggested that the hyperpolarization induced by opening potassium channels could be the mechanism suppressing Wnt/β-catenin signaling (Rapetti-Mauss et al., 2020). High expression of *KCNQ1* is associated with high expression of E-cadherin and epithelial phenotype of a colorectal cancer cell (Rapetti-Mauss et al., 2017; Rapetti-Mauss et al., 2020). In turn, suppression of *KCNQ1* is associated with redistribution of β-catenin and E-cadherin from the membrane to the cytosol and epithelial-to-mesenchymal transition (a process associated with tumor development and progression), suggesting tumor suppressor activity for *KCNQ1*. In addition to being a regulator of Wnt/β-catenin signaling, *KCNQ1* is a target gene in this pathway, as β-catenin together with TCF4 (a regulator of target gene expression in the Wnt pathway) can suppress *KCNQ1* expression (Rapetti-Mauss et al., 2020; Rapetti-Mauss et al., 2017).

KCNQ1 is present in the membrane of the somatotrope cell (Tommiska et al., 2017), and E-cadherin is expressed in somatotropinomas (Fougner et al., 2010; Venegas-Moreno et al., 2019). It has been reported that absent or minimal membranous expression of E-cadherin due its translocation to the nucleus correlates with large size and partly with high invasiveness of somatotropinomas (Fougner et al., 2010; Venegas-Moreno et al., 2019). Furthermore, downregulation of Wnt-pathway inhibitors in somatotropinomas has been observed (Elston et al., 2008; Elston et al., 2010), and some genes implicated in acromegaly or gigantism are linked to Wnt/β-catenin signaling in similar manners as *KCNQ1*. For instance, MEN1 interacts with β-catenin and TCF4 to suppress their activity, whereas the loss of MEN1 promotes Wnt/β-catenin signaling and cell proliferation (Brandi et al., 2021). In addition, *CDC73*, a candidate gene for somatotropinoma formation, interacts with β-catenin and can inhibit Wnt/β-catenin signaling (James et al., 2009; Westin, 2016). Thus, the possibility exists that *KCNQ1* could participate in somatotropinoma formation through E-cadherin and Wnt-signaling.

KCNQ1 is expressed in the developing and mature pituitary (Scagliotti et al., 2021), and in the developing neural crest (Morokuma et al., 2008). The findings by Ueharu et al. (2017) suggest that neural crest cells invade the pituitary during development, and finally account for a proportion of secretory pituitary cells in the adult. Modulation of KCNQ1 function in a neural crest-derived cell lineage conferred a hyperproliferative invasive phenotype in *Xenopus laevis* and implicated that KCNQ1 potassium channel modulation could affect not only early cell population, but also mature neural crest cells or their derivatives (Morokuma et al., 2008). These findings might suggest a possible involvement of *KCNQ1* in the development of adenomas from neural crest-derived somatotropes.

KCNE2 forms complexes with KCNQ1 not only in the pituitary (Tommiska et al., 2017) but also, for instance, in gastric epithelial cells and (likely) in pancreatic β cells, in both of which KCNE2 is required for the secretion of gastric acid or insulin (Abbott & Roepke, 2016; Abbott, 2015; Lee et al., 2017). *Kcne2* is also implicated in the proliferation of gastric epithelial- as well as hepatocellular cancer cells as a target of miR-584-5p, and reduced expression of the protein is detected in human gastric cancer and hepatocellular carcinoma (Roepke et al., 2010; Yanglin et al., 2007, Wei et al., 2019). In *KCNE2*, a heterozygous c.22A>G, p.(Thr8Ala) missense variant was identified in three patients. The allele frequency of this variant was significantly higher among the patients compared to the gnomAD control population. The variant has previously been associated with congenital or drug-induced long QT syndrome, cardiac-related sudden death, and the QT interval duration in the healthy (Crump & Abbott, 2014; Abbott, 2013; Paulussen et al., 2004; Park et al., 2003; Frangiskakis et al., 2010; Aydin et al., 2005; Jongbloed et al., 2002; Sesti et al., 2000). However, the associations with inherited arrhythmogenic syndromes, sudden cardiac death, and the QT interval duration are somewhat controversial (Raju et al., 2019; Marcondes et al., 2018; Giudicessi et al., 2018; Sanchez et al., 2016; Campuzano et al., 2020; Albert et al., 2010; Marjamaa et al., 2009; Sudandiradoss & Sethumadhavan, 2008; Arnestad et al., 2007; Gouas et al., 2007; Larsen et al., 2001).

All patients (346, 861, and ST6) harboring the p.(Thr8Ala) variant had typical somatotropinomas without significant abnormalities. Of the three patients, electrocardiograms were available for two (861 and ST6). Patient 861 had a normal QT interval in the electrocardiogram, whereas patient ST6 showed a prolonged QT interval during transient diabetic ketoacidosis. During transient diabetic

ketoacidosis, prolonged QT interval is frequent (Perez et al., 2021; Talebi et al., 2016; Youssef & Farid, 2012), and in patient ST6, the QT returned to and stayed normal after ketoacidosis recovery.

Taken together, two coding variants in *KCNQ1* and one coding variant in *KCNE2* were identified. It seems unlikely that these germline variants are associated with somatotropinoma formation. The patient cohort was however limited in size, and therefore additional screenings of *KCNQ1* and *KCNE2* for germline or somatic variants in larger cohorts including familial cases are needed to validate this conclusion. The potential connection of the KCNE2 p.(Thr8Ala) variant to acromegaly also requires further studies. Of note, this study (Iivonen et al., 2018) focused only on two candidate genes for somatotropinoma formation, *KCNQ1* and *KCNE2,* and all genes implicated in familial acromegaly were not included in the genetic screening. Thus, some of the patients could harbor variants in known familial acromegaly genes, or somatic variants contributing to somatotropinoma development. Future studies with larger patient cohorts and modeling *KCNQ1* and *KCNE2* expression in somatotropinomas are needed to elucidate the role of these genes, if any, in the development of GH excess.

6.2 Defects of *MKRN3* in CPP

In this study, two families with central precocious puberty (CPP) and novel *MKRN3* variants were described. As pedigrees of both the Finnish and the Polish families included several affected persons, the families underwent genetic testing. Subjects 1-3 had inherited the variants from their fathers consistently with the maternal imprinting of the gene. Likewise, the Finnish father had likely inherited the variant from his father and the Polish father from his mother. Both variants, c.939C>G, p.(Ile313Met) in the Finnish family and c.1237_1252del, p.(Gly413Thrfs*63) in the Polish family, were absent from gnomAD and were predicted to have deleterious effects on the protein by three *in silico* tools. In addition, the Finnish variant was absent in SISu.

The p.(Ile313Met) variant which, to the best of our knowledge, is the first *MKRN3* variant described in a Finnish CPP family so far, lied in the C3HC4 RING zinc finger domain. The majority of *MKRN3* variants implicated in CPP lie in this domain, with at least nine other missense variants, two of which affect nearby codons; one frameshift, and one nonsense variant reported (**Figure 7**) (Liu et al., 2020; Maione et al., 2020; Seraphim et al., 2021; Zubkova et al., 2021). CPP-associated missense variants affecting the RING finger domain impair the E3 ubiquitin ligase activity and auto-ubiquitination of MKRN3, as well as the ability of the protein to repress *KISS1* and *TAC3* promoters (Abreu et al., 2020; Li et al., 2020). Furthermore, variants in this domain can reduce MKRN3-mediated ubiquitination of MBD3, a transcriptional activator of *GNRH1,* as well as PABPC1, which regulates the stability of *GNRH1* mRNA (Li et al., 2020; Li et al., 2021). Some of these molecular mechanisms may have caused CPP in patient 1.

The variant in the Polish family, p.(Gly413Thrfs*63), lied in the C-terminal C3H1 zinc finger domain, where at least one frameshift, one nonsense, and five missense variants have been reported in patients with CPP (**Figure 7**) (Seraphim et al., 2021; Maione et al., 2020). Missense variants disrupting the C-terminal zinc finger domain can disturb RNA binding and partly impair MKRN3-mediated repression of *KISS1* promoter (Abreu et al., 2020; de Vries et al., 2014). In addition, variants

affecting the C3H1 domain can, to a lesser extent than variants in the RING finger domain, reduce the E3 ligase activity and ubiquitination of MBD3 and PABPC1 (Abreu et al., 2020; Li et al., 2020; Li et al., 2021). However, the Polish family variant was predicted to lead to a premature stop codon and might thus have more severe consequences. As CPP-associated variants can be present throughout the *MKRN3* sequence and differ in severity and predicted functional effects, it is likely that they can compromise MKRN3 function by several, perhaps partly overlapping, mechanisms (Seraphim et al., 2021).

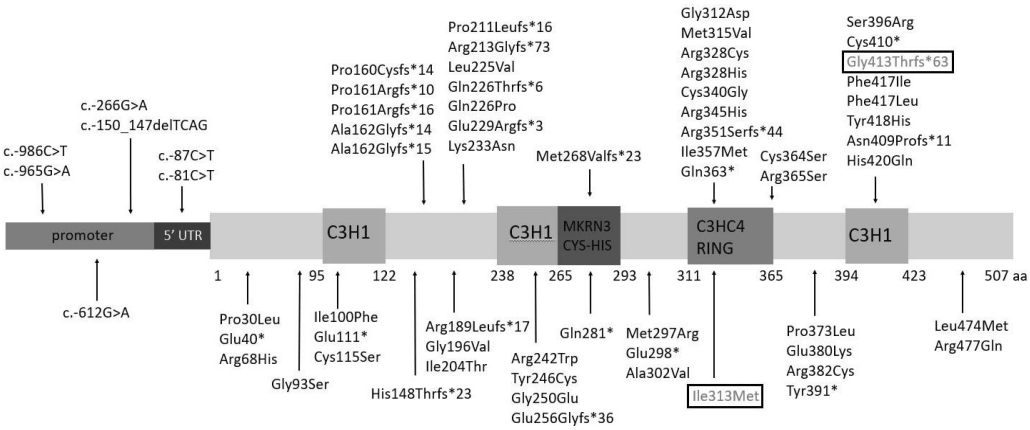

Figure 7. The human MKRN3 protein with the CPP-associated variants reported by the time of writing (not in scale). C3H1: C3H1-type zinc finger motif, MKRN3 CYS-HIS: Makorin-type Cys-His motif, C3HC4 RING: C3HC4 RING-type zinc finger motif. Adapted from Valadares et al., 2019; Chen et al., 2019; and Maione et al., 2020. The variants identified in this study (Varimo & Iivonen et al., 2021) are marked in boxes in light grey. In addition, the variants reported in Liu et al., 2020, Neocleous et al., 2021, Yin et al., 2021, Zubkova et al., 2021, and Seraphim et al., 2021 are displayed. The promoter and 5'UTR region mutations are indicated in relation to the translation initiation codon. Modified from Varimo & Iivonen et al. (2021).

The girls (patients 1 and 3) had thelarche at the ages of 6 and 4.5 years, whereas the boy (patient 2) showed signs of advanced puberty at the age of 9 years (with pubarche at the age of 7 years). The ages at the onset of puberty are similar to those reported in other girls (median 6.0, range 3.0 to 7.8. years) and boys (median 8.5, range 5.9 to 9 years) and reflect the approximate difference of two years in the timing of puberty between boys and girls who carry *MKRN3* variants (Valadares et al., 2019; Seraphim et al., 2021; Bessa et al., 2017). This study reports the long-term effects of an *MKRN3* variant in a boy for the first time. Boys are typically less severely affected than girls, and some boys with defects in *MKRN3* may experience puberty onset at an age that is only slightly advanced compared to the lower age limit for normal onset (Maione et al., 2020). Here, patient 2 presented with relatively mild advancement of pubertal timing, and as a potential consequence, he reached an expected adult height despite he received no treatment for CPP. His bone age was approximately two years ahead of his chronological age. Unexpectedly, his growth showed no signs of acceleration at the time of diagnosis, although his testes had reached the adult size at the age of 9 years. In contrast to patient 2, the father of the Finnish family, who had CPP and a missense variant in *MKRN3*, was relatively short.

Patients 2 and 3 had high BMIs in early childhood. Previously, overweight and obesity have been reported at the puberty onset in CPP patients with and without *MKRN3* variants (Ramos et al., 2020). In mice, *Mkrn3* seems to function independently of leptin action (Roberts et al., 2020). However, the potential roles of E3 ubiquitin ligases as metabolic regulators are an emerging field of study (Sun-Wang et al., 2021). Serum MKRN3 levels have been reported to correlate with HbA1c, plasma glucose, and insulin levels (Varimo et al., 2016). Furthermore, MKRN3 is predicted to interact with proteins implicated in insulin signaling and TP53-regulated cell metabolism, including MKRN1 (Yellapragada et al., 2019). *MKRN1*, the ancestral gene for and sharing a high structural similarity with *MKRN3*, has metabolic functions linked to its E3 ubiquitin ligase activity (Naulé & Kaiser, 2019). For instance, Mkrn1 acts as an E3 ubiquitin ligase for AMP-activated protein kinase (AMPK). In mice, loss of *Mkrn1* leads to stabilization and activation of AMPK, promoting glucose consumption and suppressing lipid accumulation in the adipose tissues and liver (Lee et al., 2018). The structural similarity and interaction with MKRN1 suggest that MKRN3 could play a role in the regulation of metabolic processes, but this warrants further elucidation.

Inactivating variants in *MKRN3* can be found in up to 46% of familial cases (Roberts & Kaiser, 2020), and this study adds two families to the existing literature. Perhaps future investigations will reveal whether the prevalence of *MKRN3* variants among Finnish CPP families differs from that in other Western countries. Potential long-term health consequences of *MKRN3* variants are currently unknown. Further studies are required to evaluate whether *MKRN3* variants have any predictive value in clinical management, and whether missense and severe variants bear different long-term outcomes, if any.

6.3 The roles of miRNAs in CHH and KS

Variants in microRNA-encoding genes can alter miRNA transcription, splicing, processing, or target specificity. Alterations in these processes can lead to aberrant miRNA expression, biased production of the mature canonical miRNA and different isomiRs (isoforms of the mature miRNA), loss or gain of target genes, and eventually disease (de Carvalho et al., 2019; Borghini & Andreassi, 2018; Lewis et al., 2021). For instance, a neomorphic variant in *MIR140* results in abundant miR-140-5p expression and repression of new target genes instead of the original ones. This variant underlies familial skeletal dysplasia (Grigelioniene et al., 2019). Another *MIR140* variant altering miR-140 processing is associated with non-syndromic cleft palate (Schoen et al., 2017). Variants in *MIR96* seed region, which disturb miRNA processing and change the target gene spectrum, are implicated in autosomal dominant nonsyndromic hearing loss (Mencía et al., 2009; Lewis et al., 2021). In turn, *MIR499A/-B, MIR146A*, and *MIR1304* variants in RASopathy patients cause alterations in miRNA secondary structures and expression levels (de Carvalho et al., 2019). The miRNAs are predicted to regulate genes in the RASopathy-associated pathways or -phenotypic features, suggesting the contribution of the variants to the patient phenotypes (de Carvalho et al., 2019). By the time of publication, this study was the first that investigated miRNA gene variants in CHH patients.

The miR-200 (miR-8) family miRNAs are expressed in the mouse and zebrafish olfactory epithelia. The miRNAs are essential for the olfactory progenitor cell differentiation in mice, and their knockdown produces a KS-like phenotype in zebrafish (Yang et al., 2020; Bhattacharya et al., 2017;

Garaffo et al., 2015). In mice, miR-200 miRNAs are expressed in the GnRH neurons and the pituitary, and in zebrafish, in the pituitary. In both species, they regulate sexual maturation and fertility – in zebrafish, miR-200 could even play a similar role as GnRH in mammals! (Xiong et al., 2020; Messina et al., 2016; DeVeale et al., 2021). Since the evidence from animal models indicates their importance in the olfactory system and GnRH neuron development, miR-200 family genes (*MIR141, MIR429, MIR200A, MIR200B*, and *MIR200C*) were screened for variants in KS patients. A single variant in *MIR200A*, c.42C>T (rs202051309), outside of the mature miRNA sequence, was identified in one patient. The variant was present in the gnomAD Finnish population with a frequency of 0.01996. According to VarSome (Kopanos et al., 2019) prediction, this variant is benign, and no literature on it has been published. On the one hand, given the incidence of KS in Finland (1:48 000) (Laitinen et al., 2011) the frequency is higher than expected for a causative variant in monogenic KS (Richards et al., 2015). On the other hand, variants with even higher frequencies have been suggested, quite controversially, to contribute to oligogenic KS (Mkaouar et al., 2021). In the end, the potential effects of this variant on miRNA processing, expression, or target gene regulation cannot be fully excluded. One limitation in the potential pathogenicity estimation is the relatively small cohort size in this study.

Based on the results by Ahmed et al. (2017), the performed bioinformatic analyses, and literature available before publication (Horsham et al., 2015), *MIR7-3* was screened for variants in the normosmic CHH patients. Recent literature supports the selection of a gene encoding miR-7: the miRNA was demonstrated to regulate gonadotropin secretion by mediating the effects of GnRH and estrogen in mice (He et al., 2020). Additionally, a regulatory role of miR-7 in gonadotropin secretion was observed in the pig (Li et al., 2020). During zebrafish central nervous system development, miR-7 was highly expressed in the pituitary and hypothalamus, and it suppressed Wnt/β-catenin signaling (Adusumilli et al., 2020), impairment of which has been implicated in delayed puberty and defective GnRH neuron development (Mancini et al., 2020).

The human genes encoding mature miR-7 (*MIR7-1, MIR7-2,* and *MIR7-3*) produce primary microRNAs, which undergo processing through pre-miRNAs into the mature miR-7 (Zhao et al., 2020). miR-7 expression is enriched in the hypothalamus and pituitary, and most of the human pituitary miR-7 expression is presumably attributed to *MIR7-3*, which lies in an intron of *MIR7-3HG* (MIR7-3 host gene, previously named *PGSF1, i.e.* pituitary gland specific factor 1) (Zhao et al., 2020). Predicted target genes of the mature human miR-7 include several murine and recently identified porcine miR-7 predicted target genes, such as *Glg1/GLG1*, *Ptgfrn/PTGFRN*, *Sema4c,* and *Chd3* (Ahmed et al., 2017; Li et al., 2020) as well as CHH genes, including *GNRHR, FGFR1, SEMA7A,* and *PROK2* (according to miRWalk 3.0 (Sticht et al., 2018)). However, no variants in *MIR7-3* were identified, which might imply that variants in it rarely occur in normosmic CHH patients. On the one hand, certain reports imply that miR-7 could be implicated in KS, for *TCF12,* a recently identified KS gene (Davis et al., 2020), is a miR-7 target (Adusumilli et al., 2020). On the other hand, our findings suggest that *MIR7-3* may have no implications in human CHH.

Taken together, this study suggests that variants in the examined microRNA-encoding genes are infrequent causes of normosmic CHH or KS. However, the approach was limited, as specific microRNA genes with implicated significance in animals were selected. An unbiased RNA

expression analysis from human tissues could imply that the most central human and animal miRNAs differ in the hypothalamic–pituitary–gonadal axis. In the end, the potential contribution of the examined, or other miRNAs, to the development and function of the axis in the human cannot be excluded. For instance, expression levels of miR-200 members change during the estrous cycle in the pituitary, implying their participation in the regulation of reproduction in the sheep (Ullah et al., 2020), which phylogenetically stands closer to humans than rodents do (Alvites et al., 2021). *let-7* miRNA is implicated in the onset of puberty in rodents (Cao et al., 2020), and *let-7* target gene loci are significantly associated with the age at menarche in humans (Day et al., 2017). Lastly, the pubertal increase in miR-30b expression levels detected in rats (Heras et al., 2019) has also been shown in boys (Varimo & Wang, 2021).

6.4 The first reported KS patient with a deletion in 9q31.2

The proband underwent an extensive investigation for the genetic cause of his condition with KS, developmental delay, ptosis, ventral septal defect, craniofacial abnormalities, and mild hearing loss. The targeted sequencing panel, whole exome sequencing, linked-read whole genome sequencing, nor RNA sequencing revealed coding region variants in the established KS genes. The WES disclosed a rare maternally inherited variant in *RIMBP3C* and *SARS1. RIMBP3C* (RIMS binding protein 3C) can be deleted in 22q11.2 microdeletion syndrome, in which it is associated with "pulmonary insufficiency or respiratory failure following trauma and surgery", but the role of *RIMBP3C* in the syndrome manifestation is unknown (Manno et al., 2021; Vysotskiy et al., 2021; Motahari et al., 2019). Moreover, RIMBP3C is a testis-specific protein and implicated in spermatid morphogenesis (Kent et al., 2020). In turn, *SARS1* encodes seryl-tRNA synthetase 1, a cytosolic aminoacyl-tRNA synthetase which catalyzes serine attachment to tRNA. Biallelic variants in *SARS1* have been implicated in a variable constellation of developmental delay, deafness, muscle weakness, dysmorphic facial features, febrile decompensation, microcephaly, cardiomyopathy, and seizures (Musante et al., 2017; Ravel et al., 2021). Furthermore, SARS1 inhibits angiogenesis, and heterozygous variants in the gene can underlie brain arteriovenous malformation (Pan et al., 2021; Shi et al., 2020). The *RIMBP3C* and *SARS1* variants were of uncertain significance according to the ACMG/AMP 2015 guidelines (Richards et al., 2015), and the associations of these genes to the proband's phenotype remained uncertain. Instead, a remarkable finding in the proband was a rare (allele frequency below 1/200 000) *de novo* microdeletion in the 9q31.2 region. The deletion encompassed *FKTN, TAL2, TMEM38B, ZNF462, RAD23B, KLF4,* and *MIR8081*.

The 5' breakpoint of this 2.38 Mb deletion broke *FKTN* (fukutin) in the first intron. Biallelic *FKTN* variants are implicated in dilated cardiomyopathy and hereditary muscular dystrophies with variable severity. The muscular dystrophies can manifest with muscle weakness, cardiac dysfunction, seizures, ocular anomalies, cerebral cortical dysplasia, dysphagia, and developmental delay. To the best of our knowledge, defects in *FKTN* are not associated with CHH or other phenotypic features of the proband except for the developmental delay (Ishigaki et al., 2018; Saito, 2019). Rather, the proband exhibited several manifestations of *ZNF462* haploinsufficiency or Weiss-Kruszka syndrome, including hearing loss, ptosis, developmental delay, ventricular septal defect, attention deficit disorder, and low-set ears (Kruszka, 2019; Cosemans et al., 2018; Weiss et al., 2017; Kruszka et al., 2019; Park et al., 2021). At the publication time of our study, Park et al. (2021) had reported another

patient with Weiss-Kruszka syndrome and delayed puberty (Tanner stage G2 at the age of 16). The patient, who harbored a nonsense variant in *ZNF462,* exhibited an unusual Weiss-Kruszka phenotype with GHD, flattened pituitary, and empty sella (Park et al., 2021). On the one hand, Zfp462 (murine ZNF462 ortholog) plays a role in brain morphogenesis, and it regulates the expression of Sox2 (Al-Naama et al., 2020). In the chick, Znf462 contributes to the induction of the sensory placode progenitors (Hintze et al., 2017; Seal & Monsoro-Burq, 2020). On the other hand, *Zfp462* knockout mice exhibit no abnormalities of reproduction or olfaction, and defective sense of smell has not been reported in Weiss-Kruszka patients so far (Wang et al., 2017; Kruszka et al., 2019; Cosemans et al., 2018; Weiss et al., 2017, Park et al., 2021; Talisetti et al., 2003; Ramocki et al., 2003; González-Tarancón et al., 2020), which could imply that the absence of one functional *ZNF462* allele is insufficient to cause the full KS phenotype in the proband. In OMIM, the 9q31.2 locus has received a specific annotation as MENAQ3 (menarche, age at, quantitative trait locus 3, MIM 612883), and GWAS signals associated with the timing of pubertal milestones (age at menarche, voice break, and appearance of facial hair) have repeatedly come from SNPs in the 9q31.2 region, especially in or near *TMEM38B* (Hollis et al., 2020; Busch et al., 2020; Day et al., 2017; Hou et al., 2017; Fernández-Rhodes et al., 2018). Moreover, the most significant SNP associated with fertility traits influenced by puberty has been in the vicinity of *FKTN, TAL2,* and *TMEM38B* in cattle (Tahir et al., 2021).

TMEM38B is highly expressed in the pineal gland (Hou et al., 2017), dysfunction of which might have a connection with precocious puberty (Patel et al., 2020). Based on bioinformatic analyses, *RAD23B* is associated with cleft palate in the mouse and the spermatogenic process in cryptorchid patients (Zhou et al., 2018; Suzuki et al., 2018), and *Rad23b* double knockout mice exhibit retarded growth and male infertility due to the absence of spermatogenesis (Berruti, 2021). In turn, TAL2 is required for midbrain GABAergic neuron differentiation (Makrides et al., 2018). According to miRDB (Chen & Wang, 2020), the target genes of hsa-miR-8081 include *AXL, SDHC,* and *SEMA3C*, but the miRNA is mentioned in only two publications. According to them, miR-8081 is upregulated in type 1 diabetes patients (Yi & Cheng, 2021), and holds a putative link to epithelial-to-mesenchymal transition via binding to the lncRNA NR_136400 in osteosarcoma cells (Liu et al., 2020). Despite the association of the 9q31.2 area to puberty timing, variants in *TMEM38B*, *TAL2, MIR8081,* or *RAD23B* have not been implicated in CHH.

Previous patients with 9q31.2 deletions have showed great phenotypic variability, and KS-related features have been present in a few of those published in the literature (**Figure 8**). (Of note, the most recent patient with a deletion in chr9:108,399,883–113,591,075 (hg19) (Pellino et al., 2022) exhibited no CHH-associated features and is thus excluded from **Figure 8**). In addition, the DECIPHER database (Firth et al., 2009) reports one patient with a 12.08 Mb deletion encompassing 9q31.2 and cryptorchidism, abnormality of the dentition, and hearing impairment, as well as one with a 487.97 kb deletion in 9q31.2 and hypogonadotropic hypogonadism. Unfortunately, no further information is available on these patients. Deletions in two published patients overlap with that of our proband's and are associated with CHH or delayed puberty (Ramineni et al., 2019; Xu et al., 2013). In the report by Ramineni et al. (2019), the 9q31.2 deletion segregated with delayed puberty in the family, some of whom also manifested with hearing loss, muscle cramps, delayed motor development, or short stature, or a combination of these. The overlapping region between the deletion and that of our

proband encompassed *KLF4* and the SNP rs139300691. *Klf4* is a pluripotency factor co-expressed with neural crest markers in a subpopulation of the premigratory neural crest, but its function in the cells remains unknown (Perera & Kerosuo, 2021). Moreover, the protein regulates AgRP, which inhibits KISS1 neurons via GABA (Lieu et al., 2021). KLF4 also negatively regulates Wnt-signaling by inhibiting TCF7L2 binding to β-catenin (Bou-Rouphael & Durand, 2021), which might link KLF4 to the Lin28/let-7 – NF-κB interactions (Mills et al., 2020). In turn, rs139300691 is associated with the sense of smell (Dong et al., 2017

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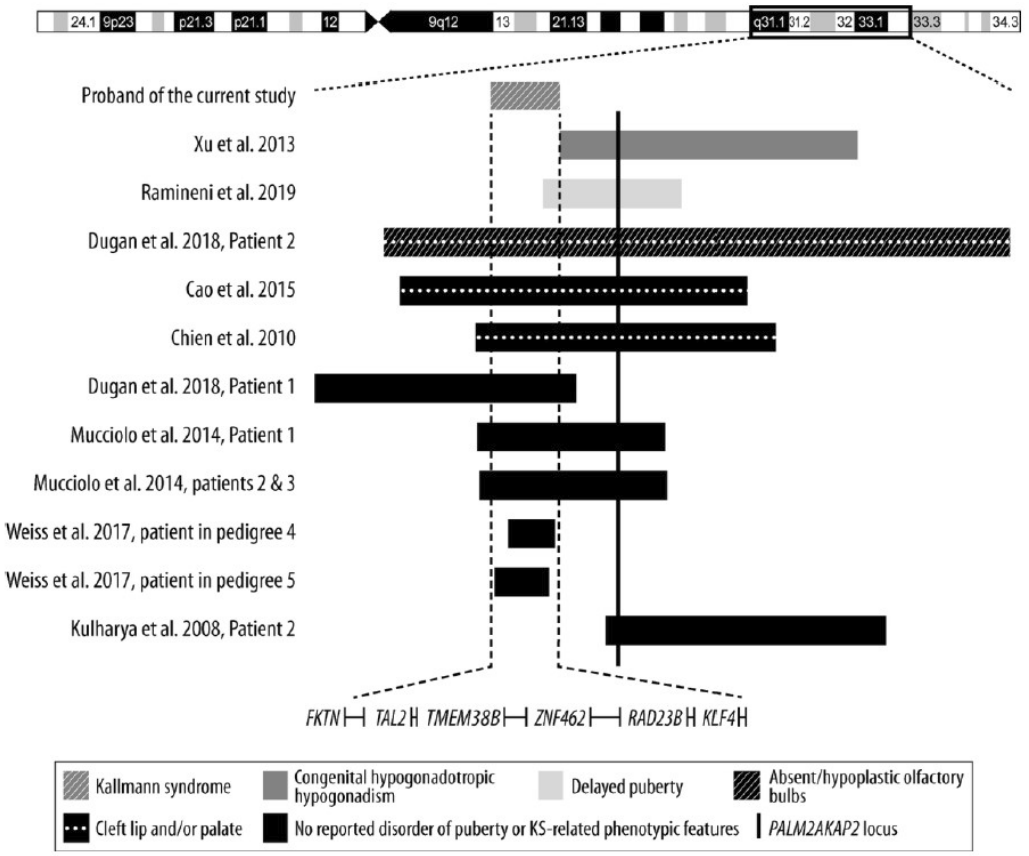

Figure 8. A diagrammatic representation of the proband's 9q31.2 deletion (in Iivonen & Kärkinen et al., 2021) in relation to other published, informative patients with deletions encompassing 9q31.2.
Five patients (Xu et al., 2013; Ramineni et al., 2019; and the three patients in Mucciolo et al., 2014), had reached pubertal age at the time of assessment. All patients, excluding the family reported in Ramineni et al. (2019) and patient 1 in Dugan et al. (2018), showed additional craniofacial phenotypes. Known CHH genes were sequenced in WES in one patient (Cao et al., 2015), and no variants in CHH genes were mentioned. The patients reported in DECIPHER (Firth et al., 2009) (see main text) are excluded. The dashed vertical lines mark the proband's 2.38 Mb deletion (chr9:108,331,353-110,707,332), which included six protein-coding genes (*FKTN*, *TAL2, TMEM38B, ZNF462, RAD23B*, and *KLF4*) and one miRNA-coding gene (*MIR8081*, not shown). Rectangles mark the relative locations and sizes of the patients' deletions, and the colors and patterns highlight Kallmann syndrome-related phenotypes. The solid vertical line marks the locus of the Kallmann syndrome candidate gene, *PALM2AKAP2*. The deletion coordinates are presented according to GRCh37

(hg19). For Kulharya et al. (2008) (patient 2), Chien et al. (2010), and Ramineni et al. (2019) patients the UCSC LiftOver tool (http://genome.ucsc.edu.) (Kent et al., 2002) was utilized to convert hg18 coordinates into hg19. The figure is modified from Dugan et al. (2018) and drawn by Helena Schmidt. Appearing originally in Iivonen & Kärkinen et al. (2021). Copyright: the authors.

The other informative patient, reported by Xu et al. (2013) was originally diagnosed with delayed puberty, but he fulfilled the criteria of CHH: he had puberty onset at the age of 18 and Tanner stage G2 at the age of 20 in the setting of normal gonadotropin levels (Galazzi & Persani, 2021). The deleted region shared by our proband and Xu's patient spanned ~35 kb and included the ribosomal pseudogene *RNA5SP293*. SNPs in or near the gene have been associated with such traits as "melanoma", "triglyserides", "platelet function", and "cholesterol" in the NCBI Phenotype-Genotype Integrator (Ramos et al., 2014), but no published literature is available on the gene itself. Moreover, the UCSC Genome Browser (Kent et al., 2002) shows that the following transcription factors have binding sites in the shared region: *GATA3, MYC, JUND, CEPBP, TEAD4, GATA2, MAFF, USF2, USF1, BHLHE40, ZNF143, ATF3,* and *SIX5.* Several of these transcription factors might have relevant functions in CHH pathogenesis. For instance, ZNF143 is predicted to regulate the *PDYN* gene transcription (Nosova et al., 2021), and it interacts with LIN28B in neuroblastoma cells (Otte et al., 2020). Potentially pathogenic biallelic variants in the gene have been detected in a single case with a disorder of intracellular cobalamin metabolism (Sloan et al., 2021). Of CHH-associated features, the case exhibited cleft palate, "hypospadias", and hearing loss before his death at 26 months of age (Pupavac et al., 2016). *Jund* is regulated by leptin in the murine tanycytes and ependymal cells in the mediobasal hypothalamus (Yoo et al., 2019), and as a component of the AP-1 transcription factor complex, it participates in the induction of *Gnrhr* transcription (Jonak et al, 2018). *Cebpb* acts as a transcriptional repressor of *Gnrh1* in the mouse (Manotas et al., 2022). *GATA2* and *GATA3* are implicated in the specification of the pituitary endocrine cell lineages (Zhang et al., 2020) and their expression also primes the induction of the neural plate border (Thawani & Groves, 2020). Usf1 and Usf2 mediate basal *Fshb* transcription in the rat (Stamatiades & Kaiser, 2018).

Deletions of transcription factor binding sites, such as those of CTCF, can alter target gene expression, disrupt promoter-enhancer interactions, and yield consequences to the phenotype (Ushiki et al., 2021; Himadewi et al., 2021). For instance, a deletion containing multiple CTCF binding sites in the *SHH* locus can impair *SHH* promoter interaction with its enhancer, reduce *SHH* expression, and result in acheiropodia, congenital limb truncation (Ushiki et al., 2021). Deletion of a CTCF binding site in another locus, the β-globin gene cluster, leads to interaction between fetal hemoglobin genes and a distal enhancer, increased expression of the genes and a cellular condition mimicking hereditary persistence of fetal hemoglobin (Himadewi et al., 2021). Trancription factor binding sites can lie megabases away from the target gene (Li & Pertsinidis, 2021; Tobias et al., 2021). However, none of the genes in the deleted region is directly implicated in KS, and the nearest potential KS gene, *PALM2AKAP2*, was efficiently expressed. How the loss the of the transcription factor binding sites might cause KS in the proband can only be speculated.

Taken together, this study (Iivonen & Kärkinen, 2021) describes the first patient, and so far, the only one, with an extremely rare *de novo* 9q31.2 deletion and KS. The 9q31.2 locus is associated with puberty timing and the sense of smell, and previous patients with deletions encompassing the region

have manifested with CHH, delayed puberty, and other KS-related features, such as olfactory bulb defect and cleft lip or -palate. The results of this study indicate that patients carrying 9q31.2 deletions should be evaluated for the presence of KS and -related features, and that KS patients with Weiss-Kruszka manifestations should be evaluated for the presence of a 9q31.2 deletion. However, among the patients with KS-related phenotypes and a 9q31.2 deletion, only those reported by Cao et al. (2015) and Park et al. (2021) had undergone sequencing of CHH genes, and no variants in the genes were mentioned. The presence of potential variants in CHH genes remains unknown in the rest of the published patients with deletions (Xu et al., 2013; Ramineni et al., 2019; Mucciolo et al., 2014; Kulharya et al., 2008; Weiss et al., 2017; Chien et al., 2010; Dugan et al., 2018) and those in DECIPHER. Moreover, several of the published patients with KS-related features were too young at the time of assessment to be evaluated for pubertal development (Kulharya et al., 2008; Weiss et al., 2017; Chien et al. 2010; Cao et al., 2015; Dugan et al. 2018).

The coding or non-coding genes or transcription factor binding sites in the 9q31.2 locus, loss of which could contribute to KS in our proband or the KS-related phenotypes in other patients with 9q31.2 deletions, are currently unknown. Deletion of the *ZNF462* gene was consistent with the proband's Weiss-Kruszka syndrome. On the one hand, the recently reported *ZNF462* nonsense variant in a patient with delayed puberty (Park et al., 2021) suggests that defects in *ZNF462* could be associated with pubertal delay. On the other hand, the region that is shared by our proband's deletion and 9q31.2 deletions of other patients with delayed puberty or CHH (in Xu et al. (2013), Ramineni et al. (2019), and the one in DECIPHER), have excluded *ZNF462.* Larger patient cohorts are required to further specify the connection between KS and the 9q31.2 locus.

Our proband carried no rare, coding variants in the known KS genes, of which 15 showed no aberrant splicing events either. However, it cannot be excluded that the proband might have carried a pathogenic variant or variants causing or contributing to his KS in non-coding regions of the other known KS genes, genes implicated in KS after our publication, or yet-undefined KS genes. The KS candidate gene, *PALM2AKAP2,* was normally expressed, and no potentially disease-causing variants in it were detected in a set of Finnish KS patients. Recently, AKAP2 has been implicated in growth plate chondrocyte functions in familial adolescent idiopathic scoliosis (Wang et al., 2021) and protection against myocardial infarction through estrogen receptor alpha -dependent signaling (Maric et al., 2021). The potential role of *PALM2AKAP2* in KS pathogenesis warrants further investigation.

7. CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis investigated the roles of variants in specific genes in disorders of growth and puberty that originate from aberrant pituitary hormone secretion. The thesis consisted of four publications, two of which examined the roles of *KCNQ1, KCNE2*, and *MKRN3* variants in abnormally activated secretion of pituitary hormones. The two other publications investigated the roles of variants in microRNA genes from the miR-200 and miR-7 families, *MIR141, MIR429, MIR200A, MIR200B, MIR200C,* and *MIR7-3*, as well as a deletion in 9q31.2 along with the KS candidate gene nearby 9q31.2, *PALM2AKAP2*, in deficient gonadotropin secretion.

The roles of the potassium channel gene *KCNQ1* and its auxiliary subunit *KCNE2* in growth hormone secretion has evoked interest, as variants in *KCNQ1* were shown to underlie GHD, and co-expression of KCNQ1 and KCNE2 resulted in diminished pituitary hormone secretion in a mouse pituitary cell line. In addition, *KCNQ1* is a regulator and a target gene in Wnt/β-catenin signaling, a relevant pathway in somatotropinoma tumorigenesis, and KCNE2 forms channels with KCNQ1 in the pituitary and secretory cells in other organs. This study showed that variants in *KCNQ1* or *KCNE2* are unlikely associated with somatotropinoma formation, but screenings of germline or somatic or both variants in larger patient cohorts with early onset and familial cases would be needed for confirmation. The possible association of the *KCNE2* c.22A>G, p.(Thr8Ala) variant with acromegaly still requires further study. The mechanism of how mutated *KCNQ1* causes GHD is under active investigation, and identification of the mechanism may further shed light on the question of whether *KCNQ1* and *KCNE2* variants could contribute to somatotropinoma formation.

Defects in *MKRN3* are at present the most frequent genetic cause of CPP, especially in Western countries and familial cases. This study showed that variants in *MKRN3* can also underlie CPP in Finnish patients. However, systematic studies on the genetics of CPP in Finland are lacking, and given the genetic curiosities of the Finnish population, it will be interesting to study whether *MKRN3* variants are equally prevalent among Finnish CPP families compared to other Western countries. In the majority of CPP patients, the genetic cause remains unidentified. CPP-associated variants in *MKRN3* can lie throughout the gene but are especially abundant in the C3HC4 ring domain-coding region, which is of particular importance for the E3 ubiquitin ligase activity. Recent investigations have revealed new target genes for MKRN3-mediated ubiquitination, which, along with *MIR30B*, could constitute novel candidate genes of CPP. In addition, this study described the first long-term observation of a boy with an *MKRN3* variant and challenged the role of GnRH analog treatment in augmenting adult height in boys with CPP due to *MKRN3* variants and mild pubertal advancement. The potential long-term health consequences of *MKRN3* variants are currently unknown, and further studies are required to evaluate whether they have any predictive value in the clinical management of CPP patients.

Animal models with a specific gene defect and a similar phenotype to the disorder of interest often suggest new candidate disease genes. The roles of miR-200 and miR-7 family members in the regulation of the hypothalamic-pituitary-gonadal axis have gathered interest, for based on animal models, these miRNAs seem to be vital in the olfactory system and GnRH neuron development or gonadotropin secretion. Animals deficient of miR-200 and miR-7 family miRNAs have recapitulated

KS and normosmic CHH, respectively. Variants in miRNA genes can cause human diseases, and the animal studies had left the roles of miR-200 and miR-7 families unclear in human CHH. This study showed that variants in the examined miRNA genes of these families are unlikely, or at least infrequent, causes of CHH in humans. It remains possible that vital functions or developmental steps in the hypothalamic–pituitary–gonadal axis are regulated by different miRNAs in humans and other animals, although the potential contribution of the examined (or other) miRNAs to the function or development of the axis in our species cannot be excluded.

Our study described the first patient with KS and a deletion in 9q31.2, a locus associated with variation in the timing of puberty and the sense of smell, in the context that previous patients with deletions encompassing the locus have exhibited normosmic CHH and KS-related features. This study also suggested that patients with 9q31.2 deletions should be carefully evaluated for the presence of KS and KS-related features, and conversely, that KS patients with manifestations of Weiss-Kruszka syndrome should be evaluated for the presence of a 9q31.2 deletion. However, the connection between KS and the 9q31.2 locus should be confirmed in further studies. Several challenges may arise in the confirmation attempts. First, large patient cohorts would be optimal for investigations, but they might be laborious to collect due to the rarity of patients with 9q31.2 deletions. Second, given the genetic complexity of CHH, not only the presence of a 9q31.2 deletion but also single nucleotide variants in the established- or CHH candidate genes (without neglecting variants in the non-coding regions) should be examined in available patients to get a comprehensive view of potential causative factors. Third, in cases where deletions encompass multiple genes, it may be challenging to evaluate how the combined loss of the genes leads to a phenotype, and further difficulty is added if additional, likely disease-causing single nucleotide variants are detected elsewhere in the genome. Thus, functional experiments or an animal model mimicking the deficiency of the 9q31.2 region would be needed to investigate the cellular and molecular mechanisms of how a 9q31.2 deletion might cause KS. Finally, the role of the candidate gene, *PALM2AKAP2*, in the pathogenesis of CHH still requires further study.

In conclusion, the publications constituting this thesis have produced new information on the association of defects in certain genes with disorders of deviant growth hormone or gonadotropin secretion. The publications investigated, for the first time, variants in *KCNQ1* and *KCNE2* in GH excess, variants in microRNA genes in human CHH, and a genomic deletion at 9q31.2 in a KS patient. One of the publications reported the long-term effects of an *MKRN3* variant in a male patient, and a variant in the gene in a Finnish CPP family, alike for the first time. Due to the great number of disorders of growth and puberty originating from pituitary dysfunction, the thesis focused on specific genes in the selected conditions. A more comprehensive analysis would require additional investigations. In the future, larger patient cohorts would be useful in studying answers to several interesting questions arising from the findings of this thesis, such as whether the tentative enrichment of the KCNE2 p.(Thr8Ala) variant in somatotropinoma patients could be replicated, whether *MKRN3* variants are present and how prevalent they are in other Finnish CPP patients, and whether pathogenic variants in the examined microRNA genes or *PALM2AKAP2* could be found in other sets of CHH patients. On the one hand, an unbiased RNA expression analysis from human tissues could show that the central human miRNAs in the hypothalamic–pituitary–gonadal axis indeed differ from those in

other animals; on the other hand, an intriguing model to further study especially the roles of miR-200 miRNAs in human GnRH neuron development would be human pluripotent stem cell-derived GnRH neurons.

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