Genetic and non-genetic causes of Isolated Growth Hormone Deficiency and Combined Pituitary Hormone Deficiency:

Results of the HYPOPIT study

Genetische en niet-genetische oorzaken van geïsoleerde groeihormoon deficiëntie en gecombineerde hypofyse hormoon deficiënties: resultaten van de HYPOPIT studie

PROEFSCHRIFT

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Voor Oma, Opa en Robert,
die wel het begin, maar helaas niet het eind van dit project mochten meemaken.

Voor Kim, Robin en Mees,
voor wie het omgekeerde geldt

Contents

Chapter 1

Introduction

1.1 Background

The key link between the brain and the peripheral endocrine system is formed by the hypothalamus and the pituitary, which coordinate the production of hormones by the endocrine glands. Deficiencies of the hormones produced by the various endocrine glands can cause major clinical problems, ranging from retarded growth only, to severe hypoglycaemias, convulsions or even death. These clinical problems demonstrate the importance of a normal function of the endocrine glands, the hypothalamus and the pituitary (Figure 1).

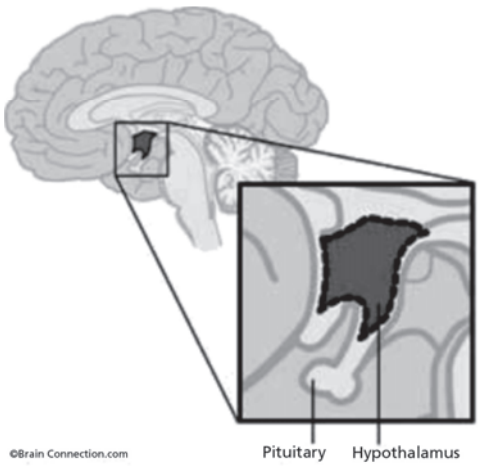

Figure 1: The hypothalamus and the pituitary gland

1.2 Embryogenesis

In the embryo, the neuroectoderm of the forebrain (prosencephalon) divides to form the telencephalon (endbrain, cortex) and the diencephalon. From the diencephalon, the primordial hypothalamus is generated from the fourth week of gestation. Around the same time, the pituitary is formed. The posterior lobe develops from neural crest cells as a downward evagination of the floor of the third ventricle of the brain. The anterior lobe develops from an evagination of ectodermal cells of the oropharynx in the primitive gut, known as Rathke's pouch (RP). Finally, the pituitary consists of a posterior lobe (PP) or neuropituitary, an anterior lobe (AP) or adenopituitary and a pituitary stalk or infundibulum (I)[1] (Figure 2).

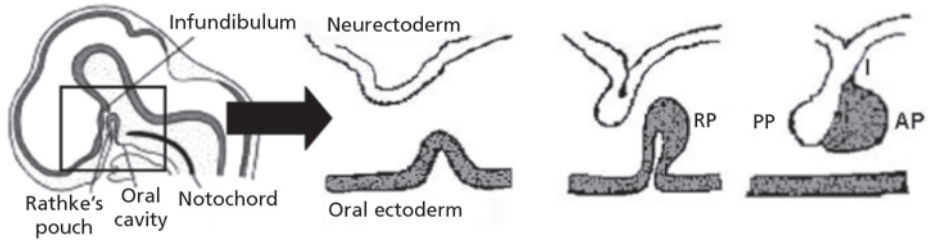

Figure 2: Embryology of the pituitary gland

Pituitary organogenesis and the development of different pituitary cell types is orchestrated by a number of genes that code for transcription factors. An increasing number of homeobox genes are sequentially expressed and act in combination with inductive signals from the future hypothalamus (Figure 3, and paragraph 1.6.3)

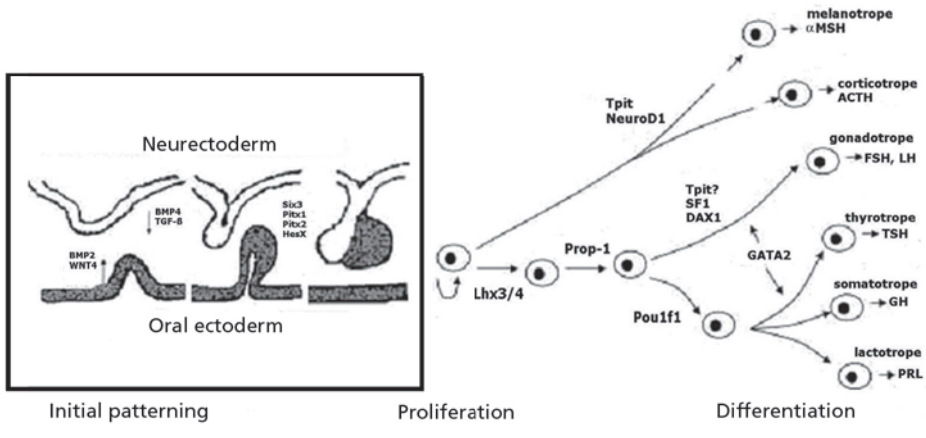

Figure 3: Signaling molecules and transcription factors involved in pituitary organogenesis (adapted from Reynaud et al, Growth Hormone and IGF research 2004, with permission)

1.3 Normal physiology of the pituitary

The action of the posterior pituitary (neuropituitary) is controlled by nerve stimulation, whereas the action of the anterior pituitary (adenopituitary) is controlled by releasing hormones from the hypothalamus and by negative feedback from the end product of its action.

The hypothalamus synthesizes Oxytocin (OXY) and vasopressin (AVP), which are stored and secreted into the bloodstream by the posterior pituitary (Figure 4). Oxytocin is necessary for uterine contraction and lactation. Vasopressin promotes the reabsorption of water by the kidney and is important for sodium and potassium homeostasis.

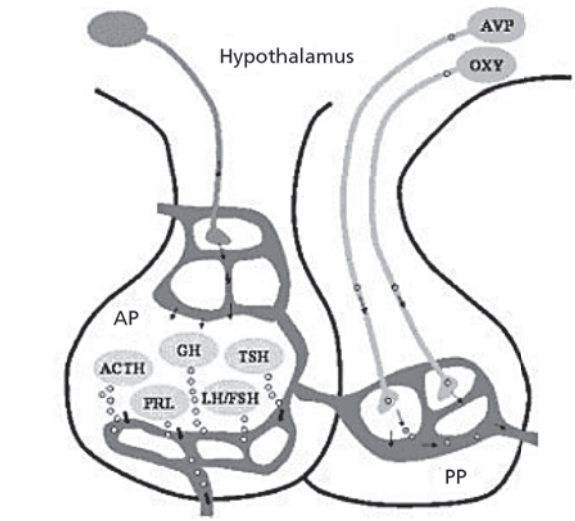

Figure 4: Different types of hormone producing cells in the pituitary (from Cheung *et al.*, Pituitary 2007, with permission)

In the anterior pituitary, thyroid stimulating hormone (TSH), or thyrotropin, causes the thyroid to secrete thyroid hormone. Adrenocorticotropic hormone (ACTH) stimulates the cortex of the adrenal gland to produce cortical hormones, particularly cortisol. Gonadotropic hormones (LH and FSH) react with ovaries and testes, to regulate the development, growth and function of these organs. Prolactin (PRL) promotes the development of glandular tissue in the female breast during pregnancy and stimulates milk production during lactation after birth of the infant. Growth hormone (GH) stimulates the growth of bones, muscles and other organs by promoting protein synthesis including production of the Insulin-like Growth Factor IGF-I[1, 2]. The endocrine axes are shown in Appendix I.

The synthesis and secretion of GH is regulated by several hormones (reviewed by Goldenberg *et al.*[3]). Growth Hormone Releasing Hormone (GHRH), produced in the hypothalamus, stimulates GH production via binding to the GHRH receptor (GHRHR), located in the anterior pituitary. GH production is negatively regulated by the Somatotropin Release Inhibiting Factor, SRIF or somatostatin, and by IGF-I. Additionally, the gastric hormone Ghrelin plays a role[3].

GH acts at the target cell through the GH receptor (GHR)[4]. After binding to GHR, GH induces activation of the JAK/STAT pathway, leading to an increased expression of IGF-I and other GH-dependent genes.

1.4 Hypopituitarism

Hypopituitarism refers to a diminished or absent secretion of one or more pituitary hormones[5]. A minority of patients has a known aetiology of hypopituitarism, like pituitary neoplastic pathology (adenoma, meningioma, metastases), trauma, infarction, infection (tuberculosis, syphilis), cranial irradiation, inflammation (sarcoidosis, Wegener's granulomatosis, lymphocytic hypophysitis) or congenital defects in the development of individual anterior pituitary cell types or hypothalamic function. However, in the majority of cases, the cause is unknown and these patients are said to have 'idiopathic' hypopituitarism.

1.5 Growth hormone deficiency

Growth Hormone Deficiency (GHD) can occur either isolated or combined with other pituitary hormone deficiencies.

IGHD

Children with GHD have proportionate short stature due to reduced linear growth. In those with isolated GHD, skeletal maturation is usually delayed in proportion to their height retardation. Other frequent findings include truncal obesity and a younger appearance than expected from their chronological age. Children with GHD often have a flattened nasal bridge and frontal bossing, their face resembling a 'doll's face' (Figure 5). IGHD patients usually have a normal pituitary on MRI, or, in a minority of the cases, a hypoplastic anterior pituitary[6].

The incidence of Isolated Growth Hormone Deficiency (IGHD) is estimated to be 1 in 3500 to 1 in 10.000 live births. In a very small number of cases, IGHD is explained by mutations in *GH1* and *GHRHR* (encoding the Growth Hormone and the Growth Hormone Releasing Hormone Receptor, respectively), and mutations in these genes are responsible for four distinct forms of familial IGHD (Table 1).

However, in the vast majority of the patients, the cause of IGHD is unknown and these cases are classified as idiopathic. Estimates indicate that between 5% and 30% of cases of idiopathic GH deficiency have first-degree relatives with retarded growth, which is suggestive of a genetic aetiology of GH deficiency.

CPHD

Combined pituitary hormone deficiency (CPHD) is any combination of two or more pituitary hormone deficiencies. Hypothyroidism, hypocortisolism, hypogonadism and hypoprolactinemia can occur in an isolated form, but often appear in combinations and in most cases, GHD is also present.

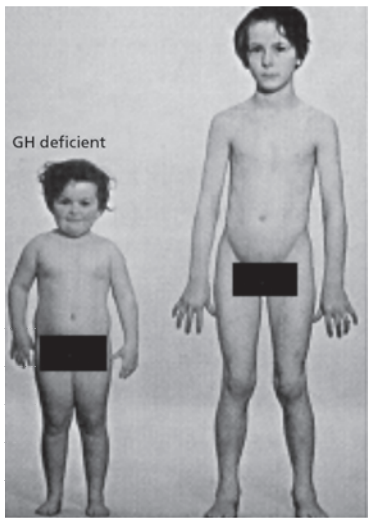

Figure 5: Typical phenotype a GH deficient child (adapted from a Colour Atlas of Endocrinology, Urban & Fischer Verlag, 1994)

In contrast to IGHD, CPHD is often accompanied by pituitary abnormalities on MRI. Hypoplasia of the anterior pituitary, an ectopic location of the posterior pituitary or an invisible stalk are frequent findings in these patients. When all three abnormalities are present, this is called the 'classic triad' of pituitary anomalies.

Although the cause of hypopituitarism is unknown in the majority of the patients, there are several hypotheses concerning its aetiology. Hypotheses related to the pathogenesis of idiopathic hypopituitarism can be divided in two major groups: the *(embryo-)genetic hypothesis,* according to which a genetic defect or a defect during the embryonic organogenesis has lead to an abnormal pituitary. The other hypothesis is the *birth trauma hypothesis,* in which a traumatic (breech-) delivery is thought to have damaged the pituitary, with hormonal deficiencies as a result. Recently, Anti Pituitary Antibodies (APA) were found in patients with idiopathic IGHD and CPHD[7,8]. As a result, the *autoimmune hypothesis* was recently added to the (embryo-)genetic and birth trauma hypotheses.

1.6 Genetic causes of IGHD and CPHD

1.6.1 Mutations and polymorphisms

A mutation is a change in genetic information, by definition occurring in less than 1% of the normal population and often related to disease. The most frequent mutations are single base changes ('point mutations') in coding regions of the genes, causing a change in the corresponding amino acid. Point mutations in parts of the gene that are important for splicing can cause production of a protein which is too short or which contains erroneous amino acids.

A polymorphism is a genetic variant, not necessarily related to disease, which appears in more than 1% of a normal population. A polymorphism that affects only one base, is called a Single Nucleotide Polymorphism or SNP.

1.6.2 IGHD

Genes known to be involved in IGHD are *GH1* and *GHRHR*, encoding the Growth Hormone and the Growth Hormone Releasing Hormone Receptor, respectively (Appendix II). These genes were discovered by somatic cell hybridization, southern blot analysis and linkage analysis in growth hormone deficient mice and humans. The expression of *GH1* is regulated by a locus control region (LCR), located 14.5 kb to 32 kb upstream of the gene[9] and by the *GH1* promoter, located directly upstream of the gene.

Defects in *GH1* and *GHRHR* account for different types of IGHD (Table 1). IGHD Type IA is mostly caused by deletions, frameshifts and nonsense mutations in *GH1.* It has an autosomal recessive mode of inheritance. In IGHD IB, splice site mutations in *GH1* and missense mutations in *GHRHR* are responsible for the GH deficiency. IGHD type II has an autosomal dominant mode of inheritance and is caused by splice site or missense mutations that have dominant-negative effects. IGHD type III is an X-linked disorder.

Table 1: Genetic and phenotypic features of different types of IGHD

IGHD type	Inheritance	Mutations	Phenotype
Type 1A	AR	*GH1*	Severe short stature, antibodies during GH treatment
Type 1B	AR	*GH1, GHRHR*	Short stature, good response to GH treatment
Type II	AD	*GH1*	Short stature, good response to GH treatment
Type III	X-linked	*BTK*	Short stature, hypogammaglobulinemia

BTK = Bruton Tyrosine Kinase, AR = Autosomal Recessive, AD = Autosomal Dominant

Polymorphisms do not directly cause disease, but they have been associated with susceptibility to diseases[10-12] and to environmental toxins[13] by affecting splicing[14], allelic expression[15], nonsense-mediated mRNA decay[16] or transcription factor binding[17]. In this thesis, we analysed polymorphisms present in the regulating regions of *GH1* and in the gene encoding the growth hormone receptor: *GHR.*

The *GH1* promoter contains SNPs that have been associated with height[18-20], bone density, bone loss and fetal growth restriction[21,22]. In addition, some *GH1* promoter SNPs have been associated with the incidence of breast and colorectal cancer[23-27]. Some of the *GH1* LCR and promoter SNPs have also been studied in relation to GH and IGF-I levels in patients with varying degrees of GH deficiency[18].

GHR contains a genetic polymorphism caused by a deletion of exon 3 *(GHR d3)*[28]. This polymorphism is present in heterozygosis in 40% of the healthy population and in homozygosis in 15%. There is a continuing controversy whether the *d3* polymorphism affects human growth and whether it could affect the response to GH treatment[29-34].

1.6.3 CPHD

The genes known to be involved in CPHD encode the pituitary transcription factors PROP1, HESX1, POU1F1, LHX3 and LHX4, which are important for DNA transcription. To start DNA transcription, transcription factors bind to short DNA sequences near a gene in order to guide and activate the polymerase (Figure 6). These short DNA sequences are often located upstream of the coding sequence of a gene, in the promoter of the gene. In order to recognize and bind to these DNA sequences, the transcription factor must partly penetrate into the major and/or minor grooves of the DNA double helix.

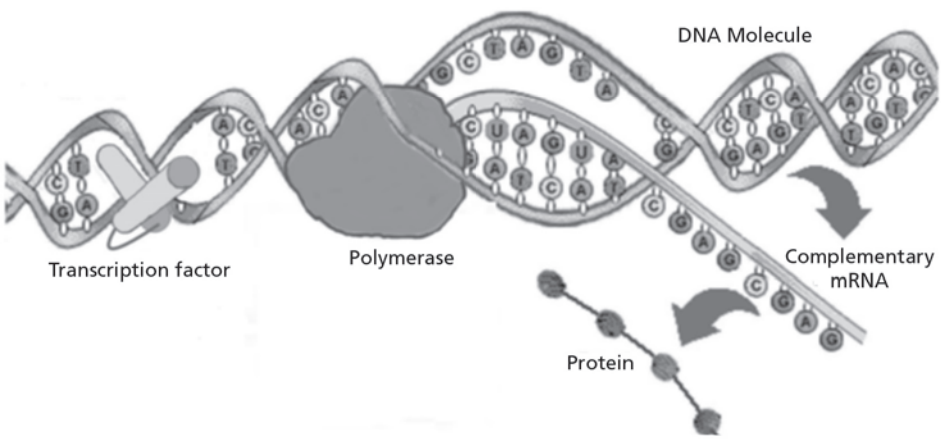

Figure 6: Transcription factors penetrate into the grooves of the DNA in order to guide and activate the polymerase

Transcription factors often have two distinct regions with different functions: a transcriptional activation domain, which activates transcription of the target genes once the transcription factor has bound to its promoter, and a DNA-binding domain. By this domain, the transcription factor binds specifically to its target gene. The pituitary transcription factors known to be important for development of the pituitary (the paired-like homeodomain transcription factors PROP1 and HESX1, the LIM homeodomain transcription factors LHX3 and LHX4 and the POU domain transcription factor POU1F1, also called PIT1) all have a so-called homeodomain as their DNA-binding domain. Homeodomains are either found alone as a DNA-binding motif or in tandem with another module, such as paired-homeodomains in PROP1, repression domain in HESX1, POU homeodomains in POU1F1 and LIM-homeodomains in LHX3 and LHX4[35-40]. The genetic and proteic structures of these transcription factors are shown in Appendix II.

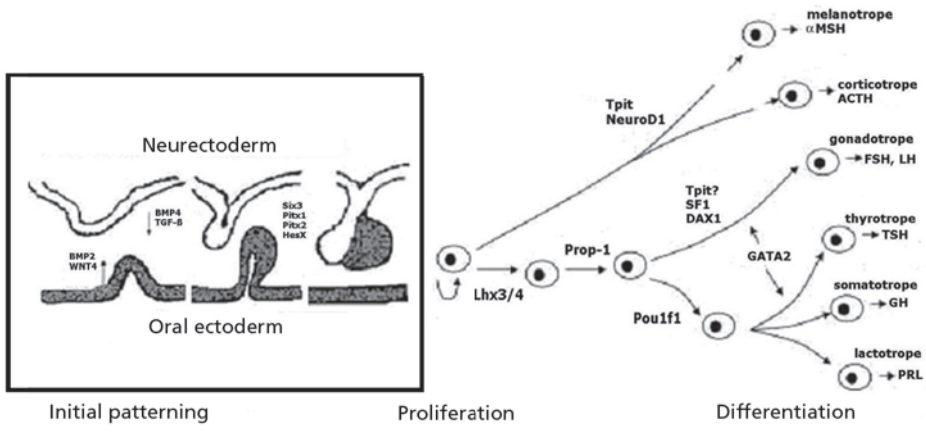

Figure 7: Signaling molecules and transcription factors involved in pituitary organogenesis (adapted from Reynaud et al, Growth Hormone and IGF research 2004, with permission)

Table 2: Hormone deficiencies generally associated with defects in different genes

	GH	PRL	TSH	LH/FSH	ACTH
PROP1	+	+	+	+	+
HESX1	+	+	+	+	+
POU1F1	+	+	+	-	-
LHX3	+	+	+	+	-
LHX4	+	+	+	+	+

PROP1 consists of a paired-like homeodomain and a transactivation domain, so it has both DNA-binding and transcriptional activation ability. PROP1 is necessary for *POU1F1* expression and thus for POU1F1 dependent proliferation and differentiation of pituitary gonadotropes, as well as somatotropes, lactotropes and caudomedial thyrotropes (Figure 7). Although mutations in *PROP1* can initially cause IGHD, they generally also result in deficiencies of TSH, PRL, LH, FSH and ACTH (Table 2). *PROP1* mutations described in the literature until 2007 are shown in Appendix II.

HESX1, like PROP1, belongs to the family of paired-like homeodomain transcription factors. HESX1 has a homeodomain and a minimal 36 amino acid N-terminal repression domain called Eh1, on which it depends for its interaction with Gro/TLE co-repressor proteins to suppress PROP1-dependent proliferation and differentiation of pituitary cell lines. As studied in mice, Hesx1 and Prop1 exhibit temporally distinct but overlapping patterns of expression over the entire period of pituitary development. Hesx1 and Prop1 heterodimerize on the same sequence element. The careful temporal regulation of their expression is critical for normal pituitary development. Hesx1 suppresses *Prop1* expression and vice versa. Premature expression of *Prop1* can block pituitary organogenesis, whereas prolonged expression of *Hesx1* with the obligate co-repressor TLE1 can block Prop1-dependent activation[41]. Although mutations in *HESX1* can initially cause IGHD, the clinical picture generally expands to CPHD with deficiencies of TSH, PRL, LH, FSH and ACTH (Table 2). *HESX1* mutations described in the literature until 2007 are shown in Appendix II.

POU1F1 is a member of the POU family of transcription factors. POU1F1 contains 2 protein domains (POU-homeo and POU-specific), both necessary for high affinity DNA binding on genes encoding growth hormone and prolactin, and a transactivation domain. The development and proliferation of somatotropes, lactotropes and caudomedial thyrotropes require *POU1F1* expression. Defects in *POU1F1* result in deficiencies of GH, PRL and TSH. Production of ACTH and LH/FSH generally remain unaffected. *POU1F1* mutations described in the literature until 2007 are shown in Appendix II.

LHX3 is important for the early formation of Rathke's pouch. It is a member of the LIM homeodomain family of transcription factors and has a direct influence on the transcription of α-glycoprotein subunit (α-*GSU*), prolactin (*PRL*), Thyrotropin-β (*TSH*-β) and *POU1F1*. LHX3 has two isoforms, LHX3a and LHX3b, which have a different ability to transactivate pituitary gene targets. Although the exact mechanisms are unknown, the different isoforms of LHX3 may play different roles

during development of the mammalian pituitary gland and other neuroendocrine systems. Mutations in *LHX3* generally cause deficiencies of GH, PRL, TSH, LH and FSH. ACTH production is usually normal. *LHX3* mutations described in the literature until 2007 are shown in Appendix II.

LHX4 is highly homologous to the human LHX3 protein and consists of a tandem pair of LIM domains and a homeodomain. LHX4 is important for cell survival in the pituitary and for the timely activation of LHX3. Inactivating mutations in *LHX4* result in deficiencies of GH, PRL, TSH, LH, FSH and ACTH. *LHX4* mutations described in the literature until 2007 are shown in Appendix II.

Although *PROP1, HESX1, POU1F1, LHX3* and *LHX4* were traditionally considered CPHD-related genes and *GH1* and *GHRHR* were considered IGHD-related genes, recently, Mullis *et al.*[42] reported CPHD in patients with mutations in *GH1.* This phenomenon might be explained by a mechanism of bystander damage from activated macrophages clearing dying somatotroph debris. Therefore, in addition to *PROP1, HESX1, POU1F1, LHX3* and *LHX4,* some *GH1* mutations might also cause CPHD.

1.8 Techniques

In this study, mutation screening was performed using the Transgenomic Wave 3500HT Denaturing High-Performance Liquid Chromatography (dHPLC) system (Transgenomic, Omaha, USA). Samples with sequence variants, detected by dHPLC, were subsequently sequenced using an ABI Prism 3100 Genetic analyzer (Applied Biosystems). When a new variant was identified, healthy Dutch controls were screened by Taqman genotyping assays in order to estimate its frequency among the normal population. After screening for mutations, multiplex ligation-dependent probe amplification (MLPA) was performed to screen for intragenic deletions and whole gene deletions. Detailed information about these techniques is given in Appendix III.

1.9 Dutch Growth Foundation

The clinical data of the GH deficient patients participating in this study were available thanks to the existence of the National Registry of Growth Hormone

Treatment in children (LRG-children), coordinated and maintained by the Dutch Growth Foundation. The Dutch Growth Foundation was founded to stimulate research into the causes of disturbances in growth and development in children, to evaluate existing and new methods of treatment and to advise medical attendants and patients. In 1992, the Dutch Growth Foundation set up the National Registry of Growth Hormone Treatment in children, where clinical data are registered for all Dutch children who received or are still receiving growth hormone treatment. The Dutch Growth Foundation performs a number of large, nation-wide studies. An important aim of these studies is to improve the quality of growth hormone treatment through scientific research. Part of the research projects focus on the cause of growth disorders, as in the HYPOthalamic and PITuitary gene study (HYPOPIT) study, which is described in this thesis.

We used the LRG data to include patients in the HYPOPIT study. All patients with childhood onset idiopathic IGHD or CPHD, who were registered in the LRG between 1992 and 2003 and who were treated in the hospitals participating in the study, were included.

IGHD was defined as a peak GH response <20 mU/L to arginine or clonidine test, or <30 mU/L combined with serum IGF-I < –2 SDS, and normal serum levels of other pituitary hormones. Patients were classified as having severe IGHD (sIGHD) or partial IGHD (pIGHD) based on a scoring system including height SDS (HSDS), maximum GH levels, IGF-I and IGFBP-3 SDS (Appendix IV).

In the CPHD group, all GH-treated patients were included who had childhood onset deficiencies of GH and one or more additional hormonal axes. Deficiencies of hypothalamic-pituitary-thyroidal, -adrenal, and -gonadal axes were defined as abnormal TRH test or TSH levels that were low or inadequate for low (F)T4; abnormal CRF / ACTH / glucagon test or ACTH levels which were low or inadequate for low cortisol and LH, FSH, estrogen / testosterone or LHRH test low for age or lack of spontaneous puberty after age 14 y. Prolactin deficiency was defined as abnormal prolactin during random or TRH testing. Reference values of the individual hospitals were used. Patients with IGHD or CPHD of known cause, such as a brain tumour, brain surgery, brain radiation and known syndromes, were excluded.

1.10 Questions and hypotheses

In the literature, the majority of the studies investigating the causes of hypopituitarism, focused on mutations in coding regions or splice sites of *GH1, GHRHR, PROP1, HESX1, POU1F1, LHX3* and *LHX4*. We could not imagine that mutations in the coding regions

of these genes were the only cause of hypopituitarism and suspected a combination of genetic, immunologic and birth-related causes, together with minor non-coding genetic variations to explain part of the cases of idiopathic hypopituitarism. Figure 10 depicts how little was known about the causes of IGHD and CPHD in Dutch patients in 2003, before the start of the HYPOPIT study.

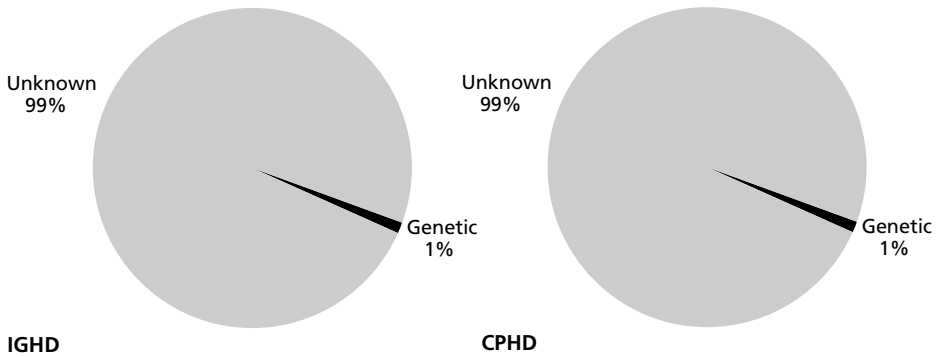

Figure 7: Causes of IGHD and CPHD as known in the Dutch patients at start of the HYPOPIT study, in 2003

Before the start of the study, we did not know the frequency of known coding mutations in IGHD and CPHD-related genes in Dutch patients with these disorders. Based on the literature, we expected to find *GH1* and *GHRHR* mutations in about 30% of the IGHD patients and mutations in *PROP1, HESX1, POU1F1, LHX3* or *LHX4* in about 25% of the CPHD patients. We did not know whether minor genetic variations outside the coding regions of the IGHD related genes were related with phenotype. We wondered whether perhaps autoimmune processes could account for the clinical picture of some of our patients. Based on the literature, we suspected that exon 3 deletion in the *GHR* would affect response to GH treatment. Based on clinical impressions, we suspected that the face of children with IGHD and CPHD might be related to their endocrinologic anomalies. We wondered whether there was an association between facial, endocrinological and MRI features.

1.11 Aims of the study

Frequency of mutations in coding regions of genes known to be involved in IGHD and CPHD

The HYPOPIT study aimed to obtain an overall picture of sequence changes in *GH1* and *GHRHR* in IGHD patients and *PROP1, HESX1, POU1F1, LHX3,* and *LHX4* in CPHD patients, respectively, and to relate them with clinical parameters. Since P89L and IVS3+1 /+2 splice site mutations in *GH1* had recently been shown to cause pituitary hormone impairment additional to GH deficiency[42], we also screened for these *GH1* mutations in our CPHD patients.

Association of non-coding genetic variations in GH1 with phenotype

In IGHD patients, whose IGHD was not explained by mutations in coding regions of *GH1* and *GHRHR,* we investigated the *GH1* locus control region and promoter SNPs and looked for associations between genotype and phenotype.

Immunologic causes of idiopathic hypopituitarism

Since the cause of IGHD and CPHD remained unknown in the majority of patients and recent articles suggested that some cases of idiopathic GHD might be explained by a silent form of autoimmune hypophysitis (AIH) based on the presence of Anti Pituitary Antibodies (APA) at high titres[7,8], we performed APA screening in 40 IGHD patients and 31 CPHD patients and related APA, when present, to clinical and morphological pituitary findings.

Aetiologic hypotheses regarding hypopituitarism

With regard to the aetiology of hypopituitarism, three major hypotheses exist: the (embryo-)genetic, birth trauma and the autoimmune hypothesis. We analysed obstetric, neonatal, biochemical, immunologic, genetic and morphologic data of 244 patients with idiopathic IGHD and CPHD, in order to assess the number of cases that could be explained by the three different mechanisms.

Effect of genetic variation of the growth hormone receptor (GHR d3) on spontaneous growth, growth during GH treatment and birth size

Next to investigating the causes of IGHD and CPHD, we were also interested in the effect of GH treatment. Since there is a continuing controversy whether the presence or absence of the exon 3 deletion (*d3+* vs. *d3-)* affects the effect of GH in human growth and whether it alters the effect of GH treatment, we performed *d3* analysis in 144 CPHD and IGHD patients born either small or appropriate for gestational age

(SGA or AGA) and related clinical data and response to GH treatment to *GHR d3+* and *d3-* genotype.

Facial and pituitary morphology in IGHD and CPHD patients
Since GHD is associated with typical phenotypic features, we analysed standardised photographs of 143 Caucasian patients with IGHD or CPHD, in order to find relations between auxological, biochemical, pituitary and facial morphometric features.

1.12 Outline of the thesis

This doctoral dissertation gives a detailed account of the various studies.

Chapters 2 and 3 describe the genetic screening of *GH1* and *GHRHR* in IGHD patients and *PROP1, HESX1, POU1F1, LHX3, LHX4* and *GH1* P89L and IVS3+1 /+2 in CPHD patients. **Chapter 4** describes the SNP analysis performed in the locus control region and promoter of *GH1* in IGHD patients without mutations in *GH1* or *GHRHR*. **Chapter 5** describes the immunologic study of Anti Pituitary Antibodies in IGHD and CPHD patients. **Chapter 6** reports the findings of the mutation screening and the immunologic study, together with the analysis of birth data, which were used to evaluate the three main aetiologic hypotheses of hypopituitarism. **Chapter 7** reports the relation between the growth hormone receptor's *d3* polymorphism and spontaneous growth, growth in response to GH treatment and birth size. **Chapter 8** describes the relation between facial characteristics and pituitary abnormalities in children with IGHD and CPHD. **Chapter 9** discusses the significance of the presented data and the mutual relationship in the context of the literature. Our final conclusions are listed, as well as recommendations for future research. Finally, **chapter 10** summarizes this dissertation in English as well as in Dutch.

Appendix I

Endocrine axes studied in this thesis

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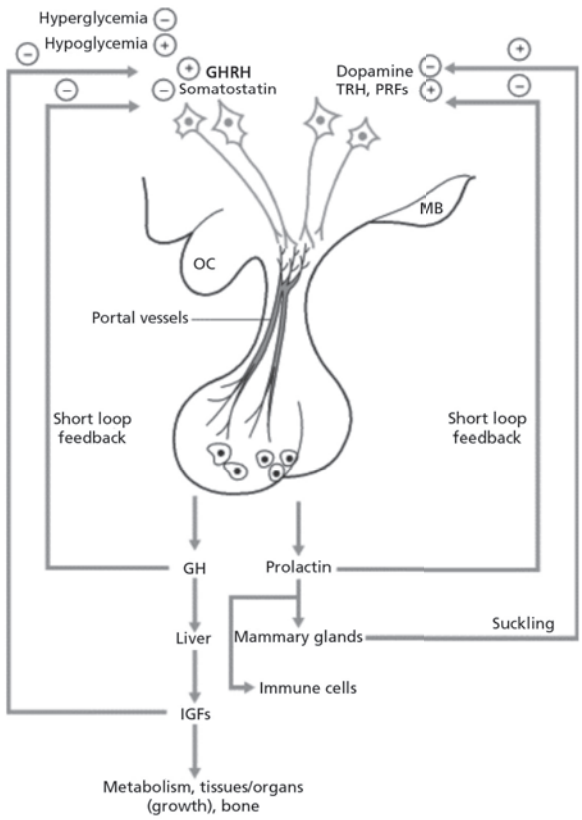


Figure I-1: The hypothalamo-pituitary-GH-IGF-I Axis

GH = Growth Hormone, GHRH = Growth Hormone Releasing Hormone, IGF = Insulin-like Growth Factor, TRH = Thyrotropin Releasing Hormone, PRF = Prolactin Releasing Factor

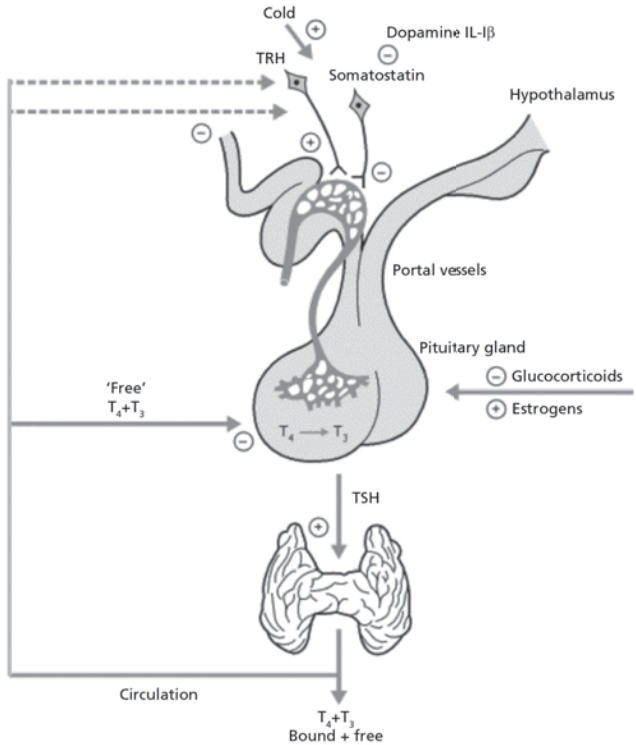


Figure I-2: The hypothalamo-pituitary-thyroidal axis

TRH = Thyrotropin Releasing Hormone, TSH = Thyroid Stimulating Hormone

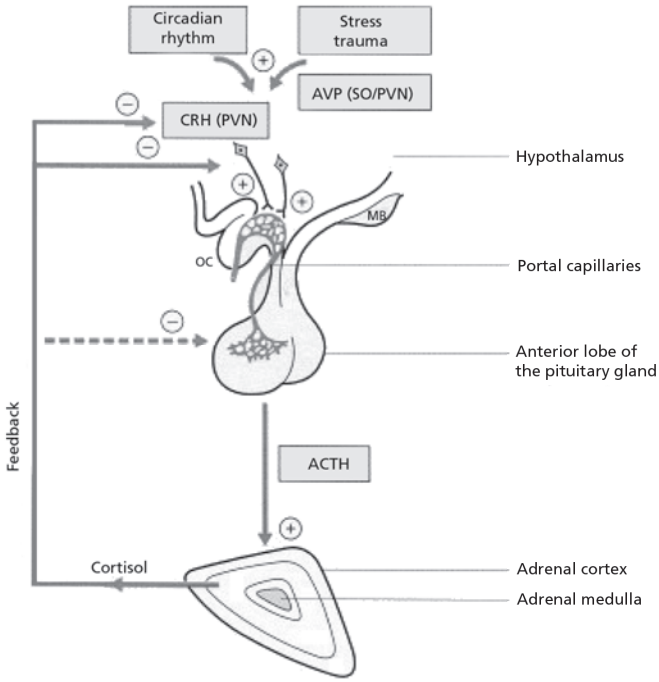


Figure I-3: The hypothalamo-pituitary-adrenal axis

AVP = Arginine Vasopressin, SO = Supra-Optic hypothalamic nuclei, PVN = Paraventricular Nucleus

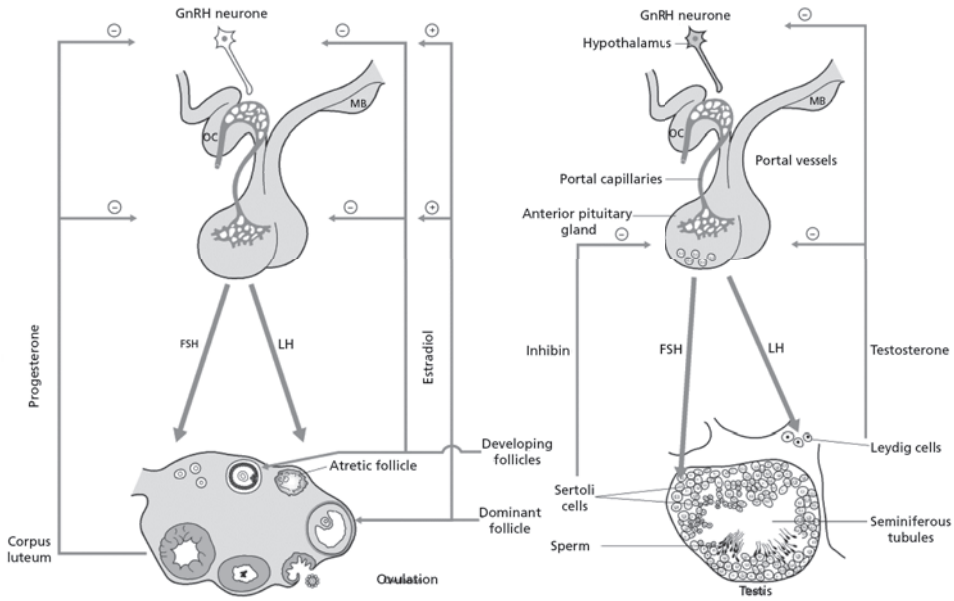


Figure I-4: The hypothalamo-pituitary-gonadal axis in females (left) and males (right)

LH = Luteinising Hormone, FSH = Follicle Stimulating Hormone

Appendix II

Genes studied in the HYPOPIT study and mutations described in the literature up to 2007

Images adapted from GROWTH AND GROWTH DISORDERS, by Berrin Ergun-Longmire and Michael P. Wajnrajch in WWW.ENDOTEXT.ORG, Chapter 1a, version February 27th, 2007, published by MDTEXT.COM,INC, South Dartmouth, MA 02748, with permission

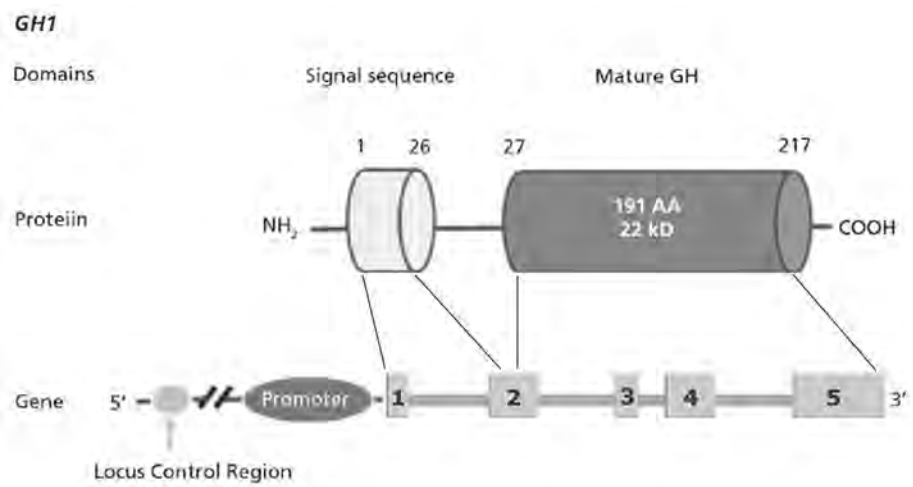


Figure II-1: The *GH1* gene

All exons and at least 20 intronic base pairs upstream and downstream of the exons were analysed by dHPLC and subsequent sequencing. The Locus Control Region and promoter were analysed by direct sequencing. AA = Amino Acid, kD = kilo Dalton, GH = Growth Hormone

Mutations in *GH1* described to date in patients with IGHD

(adapted from the Human Gene Mutation database, www.HGMD.org)

Codon change	Amino Acid change	Codon	Reference
tACA-GCA	Thr-Ala	-24	Miyata (1997) Endocr J 44, 149
CTC-CCC	Leu-Pro	-11	Millar (2003) Hum Mutat 21, 424
TGG-TAG	Trp-Term	-7	Cogan (1993) J Clin Endocrinol Metab 76, 1224
aGAG-TAG	Glu-Term	-4	Wagner (1998) Pediatr Res 43, 105
cCGC-TGC	Arg-Cys	16	Millar (2003) Hum Mutat 21, 424
ACC-ATC	Thr-Ile	27	Millar (2003) Hum Mutat 21, 424
gAAC-GAC	Asn-Asp	47	Millar (2003) Hum Mutat 21, 424
cCGC-TGC	Arg-Cys	77	Takahashi (1996) N Engl J Med 334, 432
CCC-CTC	Pro-Leu	89	Wajnrajch (2000) J Endocr Genet 1 125
cAGC-CGC	Ser-Arg	108	Millar (2003) Hum Mutat 21, 424
cAGC-TGC	Ser-Cys	108	Millar (2003) Hum Mutat 21, 424
cGTC-TTC	Val-Phe	110	Binder (2001) J Clin Endocrinol Metab 86, 3877
gACA-GCA	Thr-Ala	175	Millar (2003) Hum Mutat 21, 424
ATCg-ATG	Ile-Met	179	Lewis (2004) J Clin Endocrinol Metab 89, 1068
CGC-CAC	Arg-His	183	Miyata (1997) Endocr J 44, 149
IVS 2 as	-2	A-T	Fofanova (2003) J Clin Endocrinol Metab 88, 820
IVS 2as	-1	G-A	Millar (2003) Hum Mutat 21, 424
IVS 2as	1	G-T	Takahashi (2002) Clin Genet 61, 222
IVS 2as	5	A-G	Moseley (2002) J Clin Endocrinol Metab 87, 847
IVS 3ds	1	G-A	Cogan (1995) J Clin Endocrinol Metab 80, 3591
IVS 3ds	1	G-C	Binder (1995) J Clin Endocrinol Metab 80, 1247
IVS 3ds	2	T-C	Fofanova (2006) Bull Exp Biol Med 141, 347
IVS 3ds	5	G-C	Hayashi (1999) Growth Horm IGF Res 9, 434
IVS 3ds	5	G-A	Missarelli (1997) Hum Genet 101, 113
IVS 3ds	6	T-C	Phillips (1994) J Clin Endocrinol Metab 78, 11
IVS 3ds	6	T-G	Katsumata (2001) Growth Horm IGF Res 11, 378
IVS 3ds	28	G-A	Cogan (1997) Hum Mol Genet 6, 909
IVS 4ds	-1	G-A	Fofanova (2006) Bull Exp Biol Med 141, 347
IVS 4ds	1	G-C	Cogan (1993) J Clin Endocrinol Metab 76, 1224
IVS 4ds	1	G-T	Phillips (1994) J Clin Endocrinol Metab 78, 11
IVS 4ds	5	G-C	Leiberman (2000) Am J Med Genet 90, 188
Del GCCTG^CTCTGcCTGCCCTGGC		-11	Duquesnoy (1990) Am J Hum Genet 47 A110
Del AGAAACAGGTgggggCAACAGTGGG			Millar (2003) Hum Mutat 21, 424
Del CCCCAGGCGGggatgggggagacctgtaGTCAGAGCCC			Cogan (1997) Hum Mol Genet 6, 909
Del TCTGT^TTCTCagAGTCTATTCC		54	Igorishi (1993) Hum Mol Genet 2, 1073
Del 45 kb, entire gene + GH2,CSH1,CSHL1			Akinci (1992) J Clin Endocrinol Metab 75, 437
Del 45 kb, entire gene + GH2,CSH1,CSHL1			Ghizzoni (1994) Pediatr Res 36, 654
Del 45 kb, entire gene + GH2,CSH1,CSHL1			Baroncini (1993) Hum Mol Genet 2, 2151
Del 45 kb, entire gene + GH2,CSH1,CSHL1			Wagner (1998) Pediatr Res 43, 105
Del 6.7 kb			Phillips (1981) Proc Natl Acad Sci U S A 78, 6372
Del 7 kb			Aguirre (1993) Rev Med Chil 121, 982
Del 7.1 kb			He (1990) J Med Genet 27, 151
Del 7.6 kb			Laron (1985) Isr J Med Sci 21, 999
Double del. 6.7 kb *GH1*, ~32kb *GH2,CSH1,CSH2*			Goossens (1986) J Clin Endocrinol Metab 62, 712

as = acceptor site, ds = donor site, del = deletion

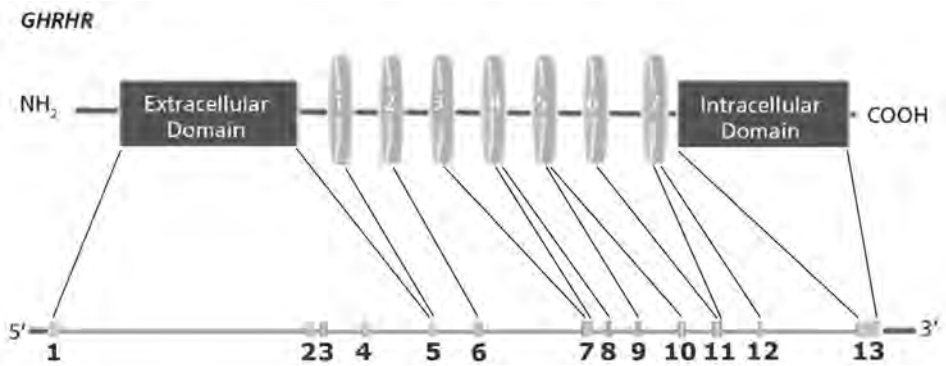


Figure II-2: The *GHRHR* gene

All exons and at least 20 intronic base pairs upstream and downstream of the exons were analysed by dHPLC and subsequent sequencing.

Mutations in *GHRHR* described to date in patients with IGHD

(adapted from the Human Gene Mutation database, www.HGMD.org)

Codon change	Amino acid change	Codon	Reference
aCAA-TAA	Gln-Term	43	Salvatori (2002) Eur J Endocrinol 147, 591
cGAG-TAG	Glu-Term	72	Wajnrajch (1996) Nat Genet 12, 88
CAT-CTT	His-Leu	137	Salvatori (2001) Clin Endocrinol (Oxf) 54, 681
CTC-CAC	Leu-His	144	Salvatori (2001) J Clin Endocrinol Metab 86, 273
GCG-GTG	Ala-Val	176	Carakushansky (2003) Eur J Endocrinol 148, 25
GCA-GAA	Ala-Glu	222	Salvatori (2001) J Clin Endocrinol Metab 86, 273
TTC-TGC	Phe-Cys	242	Salvatori (2001) J Clin Endocrinol Metab 86, 273
cAAG-GAG	Lys-Glu	329	Salvatori (2002) Mol Endocrinol 16, 450
1ds	1	G-A	Salvatori (1999) J Clin Endocrinol Metab 84, 917
3ds	1	G-A	Salvatori (2002) Eur J Endocrinol 147, 591
7ds	1	G-C	Roelfsema (2001) J Clin Endocrinol Metab 86, 2459
12ds	2	T-A	Alba (2004) Clin Endocrinol (Oxf) 60, 470
TGTGTCCCCTTGGCTAGCTCCTGCCTATGC(A-C) AACAGCCACCTGAGAAGGGGAAGCAGAGGG -124 relative to initiation codon			Salvatori (2002) Mol Endocrinol 16, 450
Del TGGAG^CTGGGactggGTTCCTTCTT		362	Salvatori (2001) Clin Endocrinol (Oxf) 54, 681

ds = donor site, del = deletion

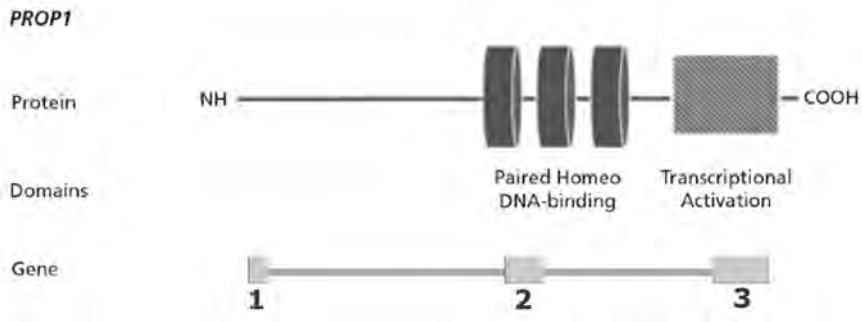


Figure II-3: The *PROP1* gene

All exons and at least 20 intronic base pairs upstream and downstream of the exons were analysed by dHPLC and subsequent sequencing.

Mutations in *PROP1* described to date in patients with CPHD

(adapted from the Human Gene Mutation database, www.HGMD.org)

Codon change	Amino Acid change	Codon	Reference
cCGC-TGC	Arg-Cys	73	Duquesnoy (1998) FEBS Lett 437, 216
CGC-CAC	Arg-His	73	Vallette-Kasic (2001) J Clin Endocrinol Metab 86, 4529
aCAG-TAG	Gln-Term	83	Sertedaki (2004) Hum Genet 115, 174
TTT-TCT	Phe-Ser	88	Osorio (2000) J Clin Endocrinol Metab 85, 2779
cCGA-TGA	Arg-Term	99	Vallette-Kasic (2001) J Clin Endocrinol Metab 86, 4529
CGA-CAA	Arg-Gln	99	Vieira (2003) J Clin Endocrinol Metab 88, 38
gTTC-ATC	Phe-Ile	117	Wu (1998) Nat Genet 18, 147
cCGC-TGC	Arg-Cys	120	Wu (1998) Nat Genet 18, 147
CGC-CAC	Arg-His	120	Mody (2002) Best Pract Res Clin Endocrinol Metab 16, 421
2as	-2	A-T	Duquesnoy (1998) FEBS Lett 437, 216
del CACGGTG^GACtcgagtgctccacCCTGC AGAAG		37	Agarwal (2000) J Clin Endocrinol Metab 85, 4556
del CCTGGT^GCAGgaGGGGGGAGAT		49	Fofanova (1998) J Clin Endocrinol Metab 83, 2601
del CTGGT^GCAGGaGGGGGGAGAT		49	Krzisnik (1999) J Endocr Genet 1 9
del AGGAGGG^GGGaGATCAAGGTT		52	Tatsumi (2004) Clin Endocrinol (Oxf) 61, 635
del GGCCCGA^GAGagTCTTGCCCGG		100	Wu (1998) Nat Genet 18, 147

as = acceptor site, del = deletion

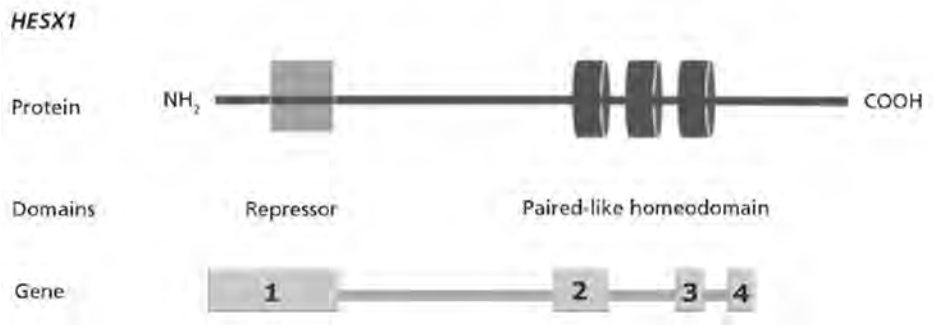


Figure II-4: The *HESX1* gene

All exons and at least 20 intronic base pairs upstream and downstream of the exons were analysed by dHPLC and subsequent sequencing.

Mutations in *HESX1* described to date in patients with CPHD and/or pituitary anomalies

(adapted from the Human Gene Mutation database, www.HGMD.org)

Codon change	Amino acid change	Codon	Phenotype	Reference
CAGg-CAC	Gln-His	6	Pituitary hypoplasia	Thomas (2001) Hum Mol Genet 10, 39
ATC-ACC	Ile-Thr	26	Pituitary hypoplasia	Carvalho (2003) J Clin Invest 112, 1192
AAT-AGT	Asn-Ser	125	Pituitary hypoplasia	Parks (1999) J Clin Endocrinol Metab 84, 4362
gCGT-TGT	Arg-Cys	160	Septo-optic dysplasia	Dattani (1998) Nat Genet 19, 125
TCA-TTA	Ser-Leu	170	Pituitary hypoplasia	Thomas (2001) Hum Mol Genet 10, 39
cACA-GCA	Thr-Ala	181	Pituitary hypoplasia	Thomas (2001) Hum Mol Genet 10, 39
Insertion of ~300 bp Alu element in exon 3			Pituitary hypoplasia	Sobrier (2005) Hum Mutat 25, 503

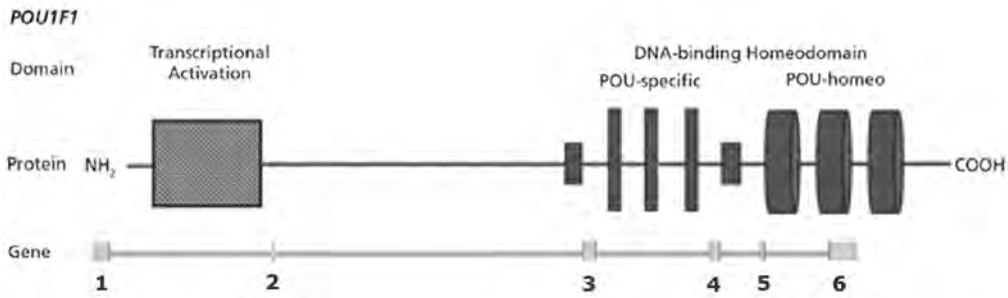


Figure II-5: The *POU1F1* gene

All exons and at least 20 intronic base pairs upstream and downstream of the exons were analysed by dHPLC and subsequent sequencing.

Mutations in *POU1F1* described to date in patients with CPHD

(adapted from the Human Gene Mutation database, www.HGMD.org)

Codon change	Amino Acid change	Codon	Reference
cCAA-TAA	Gln-Term	4	Salemi (2003) Growth Horm IGF Res 13, 264
CCT-CTT	Pro-Leu	24	Ohta (1992) Biochem Biophys Res Commun 189, 851
TTT-TGT	Phe-Cys	135	Pellegrini-Bouiller (1996) J Clin Endocrinol Metab 81, 2790
CGA-CAA	Arg-Gln	143	Ohta (1992) Biochem Biophys Res Commun 189, 851
CGA-CTA	Arg-Leu	143	McLennan (2003) Clin Endocrinol (Oxf) 58, 785
tAAA-TAA	Lys-Term	145	Cohen (2002) Endocr Rev 23, 431
gGCA-CCA	Ala-Pro	158	Pfaffle (1992) Science 257, 1118
tCAA-AAA	Gln-Lys	167	Malvagia (2003) Pediatr Res 54, 635
cCGA-TGA	Arg-Term	172	Tatsumi (1992) Nat Genet 1, 56
CGA-CAA	Arg-Gln	172	Turton (2005) J Clin Endocrinol Metab 90, 4762
GAA-GGA	Glu-Gly	174	Brown (1998) Horm Res 49, 98
aTGG-CGG	Trp-Arg	193	Hendriks-Stegeman (2001) J Clin Endocrinol Metab 86, 1545
CTG-CAG	Leu-Gln	194	McLennan (2003) Clin Endocrinol (Oxf) 58, 785
aAAA-GAA	Lys-Glu	216	Cohen (1999) Mol Endocrinol 13, 476
gGAG-AAG	Glu-Lys	230	Gat-Yablonski (2002) J Pediatr Endocrinol Metab 15, 325
cTTT-CTT	Phe-Leu	233	Rainbow (2005) Clin Endocrinol (Oxf) 62, 163
aCCT-TCT	Pro-Ser	239	Pernasetti (1998) J Clin Endocrinol Metab 83, 2079
aGAA-TAA	Glu-Term	250	Irie (1995) Endocr J 42, 351
cCGG-TGG	Arg-Trp	265	Bircan (2001) J Endocr Genet 2 61
aCGG-TGG	Arg-Trp	271	Radovick (1992) Science 257, 1115
del GGATG^GCTGAaGAACTGAATC		248	Hendriks-Stegeman (2001) J Clin Endocrinol Metab 86, 1545
del GAGAA^AAACGgGTGAAAACAA		270	Blankenstein (2001) Horm Res 56, 81
Ins AGAAGTA^GTAaAGAGTTTGGT 258		778	Turton (2005) J Clin Endocrinol Metab 90, 4762

del = deletion, ins = insertion

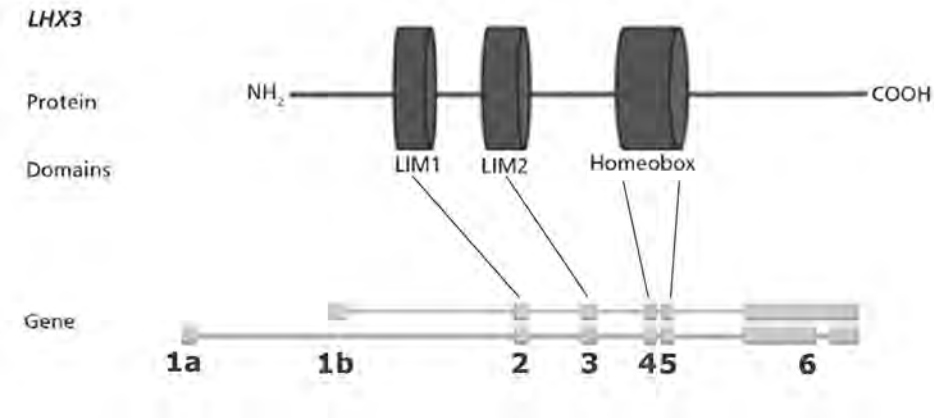


Figure II-6: The *LHX3* gene

All exons and at least 20 intronic base pairs upstream and downstream of the exons were analysed by dHPLC and subsequent sequencing.

Three mutations described to date:

- LHX3a Y111C (LHX3b Y116C) (Netchine *et al*, Nature Genetics 2000, 25(2):182-6)

- 23 bp deletion in exon 3 (Netchine *et al*, Nature Genetics 2000, 25(2):182-6)

- g.159delT (Bhangoo *et al*, JCEM 2006, 91(3) 747-753)

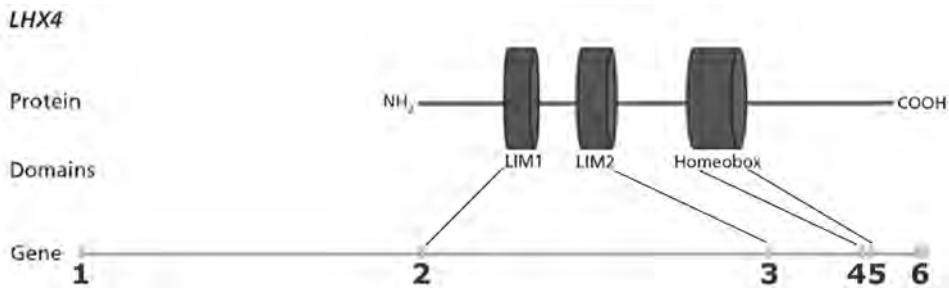


Figure II-7 The *LHX4* gene

All exons and at least 20 intronic base pairs upstream and downstream of the exons were analysed by dHPLC and subsequent sequencing.

Five mutations described to date:

- IVS4-1 G>C (Machinis et al, Am J Hum Genet 2001, 69, 961)
- R84C, L190R, and A210P (Pfaeffle et al, JCEM, epub december 2007)
- P366T (Tajima et al, Endocrine Journal 2007;54(4):637-41.)

Appendix III

Techniques used in the HYPOPIT study

dHPLC *Denaturing High-Performance Liquid Chromatography, Wave 3500HT Transgenomic, Omaha, USA.*

DHPLC is a highly sensitive method for detecting single nucleotide polymorphisms (SNPs), mutations, and other DNA sequence variations. Before passing DNA samples through the dHPLC, heteroduplexes are formed by heating the PCR products. Heating to 95 degrees produces denaturation (separation of DNA strands) and cooling down produces recombination of single strands into new pairs of either two identical strands (called matched DNA or homoduplex) or two non-identical strands (called mismatched DNA or heteroduplex). Notably, heteroduplexes are only formed when two different strands are present in the sample, like a mutation in heterozygosis in the sample of an individual patient, or a mutation in homozygosis in the individual's DNA mixed with wild-type DNA in homozygosis from control DNA.

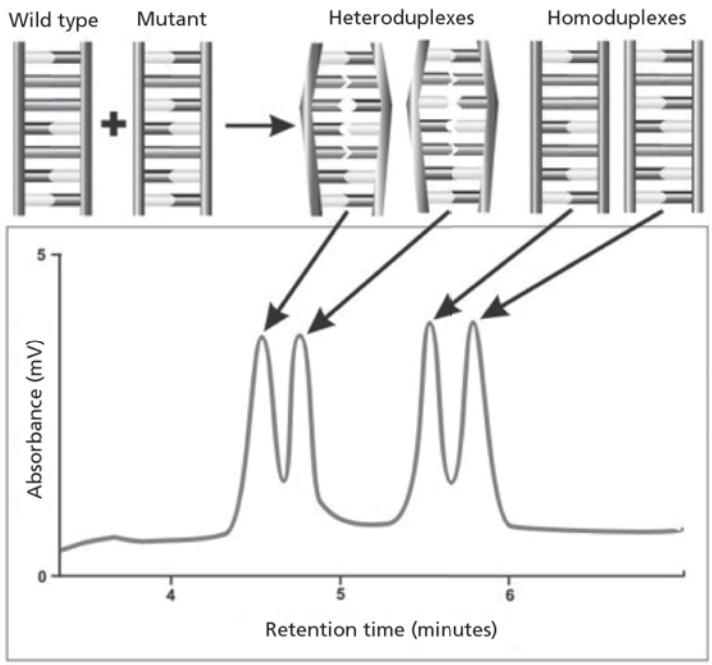


Figure III-1: Chromatogram showing peaks produced by hetero- and homoduplexes eluting off the dHPLC column

DNA passes through a dHPLC column that binds DNA. Separation of heteroduplexes from homoduplexes is accomplished under partially denaturing conditions. Mismatched DNA has a weaker binding to the dHPLC column and elutes off before the homoduplexes elute off. The UV detector detects DNA that passes by and produces corresponding chromatograms ('peaks'): two for the homoduplexes and two for the heteroduplexes (under ideal conditions) (Figure III-1). Sensitivity of dHPLC depends on the accuracy and precision of temperature[43-48]. Correct partial denaturation temperatures differentiate between double-stranded DNA sequence matches and mismatches. Temperature precision and control are key to decreasing false-negatives, and ensuring consistent and accurate dHPLC results. We analysed samples at at least two different temperatures, selected using Transgenomic NavigatorTM software. By analysis at various temperatures, the chance of missing a mutation is diminished to less than 1%[43-48].

Sequencing *ABI Prism 3100 Genetic analyzer (Applied Biosystems)*
Any heteroduplex detected by dHPLC was subsequently sequenced, after cleaning the PCR product with Exo/SAP. Prior to sequencing, a mix is added to the PCR product, which contains Taq DNA polymerase, a primer that can hybridize at the desired location on only one complementary strand of the DNA (as opposed to both strands in PCR), and all four of the nucleotide bases (A, T, C, G). In addition, small amounts of fluorescence labeled dideoxynucleotides (A, T, C, G) are added to the mixture. Dideoxynucleotides are human-made nucleotides whose sugar component is slightly different from that of the nucleotides that make up DNA; there is no OH on the 3' carbon. Dideoxynucleotides can be picked up and added to a growing DNA chain, but, as a result of this structural difference another nucleotide cannot be added at its 3' end. Consequently, if one of the dideoxynucleotides is added to a growing chain of nucleotides, the strand will be terminated. Each dideoxynucleotide is labeled with a different fluorescent compound so that it will give off an identifying color in a laser beam.

After 20-30 cycles of the PCR heating and cooling, the resulting mixture contains a series of fragments of different lengths depending on how many bases had been added to the chain before one of the dideoxynucleotides blocked further growth. The mix of billions of short fragments from the sequencing reactions is loaded into capillary tubes that contain a gel solution that serves as a sieving matrix.

Samples were run on ABI Prism 3100 Genetic analyzer (Applied Biosystems), an example of the sequence results is shown in figure III-2.

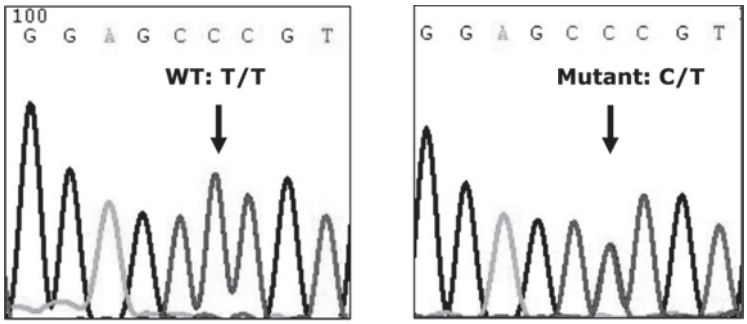
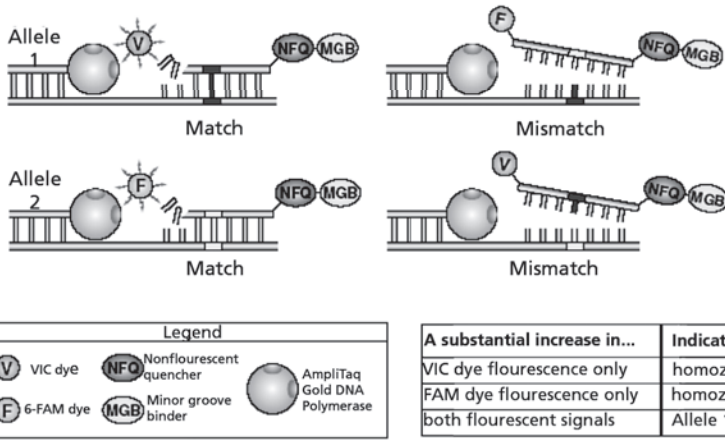


Figure III-2: Example of a sequencing result, showing the heterozygous base change of T to C in the P89L mutation, compared to wild-type (WT) DNA

Taqman *TaqMan® SNP Genotyping Assays, Applied Biosystems*
When a new variant was found in a patient, a healthy control group was genotyped for this variant in order to determine whether it was a mutation (frequency in healthy controls <1%) or a polymorphism (frequency >1%).

TaqMan SNP Genotyping Assay is based on the design of two TaqMan probes, specific for the wild type allele and the mutant allele. Each of the two probes is labelled with a different fluorescent reporter (usually FAM and VIC), and each is designed with the gene mutation affecting the middle part of the probe sequence. Each of the reporters is quenched ('silenced') by TAMRA (6-carboxy-tetramethyl-rhodamine) attached via a linker arm located at the 3' end of each probe. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence.

During PCR, forward and reverse primers hybridize to a specific sequence of the target DNA. The TaqMan probe hybridizes to a target sequence within the PCR product. The AmpliTaq Gold enzyme cleaves the TaqMan probe with its 5´–3´ nuclease activity. The reporter dye and quencher dye are separated upon cleavage (Figure III-3). The 3' end of the TaqMan probe is blocked to prevent extension of the probe during PCR. The separation of the reporter dyes from the quencher dye results in increase in fluorescence for each of the FAM and VIC reporters. Both primer and probe must hybridize to their targets for amplification and cleavage to occur. The fluorescence signals are generated only if the target sequences for the probes are amplified during PCR. The increase in fluorescence is measured, and is a direct consequence of target amplification during PCR.

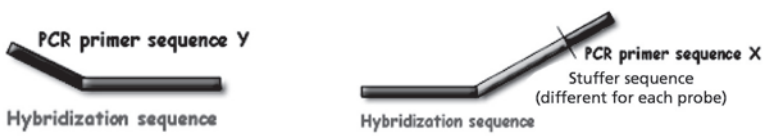


A substantial increase in...	Indicates...
VIC dye flourescence only	homozygosity for Allele 1.
FAM dye flourescence only	homozygosity for Allele 2.
both flourescent signals	Allele 1-Allele 2 heterozygosity.

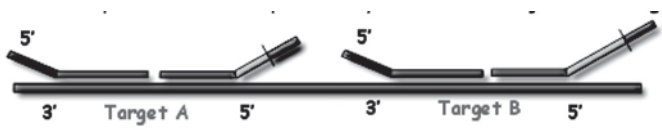
Figure III-3: The principle of Taqman genotyping

MLPA *SALSA P216 GHD kit, MRC-Holland, Amsterdam, the Netherlands*
After screening for mutations, multiplex ligation-dependent probe amplification (MLPA) was performed to screen for intragenic deletions and whole gene deletions. Briefly, ligation products are amplified by PCR by means of 6-FAM labelled universal primer pairs. Amplification products are identified and quantified by capillary electrophoresis on an ABI 3100 genetic analyzer, using GENESCAN software (version 3.7) from Applied Biosystems (FosterCity, CA, USA). A schematic overview of the MLPA technique is shown below.

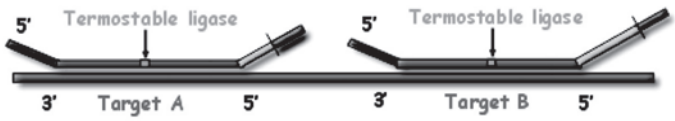
1.The MLPA probemix is added to denatured genomic DNA



2.The two parts of each probe hybridise to adjacent target sequences

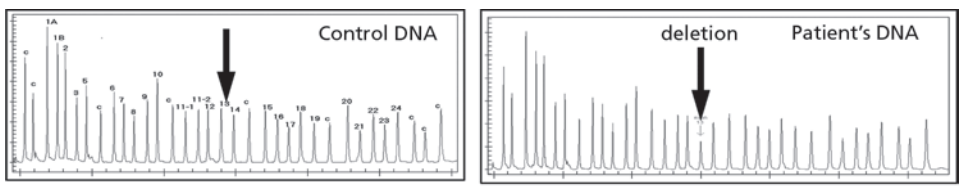


3. Probes are ligated by a thermostable ligase



4. A universal primer pair is used to amplify all ligated probes. The amplification product of each probe has a unique length.

5. Each peak is the amplification product of a specific probe. Samples are compared to a control sample. A difference in relative peak height or peak area indicates a copy number change of the probe target sequence



Appendix IV

Inclusion criteria for IGHD patients participating in the HYPOPIT study

		Score
First degree relative with GHD		3
First degree relative with height SDS <-3		2
Height SDS at start GH treatment	< -3.0	2
	-2.0 to –3.0	1
Arginin test before start or after stop GH treatment	In children < 1 y: < 30 mU/L	2
	In children > 1 y: < 20 mU/L	2
	In adults < 5 mU/L	2
Clonidin test before start or after stop GH treatment	In children < 1 y: < 30 mU/L	2
	In children > 1 y: < 20 mU/L	2
	In adults < 5 mU/L	2
Other GH test before start or after stop GH treatment	In children < 1 y: < 30 mU/L	1
	In children > 1 y: < 20 mU/L	1
	In adults < 5 mU/L	1
IGF-I SDS before start or after stop GH treatment	< -3.0	2
	-2.0 to –3.0	1
IGF-BP3 SDS before start or after stop GH treatment	< -3.0	2
	-2.0 to –3.0	1
Inclusion criteria:	**Severe IGHD (sIGHD):**	6 points (or 5 if patient had missing test results due to diagnose before 1980 or in another country)
	Partial IGHD (pIGHD):	At least one GH test < 30 mU/L and 2 other points
Exclusion criteria:		Normal GH test or both IGF-I SDS and IGF-BP3 SDS > 0

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Chapter 2

Genetic Screening of a Dutch Population with Isolated Growth Hormone Deficiency

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Abstract

Objective: Five to 30% of cases of idiopathic isolated growth hormone deficiency (IGHD) have first-degree relatives with short stature, which is suggestive of a genetic aetiology. The HYPOPIT study aimed to obtain an overall picture of *GH1* and *GHRHR* defects in a Dutch IGHD cohort and to relate them with clinical parameters.

Design, patients and measurements: Genetic analysis was performed of exons and exon-intron boundaries of *GH1* and *GHRHR* in 89 Caucasian IGHD patients from 82 families, by using dHPLC, DNA sequencing and MLPA. In addition, we performed functional studies on novel identified *GH1* exonic variants.

Results: Five different heterozygous *GH1* mutations were present in five out of 82 participating families (6.1%), whereas no mutations in *GHRHR* were found. Patients with IGF-I SDS <-4.0 and peak GH levels <5.7 mU/L had a mutation frequency of 40%, in contrast to 6.8% in patients with only one criterion, and 0.0% in patients with none of these criteria (p=0.00007). Five new *GH1* and two *GHRHR* variants were also identified; two of them (*GH1* F92L and D153H) caused a marked reduction of GH secretion *in vitro*.

Conclusion: *GH1* and *GHRHR* mutations are rare in Caucasian Dutch IGHD patients, which suggests the involvement of other genetic determinants in the aetiology of IGHD. The overall phenotype of patients with *GH1* mutations is more severe than that of patients without *GH1* sequence changes. IGF-I <-4.0 and peak GH levels <5.7 mU/l are strong predictors of *GH1* mutations in the studied population.

Introduction

Growth hormone deficiency is caused by insufficient GH secretion, resulting in a decreased production of GH-dependent growth factors. The incidence of isolated GH deficiency (IGHD) is estimated to vary between 1 in 3,500 to 1 in 10,000 live births[1-5]. Estimates indicate that between 5% and 30% of idiopathic IGHD cases have first-degree relatives with short stature, which is suggestive of a genetic aetiology[6]. The genetic background of IGHD is largely unknown, while mutation frequencies of known involved genes vary substantially among the populations described in the literature; *GH1* and *GHRHR* mutation frequencies reported from different populations vary between 0 and 32%[7-11]. Since there are few data for Dutch patients, the HYPOPIT study was initialised with the aim of obtaining a realistic overall picture of known and new genetic defects and variations in the Dutch IGHD population. The unique database of the Dutch National Registry of Growth Hormone Treatment, in which clinical and laboratory parameters of 2500 GH-treated Dutch patients have been registered, gave us the opportunity to realise the second aim of the study: to relate genetic variants to clinical and laboratory parameters in order to predict gene defects by phenotypic characteristics in future patients.

Patients and Methods

Patients

IGHD patients from the Endocrinology departments of six university- and two non-university Hospitals, who were registered in the Dutch National Registry of Growth Hormone Treatment between 1992 and 2003, were included in the study. IGHD was defined as a peak GH response <20 mU/L to arginine or clonidine test, or <30 mU/L combined with serum IGF-I <–2 SDS and normal serum levels of other pituitary hormones. Patients were classified as having severe IGHD (sIGHD) or partial IGHD (pIGHD) based on a scoring system including height SDS (HSDS), maximum GH levels, IGF-I and IGFBP-3 SDS. GH, IGF-I and IGFBP-3 measurements were centrally performed in one laboratory with published reference values[12]. For the remaining 20%, the laboratory-specific reference values for IGF-I and IGFBP-3 were used to calculate the SDS. Exclusion criteria were as follows: GH deficiency of known cause, such as a brain tumour, brain surgery, brain radiation, known syndromes and non-Caucasian ethnicity. To obtain an unbiased representative Dutch GH-deficient cohort, we applied strict rules such that of each participating centre, either all IGHD patients treated between 1992 and 2003, or none, could participate. Of 317 IGHD patients

who were registered in the Dutch National Registry of Growth Hormone Treatment between 1992 and 2003, 130 patients were excluded because they were treated in 15 different regional hospitals not participating in the study. In addition, we excluded 40 patients who were of self-reported non-Caucasian ethnicity and 34 patients who did not meet the inclusion criteria. Twenty-four patients were excluded because DNA isolation failed. Two patients were already known to have a mutation in *GH1*[13] and were excluded from genetic screening, but included in the statistical analyses to prevent bias. Hence, genetic analysis was performed in the 87 remaining patients. Approval was obtained from the medical ethical committees of all participating hospitals. Informed consent was obtained from all participating patients and their parents or legal guardian, if patients were aged less than 18 years.

Methods

Genomic DNA was extracted from peripheral venous blood samples according to standard procedures. All exons and exon-intron boundaries of *GH1* (Ensembl ID: ENSG00000189162) and *GHRHR* (Ensembl ID: ENSG00000106128) were individually amplified by PCR using specific primers (Table 1). The entire *GH1* intron 3 which is crucial for the splicing of *GH1* mRNA and that harbours several mutations known to cause type II IGHD[14] was also routinely amplified and screened.

GH1 and *GHRHR* mutation screening was performed by denaturing High-Performance Liquid Chromatography (dHPLC) using a Transgenomic Wave 3500HT system (Transgenomic, Omaha, USA). For mutation detection each amplicon was processed at, at least, two different melting temperatures. PCR and dHPLC conditions are shown in Table 1. For detection of homozygous variants, mixes of PCR amplified patient-control samples (1:1) were also analysed. Any detected heteroduplex was subsequently sequenced, using an ABI Prism 3100 Genetic analyzer (Applied Biosystems). The frequency of any newly identified variant was estimated in the normal population by screening 200 chromosomes from healthy Caucasian controls with heights between -2 and +2 SDS by Taqman Genotyping assay. In the case of missense mutations, the degree of conservation of the affected residue was examined (http://www.ensembl.org) as an estimate of its potential pathogenicity. For paternity testing, we used the 16-marker AmpFISTR Identifiler PCR Amplification Kit (Applied Biosystems). The potential functional impact of newly found variants was assessed *in silico* using online tools such as Exonic Splice Enhancer (ESE) finder (http://rulai.cshl.edu/tools/ESE/ESEbkgr.html)[15] and Alternative Splice Site Predictor (http://es.embnet.org/~mwang/evaluation.html)[16], as well as online genomics and proteomic information like http://ensembl.org, http://expasy.org/, http://hapmap.org, and http://www.ncbi.nlm.nih.gov.

Table 1

Gene and exon name	Sense primer 5'-3'	Antisense primer 5'-3'	Ann T° °C	Amplicon (bp)	dHPLC analysis Temp °C
PCR primers					
hGH1 Ex 1 F3R3	ACAGGTGGGGGCAACAGTG	CAGAGGGCAACAGAGGGAG	57.9	387	61.0 / 61.4 / 61.8
hGH1 Ex 2 F2R3	GCCATGTAAGCCCAGTATTTG	TCTGCCTGCATTTTCGCTTCG	57.9	430	59.7 / 61.9 / 62.7
hGH1 Ex 3 F1R1	CGCTGGGAAATAAGAGGAGG	AAGGACGGGCATTGGCTGTG	57.4	355	59.7 / 60.7
hGH1 Ex 4 F1R1	CCGTGAGTGGATGCCTTCTC	GTGAGTTCTCTTGGGTCAGG	57.7	374	62.1 / 62.6 / 64.0
hGH1 Ex 5 F1R2	TGAGAAAGGGAGGGAACAGT	CCCGAATAGACCCCGCAGGC	57.0	474	56.9 / 59.5 / 61.4 / 63.2
GHRHR Ex 1 F2R2	GGGTGCGGTGGAAACGGCTG	AGCAGGAGAAGGGCAGTAGGGTC	61.3	258	62.7 / 63.7 / 64.7
GHRHR Ex 2F1- 3R1	CCACAGAGCCCAGAAAGACACCC	GCCACTTCCAGATGAAAGCACCT	60.4	453	60.0 / 61.0
GHRHR Ex 4 F2R2	GCAGCCCAGCTCACCACTC	TCACTGCTCTCTGCTGGAAAACT	59.4	404	59.7 / 61.2 / 62.4
GHRHR Ex 5 F1R1	AGGCTTCACCTGCTTGATT	GATCTGGGAAGTTCATGGC	54.3	284	59.0 / 60.0
GHRHR Ex 6 F2R2	GAGGCAGGGACCAGATATTC	AGTCCAGGAAAGGCGTGAGG	57.0	553	58.9 / 60.9 / 61.9
GHRHR Ex 7 F1R2	GTAGAGGAGACTGGGATGGG	TGGAGGGGCCTTTATGTAAA	58.2	303	62.3 / 63.3 / 63.8
GHRHR Ex 8 F1R1	GATCTCAGAGTCAAGGATGC	CTGTCCACTCCACACCCCAT	55.2	179	63.0 / 64.0
GHRHR Ex 9 F2R2	CCTTTGGGTGAGACCTTAACTGG	TCACAGAGAATCAGTGGCAGAGC	59.1	367	60.0 / 61.6 / 62.2
GHRHR Ex 10 F2R2	CCTGCACATTCTCACGTCTCAAG	ATCCCTCTCCCCAGGCTCCCATA	57.8	475	58.1 / 59.1 / 60.1
GHRHR Ex 11 F2R2	TGTGGGGAGGTGGCGTTTC	AGCACCCTCAATGGAAAGA	55.9	310	60.1 / 60.6 / 61.5
GHRHR Ex 12 F4R4	TAAATTCCCCTTTCAATCA	CCACCTCCTGAAGAGTAGC	54.8	422	58.6 / 59.6 / 60.6
GHRHR Ex 13 F3R3	CTGCACCTTAGTCTCATTGG	AGGAATGATGAACGCAACTC	56.5	584	58.9 / 60.5 / 62.7
Mutagenesis primers (5'-3')					
F92L Forward[1]	CTGGAGCCCGTGCAGCTCCTCAGGAGTGTC				
F92L Reverse[1]	GACACTCCTGAGGAGCTGCACGGGCTCCAG				
D153H Forward[1]	GACACAAACTCACACAACCATGACGCACTACTCAAGAAC				
D153H Reverse[1]	GTTCTTGAGTAGTGCGTCATGGTTGTGTGAGTTTGTGTC				

[1] underscore indicates base change

GH1 and *GHRHR* intragenic and whole gene deletion / duplication analysis was performed by Multiplex Ligation-Dependent Probe Amplification (MLPA) using the SALSA P216 GHD kit provided by MRC-Holland (http://www.mlpa.com), according to the provider's protocol. Briefly, the ligation products were amplified by PCR by means of 6-FAM labelled universal primer pairs. Amplification products were identified and quantified by capillary electrophoresis on an ABI 3100 genetic analyzer, using GENESCAN software (version 3.7) from Applied Biosystems (FosterCity, CA, USA). The peak area of the PCR products was determined by GeneMarker software V1.51, which normalizes peak intensities and compares them with those of healthy controls included in the analysis. In order to correct for the peak intensity variation over size, an exponential function $a*e^{-bz}$ was used to fit to the square root of peak intensities, where z is size and a and b are fitting constants.

Functional analysis of new variants

A human *GH1* cDNA clone was obtained from RZPD (Deutsches Ressourcenzentrum fur Genomforschung GmbH, Berlin, Germany) and subcloned into the mammalian expression vector pSG5 (Stratagene). The newly identified *GH1* variants F92L and D153H (Table 4) were introduced into the cDNA using the QuickChange Site-Directed Mutagenesis protocol (Stratagene) and confirmed by sequencing. The oligonucleotides used for site-directed mutagenesis are shown in Table 1. The different mutant *GH1* cDNAs were expressed in COS1 cells after FuGENE-mediated plasmid DNA transfection according to the manufacturer's protocol (Roche). To study the effects of the new variants in homozygosis, we transfected 500 ng of the mutant plasmid and compared the GH secretion of the various mutant cells with that of cells transfected with wild-type (wt), and of those transfected with the empty vector. To study the effects in heterozygosis, we transfected 250 ng of the mutant combined with 250 ng wild-type. To normalise the transfection efficiency, cells were transfected in duplicate under identical reaction conditions. In co-transfection experiments, cells were also transfected with empty vector to keep the total amount of DNA constant in a given condition. After 16, 21, 40 and 46 hours, an aliquot of the medium was harvested and after 46 hours the cells were harvested. GH concentration in COS1 cells and medium was determined using a nonisotopic, automatic chemiluminescence immunoassay system (Immulite; Diagnostic Products Corp., Los Angeles, CA) using rabbit polyclonal antibodies with an analytical sensitivity of 0.01 µg/liter. The intra- and interassay coefficients of variation (CV) of this assay are 6.0 and 5.7%, respectively.

Statistical analysis

The genetic and clinical data of the 89 patients were analysed using SPSS 11.0®. The independent T-test was used to compare phenotypic data between patients with and without mutations in *GH1* and/or *GHRHR*. Logistic regression analysis was used to assess whether clinical parameters could predict mutation risk and ROC curves to find 'cut off' values for laboratory parameters, which distinguish children with high mutation risk from children with low mutation risk. Data from one patient younger than one year were excluded from the correlation between peak GH levels (mean of arginine and clonidine tests) and mutation risk assessment since different reference values apply to patients at that age.

Results

The 89 participating patients (29 females and 60 males) originated from 82 non-related Dutch families of Caucasian ethnicity, of which six had two affected children and one family had three affected children. In one family, one child, the mother and the mother's uncle were affected. Consanguinity was anamnestically excluded in all of the participating families. The clinical and laboratory parameters of the 89 IGHD patients are summarised in Table 2.

An IVS3+1G>C splice site mutation in *GH1* was previously detected in heterozygosis in one family with two affected children[13]. Two *GH1* splice site mutations (IVS3+1G>A, IVS3+1G>T) and two missense mutations, c.344C>T (P89L) and c.626G>A (R183H) (all of which have been previously described[8,17]) were identified in heterozygosis in six patients with sIGHD. The heterozygous IVS3+1G>A mutation was found in a patient whose parents and two siblings were of normal height and did not have the mutation. Paternity was confirmed by appropriate testing, indicating that it was a *de novo* mutation.

The phenotypic data and growth charts of the patients with established *GH1* mutations are summarised in Table 3 and Figure 1, respectively. Logistic regression showed that, independently of height, the chance of detecting a *GH1* mutation was significantly related to IGF-I SDS (p=0.022) and mean peak GH levels during arginine and clonidine tests (p=0.019). IGF-I SDS and peak GH levels ROC curves values were 0.81 (p=0.007) and 0.83 (p=0.004), respectively, indicating that IGF-I SDS and peak GH levels are good predictors for detecting a *GH1* mutation. Best sensitivity (Sn) / specificity (Sp) combination was found using cut-off values of IGF-I SDS <–4.0 (Sn 72% and Sp 86%) and peak GH levels <5.7 mU/L (Sn 72% and Sp 85%). When height was not taken into account, children with an IGF-I SDS <-4.0 and peak GH levels <5.7

mU/L (11% of the population) had a *GH1* mutation frequency of 40%, in contrast to 6.8% in children with only one criterion, and 0.0% in children with none of these criteria (p=0.00007). No copy number variation was detected by MLPA analysis in any of the examined patients DNA samples.

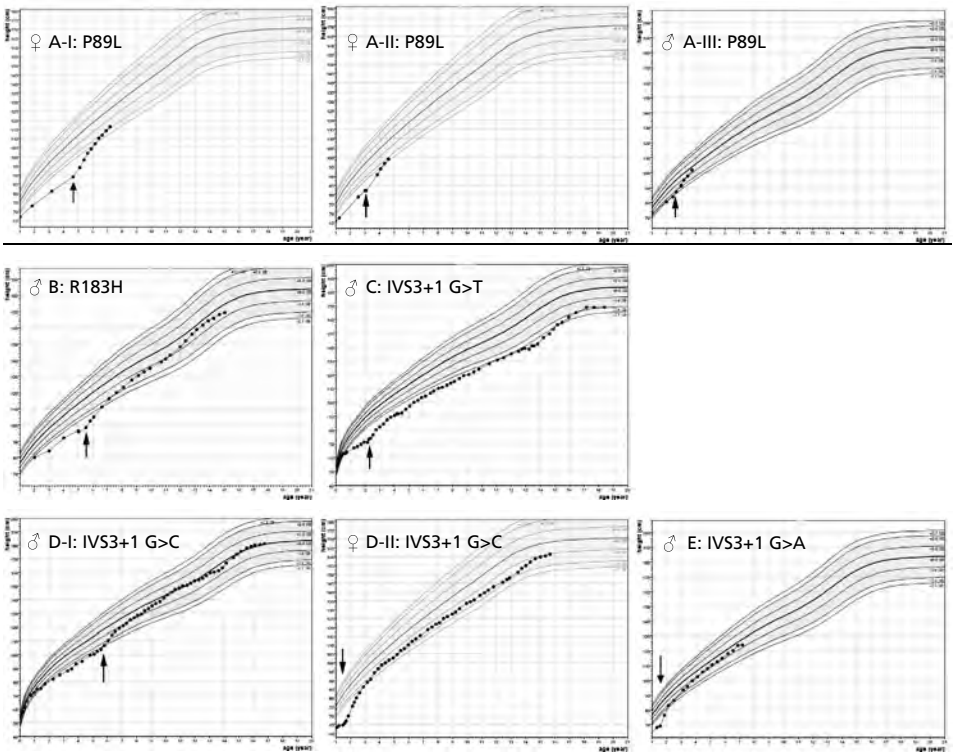


Figure 1: Growth charts of patients with *GH1* mutations. Patients are numbered according to Table 3. Arrows indicate start of GH treatment.

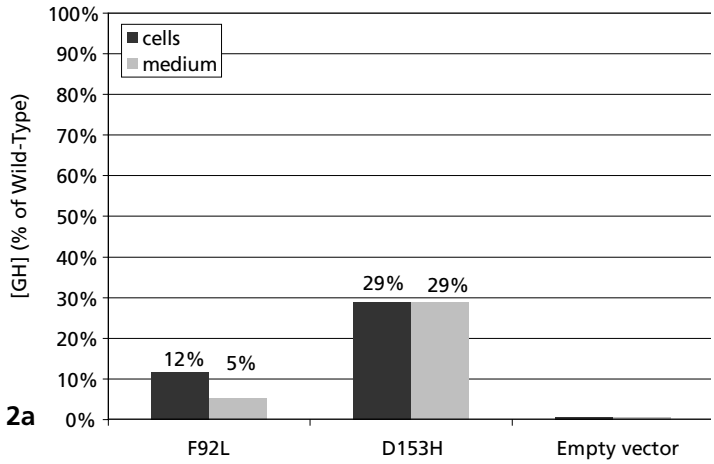
Apart from the previously described mutations, we found five new sequence variants in *GH1* and two in *GHRHR* of which, to our knowledge, the clinical significance has not been studied previously. Characteristics of these variants and clinical data of the patients carrying these new variants are shown in Table 4. To assess the potential functional impact of the new *GH1* variants, COS1 cells were transfected with expression vectors encoding wild-type (wt) *GH1* and / or the novel exonic *GH1* variants. There was a marked reduction in GH concentration in cells (Figure 2a) and in medium (Figure 2b) of cells transfected with F92L or D153H. When wt *GH1* and mutant *GH1* were cotransfected, mimicking a heterozygous state, GH concentrations

in medium were below or equal to that observed in cells cotransfected with the wt/empty vector construct (data not shown). This indicates that the mutant alleles were not contributing to GH secretion. In medium of cells cotransfected with the wt/D153H construct, GH concentrations were lower than in medium of cells cotransfected with the wt/empty vector construct. However, GH concentrations inside the wt/D153H cells, although low, were higher than those inside the wt/empty vector cells. Low GH concentrations inside the cells, combined with even lower GH levels secreted in medium, indicate that D153H may not only reduce the production of GH, but also the secretion of wt GH out of the cell.

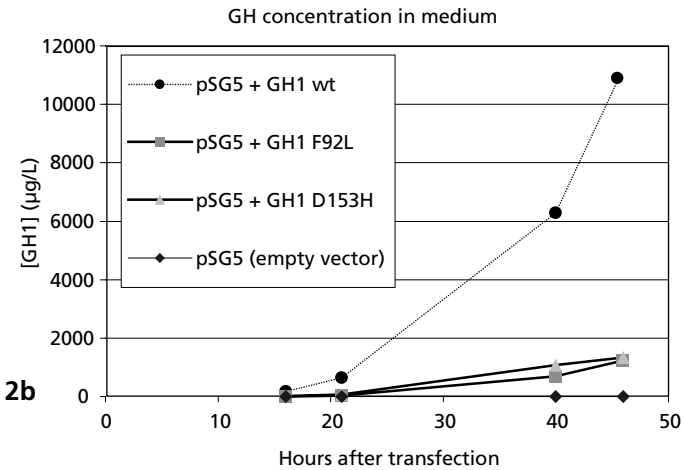
Table 2: Differences in clinical and laboratory parameters between patients with and without mutations in *GH1*.

	***GH1* Mutation** **n=8**	**No *GH1* mutation** **n=81**	***p***
Gender (M/F)	5 / 3	55 / 26	
BW SDS	-0.37 (0.8)	-0.28 (1.3)	NS
BL SDS	-0.2 (1.1)	-1.0 (1.4)	NS
Gestational age	39.1 (1.1)	38.8 (2.7)	NS
HSDS at start of GH	-4.3 (1.1)	-3.2 (0.8)	0.001
GH peak during arginine test (mU/L)	3.9 (2.3)	10.5 (6.4)	0.015
GH peak during clonidine test (mU/L)	4.9 (2.2)	11.5 (5.6)	0.048
IGF-I SDS	-5.5 (1.8)	-3.2 (2.3)	0.013
IGFBP-3 SDS	-5.4 (3.5)	-4.3 (5.4)	NS
MRI abnormalities	5 patients: normal MRI, 3 patients: MRI NA	27 pts: normal MRI, 36 pts: abnormal MRI, of which 2 triad[1]	0.048
FDR with GHD	75%	14%	0.001
Micropenis	60%	11%	0.021
Neonatal jaundice	23%	17%	NS
Hypoglycaemia	29%	11%	NS

Data are expressed as mean (SD), BW= birth weight, BL= birth length, [1]triad= hypoplastic anterior pituitary, ectopic posterior pituitary and interrupted/ invisible stalk, FDR = first-degree relatives, NA = not available



2a



2b

Figure 2: a) GH concentration as percentage of wild-type, in COS1 cells and medium 46 h after transfection with new *GH1* exonic variants F92L and D153H. [GH] = GH concentration **b)** GH levels in cell culture medium measured after 16, 21, 40 and 46 hours of transfection with F92L and D153H *GH1*.

Wt = wild-type, [GH] = GH concentration, pSG5 = expression vector used for transfection of wild-type and mutant *GH1* cDNAs into COS1 cells.

Table 3: Clinical data of families with mutations in *GH1*

Pt	Mutation *GH1*	Height F cm (SDS)	Height M cm (SDS)	FDR with GHD	Age start GH	HSDS start GH	CA-BA (y)	GH (mU/L)	IGF-I SDS	IGFBP-3 SDS	MRI	BW (kg)	BL (cm)	Physical exam	NS
AI	**P89L**	175 (-1.0)	147* (-3.5)	M and 2 S	4.9	-4.7	1.9	5.1 a 3.3 c	-9.0	-	Normal	2.7	48	IAF, FB	NJ
AII	**P89L**	175 (-1.0)	147* (-3.5)	M and 2 S	3.3	-4.3	0.3	7.4 a 10.1g	-6.1	-2.0	-	2.7	-	IAF, FB	-
AIII	**P89L**	175 (-1.0)	147* (-3.5)	M and 2 S	2.4	-2.7	0.9	5.6 a 0.9 g	-4.7	-4.7	-	2.8	-	-	-
B	**R183H**	178 (-0.6)	157* (-1.9)	1 normal S, M no symptoms of GHD	5.5	-3.9	-	7.0 a 4.0 c	-6.0	-9.0	Normal	3.7	52	IAF, FB, GHDa	-
C	**IVS3+1 G>T**	142* (–5.5)	166 (-0.5)	F and uncle	2.3	-5.4	-	2.4 a 1.0 a	-2.2	-	-	3.5	51	IAF	-
D1	**IVS3+1 G>C**	163 (-2.7)	152* (-2.8)	M and S	5.6	-2.8	0.5	1.6 a 0.5 g	-3.4	-	Normal	3.6	53	GHDa	-
D2	**IVS3+1 G>C**	163 (-2.7)	152* (-2.8)	M and S	1.7	-5.8	-	1.6 a 0.5 l	-5.5	-	Normal	3.1	50	GHDa	-
E1	**IVS3+1 G>A *de novo***	183 (+0.1)	170 (0)	2 normal S	1.6	-4.8	1.0	0.97g	-4.1	-5.1	Normal	3.1	-	IAF, FB	HG

F=father, M=mother, S=Sibling, FDR = first-degree relatives, BW= birth weight, BL= birth length, NS= neonatal signs, NJ=neonatal jaundice, HG=hypoglycaemias, IAF=increased abdominal fat, FB= frontal bossing, GHDa= GHD appearance, CA-BA= bone age delay at start GH, GH tests: a=arginine test, c= clonidine test, g=glucagon test, l= L-dopa-propranolol test, - = not available *parent with same mutation.

Table 4: New *GH1* and *GHRHR* sequence variants found in the HYPOPIT study

	Variant	MAF in healthy controls	Present in healthy FDR	Height F cm (SDS)	Height M cm (SDS)	Age start GH (y)	HSDS start GH	GH (mU/L)	IGF-I SDS	Sex
GH1	**c352 T>C (F92L)**	0%	+	195* (1.7)	171 (0.2)	10.0	-3.2	7.2	-5.8	female
	c535 G>C (D153H)	0%	- [1]	171 (-1.6)	166 (-0.6)	16.0	-1.7	11.2	-1.5	female
	c294 C>T (N72N)	0%	+	178* (-0.6)	172 (0.4)	4.9	-2.9	20.5	-0.6	male
	c597 C>G (V173V)	0%	- [1]	178 (-0.6)	157 (-2.0)	8.1	-3.1	13	-1.5	male
	IVS1-70 C>T	0%	+	174 (-1.2)	167* (-0.4)	3.3	-3.5	21.0	-6.5	female
GHRHR	**c53C>T (P-5L)**	0%	+	178 (-0.6)	170* (0.3)	2.1	-3.6	5.3	-3.4	male
	IVS7+12 G>A	2%		175 (-1.0)	158 (-1.8)	11.1	-3.5	11.0	-1.9	female
	IVS7+12 G>A	2%		183 (0.1)	161 (-1.4)	3.9	-3.7	5.1	-3.5	male

MAF = minor allele frequency, F = father, M = mother, [1] father was not available for analysis, FDR = first-degree relatives, * = parent with same variant

Discussion

The HYPOPIT study was performed to investigate the frequency of *GH1* and *GHRHR* mutations in the Dutch IGHD population, as well as to relate gene defects with clinical and laboratory parameters. For this purpose, we screened a representative, unbiased Dutch IGHD cohort of 82 participating families with 89 affected Dutch patients of Caucasian ethnicity.

Five different *GH1* mutations, which were formerly reported to cause IGHD (P89L, R183H, IVS3+1 G>T, IVS3+1 G>C and IVS3+1 G>A) were identified in eight of the 89 participating patients. This brings the *GH1* mutation frequency in our Dutch IGHD cohort to 9.0% of the patients and 6.1% of the participating families. No mutations in *GHRHR* were found. These results are in accordance with *GH1* and *GHRHR* mutation frequencies reported from other populations (0-32%)[7-11]. Although eight families had more than one affected member, *GH1* mutations were found in only three of these families, thus suggesting the existence of additional genetic determinants of IGHD, other than mutations in *GH1* and *GHRHR*. We are convinced that this low *GH1* and *GHRHR* mutation frequency is realistic, since the probability of false negative results was rendered highly unlikely by the high sensitivity and reliability of the screening methods used. dHPLC is highly sensitive (96-100%[18-23]) for the detection of point mutations and small indels, and MLPA is a highly sensitive quantitative technique for the detection of copy number variation (deletions / duplications) in genomic DNA samples.

When we compared the phenotypic characteristics of IGHD patients with and without *GH1* mutations, we found that the patients with the heterozygous P89L, R183H and IVS3+1 G>A/C/T mutations had significantly lower mean IGF-I SDS and lower peak GH levels. In addition, their probability of having a micropenis or first-degree relatives with GHD was significantly higher. IGF-I SDS and peak GH levels were shown to be good predictors for mutation risk. Independently of height, children with IGF-I SDS <-4.0 and peak GH levels <5.7 mU/L had a *GH1* mutation frequency of 40%, in contrast to 6.8% in children with only one criterion, and 0.0% in children with none of these criteria (p=0.00007). This indicates that the clinical phenotype of patients with *GH1* mutations was, in general, more severe than in patients without mutations.

Nevertheless, even within the group of patients with *GH1* mutations there was phenotypic heterogeneity, with some patients who had a remarkably 'mild' growth retardation, in particular patients A-II and A-III (P89L), patient B (R183H) and patient D-I (IVS3+1G>C, Fig. 1). Binder *et al* (2001) previously suggested that children with (heterozygous) splice site mutations in *GH1* might be more severely affected than

children with (heterozygous) missense mutations[24]. Salemi *et al* (2005) reported that patients with the P89L mutation were older at diagnosis of GHD when compared to patients with splice site mutations[25]. The rather mild growth retardation shown by patient D-I, however, suggests that patients with splice site mutations may also present with milder phenotypes.

The phenotypic variability was also evident among members of the same family carrying the same mutation, as illustrated by patient B (R183H) and relatives. When the mutation was identified in the patient, he had reached adult age and had just stopped GH treatment. When his GH secretion was retested, in contrast to the very low response shown during childhood (Table 3), his arginine stimulated GH secretion peak was normal (31 mU/L) and his serum IGF-I level subnormal (162 ng/ml). His mother, carrier of the same R183H mutation, with a low-normal height (157 cm, HSDS –1.9) and a low-normal IGF-I level (130 ng/ml), had never been tested nor treated for GHD.

The presence of further hormonal deficiencies in addition to GH deficiency, as was previously described in patients with P89L and IVS3+1 G > C/T mutations[14], was excluded in the patients with *GH1* mutations in our cohort.

Whereas previous publications report that patients with *GH1* mutations might have pituitary hypoplasia on MRI[14], the patients with mutations identified in this study had a normal pituitary.

Apart from the five known *GH1* mutations, five *GH1* and two *GHRHR* variants of yet unknown clinical importance were identified. Among the five *GH1* variants, two were heterozygous non-synonymous exonic variants causing a change in the encoded amino acid (F92L, D153H), another two were heterozygous synonymous exonic variants, and the remaining one was a heterozygous intronic variant. Estimation of the F92L and D153H allelic frequency in the normal population showed that both were absent in 200 chromosomes from healthy Dutch controls. F92L has been previously described in heterozygosis in a patient with a height below the 3rd percentile and reduced GH and IGF-I levels[26]. In our study, F92L was found in heterozygosis in a patient with sIGHD, whereas D153H was found in heterozygosis in a patient with pIGHD. F92 is located at the C-terminal end of the second GH alpha helix, and the affected base (c.352T>C) is located in a predicted splice enhancer. D153H (c.535C>G) is located near the N-terminal end of helix 4, which suggests that it may be important for the folding of the protein. Conservation analysis revealed that both variants were located in a genomic region that is conserved in mouse, dog and cow. This high degree of conservation underlines the evolutionary importance of this region and suggests that sequence alterations could be of functional importance. Therefore, the functional impact of both variants was estimated by the

assessment of GH levels in COS1 cells transfected with wild-type and / or the mutant *GH1* cDNAs. Cells and medium of cells transfected with F92L or D153H *GH1* showed a marked reduction in GH concentration, indicating that the mutant alleles were not contributing to GH secretion. For D153H, low GH concentrations in the cells, combined with even lower GH levels in medium, indicate that D153H may not only reduce the production of GH, but also the secretion of wt GH into the medium. These results are suggestive of potential clinical relevance of the F92L and D153H variants. However, segregation analysis in the affected families identified non-affected F92L carriers among the family relatives (the father of the F92L proband was a 195 cm tall carrier) which suggests that F92L penetrance may be variable, or, alternatively, that F92L does not necessarily cause IGHD, but may possibly act in combination with yet unknown additional genetic or environmental factors.

The functional significance of the two novel synonymous *GH1* exonic variants was also assessed *in silico*. The heterozygous c.294C>T base substitution in exon 4 (AAC>AAT; N72N) was found in a patient with pIGHD. Although no amino acid was altered, base substitutions near the start of an exon may lead to aberrant splicing with partial or complete exon skipping, as formerly shown for the fifth base of exon 3 (c.176A>G) of *GH1*, which leads to (partial) loss of the codons for amino acids 32–71 and causes IGHD[27]. Theoretically, the c.294C>T base change might cause aberrant splicing by the same mechanism. The variant was absent in 100 healthy Dutch controls and conservation analysis revealed that it is located in a genomic region that is highly conserved in mouse, dog, cow, chimp and macaque.

The second novel synonymous variant c.597C>G (V173V) was found in heterozygosis in a patient with sIGHD. This base change is located directly adjacent to a cryptic splice donor as predicted by Alternative Splice Site Predictor[16]. Mutations that activate cryptic splice donor / acceptor sites can alter splicing of mRNA and thereby change the resulting protein. The variant was absent in 100 healthy Dutch controls and located in a highly conserved genomic region.

Finally, *in silico* analysis of the novel intronic *GH1* variant IVS1-70 C>T, present in a patient with pIGHD, revealed that it is located in a predicted splice enhancer site according to Exonic Splice Enhancer (ESE) finder[15]. Mutations that inactivate splice enhancers, may change the resulting protein by altering splicing of mRNA. IVS1-70 C>T was not detected in the 100 healthy controls, but was also detected in non-affected relatives, for which reason we believe that its functional importance is relatively small.

In addition to the five *GH1* variants identified, two novel *GHRHR* variants were found in heterozygosis. The first one, present in a patient with sIGHD, was a missense mutation c.101C>T, which causes a P18L change in the signal peptide (P-5L). The

genomic region, in which it was located, is highly conserved in larger mammals (chimp, macaque, cow, pig and sheep). Though the functional consequence of this variant has not been investigated yet, previous work by Millar *et al.*[28] showed that a missense mutation in the GH signal peptide of a patient with sIGHD impaired the ability of the signal peptide to direct GH to the Endoplasmatic Reticulum for post-translational processing, hence inhibiting its secretion. P18L was not detected in 100 healthy Dutch controls but was also present in healthy relatives. The second new *GHRHR* variant was an intronic variant (IVS7+12 G>A), located in a predicted splice enhancer. This variant was also present in healthy controls and was therefore believed to be a new polymorphism, rather than a causal variant for IGHD.

In conclusion, in the Dutch IGHD cohort *GH1* mutations are present in 6.1% of the examined families, whereas no mutations in *GHRHR* were detected. This suggests the existence of additional genetic determinants of IGHD, other than mutations in *GH1* or *GHRHR*. Although patients with *GH1* mutations presented with phenotypic variability, the overall phenotype of patients with mutations was more severe than the phenotype of patients in whom no *GH1* mutations were detected. Independently of height, children with IGF-I SDS <-4.0 and peak GH levels <5.7 mU/L, had a *GH1* mutation frequency of 40%. In addition, five novel *GH1* variants were found, of which two (F92L and D153H) were associated with a severely reduced GH secretion *in vitro*, indicating that they might be functionally relevant.

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Chapter 3

PROP1, HESX1, POU1F1, LHX3 and *LHX4* mutation and deletion screening and *GH1* P89L and IVS3+1 /+2 mutation screening in a Dutch nation-wide cohort of patients with combined pituitary hormone deficiency

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Abstract

Objective: The genetic background of combined pituitary hormone deficiency (CPHD) is largely unknown, while mutation frequencies of known involved genes vary substantially between populations. The HYPOPIT study aims to obtain an overall picture of known and new genetic defects and variations in a nation-wide cohort of Dutch CPHD patients.

Design, patients and measurements: We screened 79 CPHD patients from 78 families for mutations and deletions in exons and exon-intron boundaries of *PROP1, HESX1, POU1F1, LHX3,* and *LHX4*, by using MLPA, dHPLC and DNA sequencing. Since *GH1* P89L and IVS3+1 /+2 splice site mutations have recently been shown to cause pituitary hormone impairment in addition to GH deficiency, we also screened for these mutations.

Results: We did not find any mutation or deletion in *PROP1, HESX1, LHX3* or *LHX4*. *GH1* P89L and *GH1* IVS3+1 /+2 mutations were also absent. One patient in our cohort was formerly known to have a *POU1F1* mutation (R271W). We found three new missense mutations in *LHX3* and *POU1F1*, which were considered rare non-causal variants since they were also present in non-affected family members or healthy controls with a frequency below 1%.

Conclusion: Thorough screening for mutations and deletions in *PROP1, HESX1, POU1F1, LHX3, LHX4,* as well as screening for *GH1* P89L or *GH1* IVS3+1 /+2 mutations, did not reveal any genetic defect in our CPHD cohort, apart from one formerly known *POU1F1* mutation in one patient. Future research should focus on alternative explanations for CPHD, like other genes or environmental factors.

Introduction

The anterior pituitary is the central regulator of peripheral hormones. It contains somatotropes, lactotropes, gonadotropes, thyrotropes and corticotropes, cells producing GH, PRL, LH/FSH, TSH and ACTH, respectively. A number of transcription factors and signaling molecules are essential for normal pituitary morphogenesis and differentiation of the five hormone-producing cell types of the anterior pituitary (reviewed by Zhu 2007[1]). Defects in these transcription factors are known to cause various grades of hypopituitarism[2-8] (Table 1).

Transcription factors bind to short DNA sequences that are often located in the promoter region of the gene, upstream of the coding sequence, in order to guide and activate the polymerase to start DNA transcription. To recognize and bind to these DNA sequences, the transcription factor must often partly penetrate into the major and/or minor grooves of the DNA double helix, where their surface is complementary to the surface features of the DNA.

Transcription factors usually have two or more distinct regions with different functions. The first domain is the DNA-binding domain, which establishes binding of the transcription factor to its target DNA by making the above mentioned major groove contact. The DNA-binding domains are either found alone or together with another module. This other module can be a transcriptional activation domain as in PROP1 and POU1F1, a transcriptional repression domain as in HESX1, and a LIM homeodomain (named after its initial discovery in Lin11, Isl-1 and Mec-3 proteins) as in LHX3 and LHX4. Mutations causing defects in these functional domains can cause combined pituitary hormone deficiency (CPHD). Functional characteristics and the different domains of the transcription factors involved in CPHD are summarised in Table 1.

The genetic background of CPHD is largely unknown, while mutation frequencies of known involved genes vary substantially between populations. Therefore, the HYPOPIT study was initialised with the aim to obtain an overall picture of known and new genetic defects and variations in a nation-wide cohort of Dutch CPHD patients.

Recently, Mullis *et al.* reported pituitary hormone impairment in addition to GH deficiency in patients with P89L and IVS3+1 /+2 splice site mutations in *GH1,* which might be explained by a mechanism of bystander damage from activated macrophages clearing dying somatotrope debris[9]. Therefore, in addition to *PROP1, HESX1, POU1F1, LHX3* and *LHX4,* we screened for these *GH1* mutations.

Table 1: Summary of functional characteristics of the pituitary transcription factors studied in the HYPOPIT study

TF	Family	Length (AA)	Domains	Activity	Role in pituitary organogenesis	Associated hormone deficiencies
PROP1	Paired-like Homeo-domain	223	- Paired like homeodomain (60 AA) - C-terminal transactivation domain	- DNA binding - transcriptional activation	Necessary for *POU1F1* expression, thus for POU1F1 dependent proliferation and differentiation of pituitary gonadotropes, somatotropes, lactotropes and caudomedial thyrotropes. PROP1 suppresses *HESX1* expression. Premature expression of *PROP1* can block pituitary organogenesis	GH, PRL, TSH, LH/FSH, and ACTH
HESX1	Paired-like homeo-domain	185	- Paired like homeodomain (60 AA) - N-terminal repression domain Eh1 (36 AA)	- DNA binding - Transcriptional repression of *PROP1*	*HESX1* and *PROP1* have temporally overlapping expression patterns and heterodimerize on the same sequence element. HESX1 together with Gro/TLE corepressor proteins suppresses PROP1 dependent proliferation and differentation of pituitary cell lines. Prolonged expression of *HESX1* with the obligate co-repressors can block PROP1 dependent activation.	GH, PRL, TSH , LH/FSH and ACTH
POU1F1	POU	291	- POU domain consisting of * POU homeo (60 AA) * POU specific domain (75 AA) - Transactivation domain	- DNA binding - Transcriptional activation	Regulation of *GHRHR, GH, PRL, TSH*-β expression. Development and proliferation of somatotropes, lactotropes and caudomedial thyrotropes	GH, PRL, TSH
LHX3	LIM homeo-domain	LHX3a: 400 LHX3b: 402	- homeodomain (60 AA) - 2 LIM domains (51 and 55 AA) *Both isoforms have different amino-terminal sequences preceding the LIM motifs*	- DNA binding - Protein-protein interaction	Transcription of α-*GSU, PRL, TSH*-β, *POU1F1*. LHX3a can transactivate α-*GSU* more effectively than LHX3b. LHX3a synergizes with POU1F1, inducing transcription of *TSH*-β, whereas LHX3b does not. The differences in gene activation properties suggest different roles during pituitary development.	GH, PRL, TSH, LH/FSH
LHX4	LIM Homeo-domain	390	- Homeo-domain (60 AA) - 2 LIM domains (51 and 55 AA) *LHX4 is highly homologous to LHX3, except in the N-terminal region.*	- DNA binding - Protein-protein interaction	Cell survival in the pituitary and timely activation of LHX3. Probable regulation of *POU1F1* expression.	GH, PRL,TSH , LH/FSH and ACTH

Patients and methods

Patients

The Dutch HYPOPIT study is a multi-center study in which six of eight Dutch university hospitals and two regional hospitals participated. We included all GH-treated children and adults registered in the Dutch National Registry of Growth Hormone Treatment between 1992 and 2003, who were treated in the hospitals participating in the study and who had deficiencies of GH and one or more additional hormonal axes. Deficiencies of hypothalamic-pituitary-thyroidal, -adrenal, and -gonadal axes were defined as abnormal TRH test or TSH levels that were low or inadequate for low (F)T4; abnormal CRF / ACTH / glucagon test or ACTH levels which were low or inadequate for low cortisol and LH, FSH, estrogen / testosterone or LHRH test low for age or lack of spontaneous puberty after age 14 y. Prolactin deficiency was defined as abnormal prolactin during random or TRH testing. Reference values of the individual hospitals were used.

Patients with CPHD of known cause, such as a brain tumour, brain surgery, brain radiation and known syndromes, were excluded. To obtain an unbiased representative CPHD population, we applied strict rules: of each participating center, either all CPHD patients treated between 1992 and 2003 or none, could participate in the DNA analysis. Of the 106 CPHD patients who met the inclusion criteria and agreed to participate in the study, twenty-eight patients were excluded because DNA isolation failed. One patient, who was diagnosed with a R271W mutation in *POU1F1* between 1992 and 2003[10], was excluded from further DNA analysis but included in the statistical analysis. We performed mutation screening in the 78 remaining patients. Approval was obtained from the Medical Ethics Committees of all participating hospitals. Informed consent was obtained from all participating patients and their parents, if patients were less than 18 years of age.

Methods

Genomic DNA was extracted from samples of peripheral venous blood according to standard procedures. All coding exons and intron-exon boundaries of *PROP1, HESX1, POU1F1, LHX3* and *LHX4* were individually amplified by PCR. *GH1* exon 3 and exon 4 including intron-exon boundaries, were individually amplified by PCR in order to screen for IVS3 +1/ +2 splice site mutations and the P89L mutation, respectively. Mutation screening of *PROP1, HESX1, POU1F1, LHX3, LHX4* and exon 3 and 4 of *GH1* was performed using the Transgenomic Wave 3500HT Denaturing High-Performance Liquid Chromatography (dHPLC) system (Transgenomic, Omaha, USA). Each amplicon was processed through the WAVE system for mutation detection at,

at least, two different melting temperatures (PCR and dHPLC conditions are available on request). For detection of homozygous variants, mixes of PCR amplified patient-control samples (1:1) were also analysed for *PROP1, HESX1, POU1F1, LHX3* and *LHX4*. Any detected heteroduplex was subsequently sequenced, using an ABI Prism 3100 Genetic analyzer (Applied Biosystems). When a new variant was identified, 150 healthy Dutch controls were screened by Taqman genotyping assays in order to estimate its frequency among the normal population. In case of missense mutations, the degree of conservation of the affected residue was examined (at http://www.ensembl.org) as an estimate of its potential pathogenicity.

Gene nucleotide sequences obtained from Ensembl (*PROP1*: ENSG00000175325; *HESX1* ENSG00000163666; *POU1F1* ENSG00000064835; *LHX3* ENSG00000107187; *LHX4* ENSG00000121454; *GH1* ENSG00000189162) were used. The *LHX3* gene produces two major mRNAs known as LHX3a and LHX3b[11]. We used LHX3 AA numbering according to transcript ENST00000371746.

After screening for mutations in *PROP1, HESX1, POU1F1, LHX3* and *LHX4*, multiplex ligation-dependent probe amplification (MLPA) was performed to screen for intragenic deletions and whole gene deletions. We used a specifically designed set of probes for GHD, including *PROP1* (all exons)*, HESX1* (all exons)*, POU1F1* (all exons except exon 5)*, LHX3* (all exons) and *LHX4* (all exons). The SALSA P216 GHD kit containing these probes was provided by MRC-Holland, Amsterdam, the Netherlands (http://www.mlpa.com). MLPA was carried out in all patients according to the provider's protocol. The ligation products were amplified by PCR by using the common primer set with the 6-FAM label distributed by the supplier. Amplification products were identified and quantified by capillary electrophoresis on an ABI 3100 genetic analyzer, using GENESCAN software (version 3.7) from Applied Biosystems (FosterCity, CA, USA). The peak area of the PCR products was determined by GeneMarker software V1.51, which normalizes peak intensities and compares them with those of healthy controls included in the analysis. In order to correct for the peak intensity variation over size, an exponential function $a*e^{-bz}$ was used to fit to the square root of peak intensities, where z is size, and a and b are fitting constants.

Clinical data of the patients were collected from the Dutch National Registry of Growth Hormone Treatment, where clinical and laboratory parameters of 2500 GH-treated Dutch patients have been registered. We analysed data by SPSS version 11.0, descriptive statistics.

Results

The 79 participating patients originated from 77 families (73 Caucasian Dutch, one Afro-Caribbean, one Moluccan, one Turkish and two Moroccan). In the two Moroccan families, consanguinity was present: in one family, the parents were first cousins and in the other family, the patient's parents as well as the parents of the patient's father were cousins. In one Dutch family, the parents were seventh-degree relatives. Clinical and laboratory parameters are summarised in Table 2. One patient had a formerly known R271W mutation in *POU1F1*[10]. He had TSH, GH and prolactin deficiency but normal function of the hypothalamic-pituitary-adrenal axis and normal spontaneous puberty. He was born by breech delivery, without neonatal jaundice, micropenis or neonatal hypoglycaemia. His sister and mother, who had the same *POU1F1* mutation, had a similar phenotype.

All patients had GHD and all required thyroid hormone replacement, while 79% of the patients required glucocorticoid hormone replacement and 85% of patients older than 14 years required induction of puberty. Although mutations of all genes tested can cause hypoprolactinemia (Table 1), hypoprolactinaemia was present in only 15% of the patients tested. Four patients (5%) had diabetes insipidus and 82% of the patients had an abnormal pituitary on MRI. Figure 1 shows the various pituitary anomalies in the population.

Although ectopia of the neurohypophysis is known to be absent in children with *PROP1* and *POU1F1* mutations, this anomaly was present in almost 75% of the patients tested.

We did not find any previously described mutation in *PROP1, HESX1, POU1F1, LHX3* and *LHX4,* apart from the patient with the known *POU1F1* mutation. We found three new variants causing an amino acid change. In *POU1F1*, a new heterozygous missense sequence variation was found in a Moroccan girl, whose healthy parents had a height of 172 and 155 cm, respectively. The parents were cousins, and the father's parents were also cousins. The patient was born at term by head delivery with a birth length of 49 cm and birth weight of 2900 gr. She was born with a midline palatal cleft. In the neonatal period, she suffered hypoglycaemias and respiratory insufficiency. She was treated for hypothyroidism, hypocortisolism and GHD. MRI showed a small pituitary and an invisible pituitary stalk. DNA analysis revealed a base change of C to T at base c462 of *POU1F1*, changing the positively charged, hydrophilic arginine at position 113 into the neutral, hydrophobic tryptophan (R113W). As the R113 residue is highly conserved in all species, it might be functionally important. However, the function of the region, in which R113 is located, is unknown. Among 150 healthy individuals screened, one individual had the same variant, suggesting that R113W could be a low frequency polymorphic variant with yet unknown functional consequences.

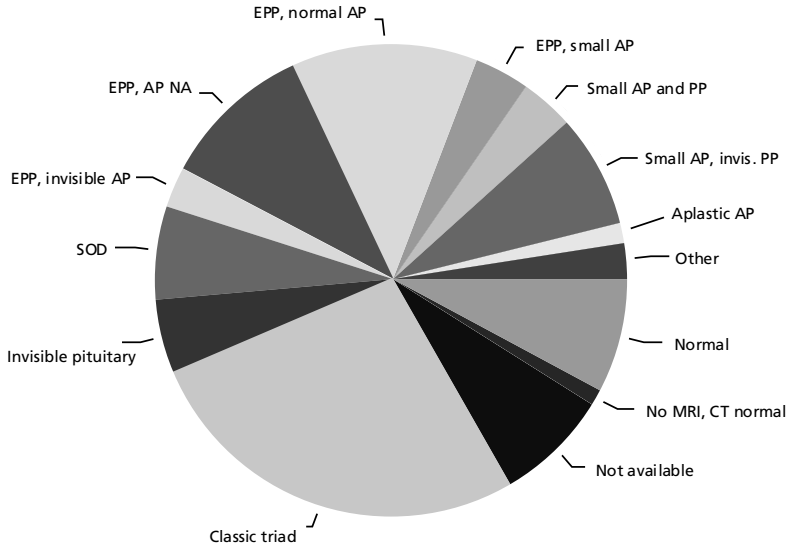

Figure 1: MRI anomalies of 79 CPHD patients participating in the study. SOD = septo-optic dysplasia, EPP = ectopic PP, AP = anterior pituitary, NA = not available, PP = posterior pituitary, invis. = Invisible, Other = one patient had a hypoplastic and interrupted pituitary stalk combined with parenchym loss of the thalamus; the other patient had an absent pituitary stalk combined with central and cortical cerebral atrophy, MRI = Magnetic Resonance Imaging scan, CT = computed tomography scan, Classic triad = combination of small AP, EPP and invisible or interrupted pituitary stalk

In two unrelated male patients, we found the same new missense variant in *LHX3.* The first patient was born by vaginal head delivery after 41 weeks of gestation with a birth weight of 3510 g, without any neonatal abnormalities. He required GH, thyroid hormone and glucocorticoid hormone replacement at the age of nine years. MRI was normal. The second patient had a more severe phenotype. He was born by vaginal head delivery after 36 weeks of gestation with a birth weight of 2300 g and birth length of 44 cm. He suffered neonatal hypoglycaemias and had prolonged neonatal jaundice. He was treated for GHD, hypothyroidism and hypocortisolism by the age of five years, and for hypogonadotrope hypogonadism at the age of 16 years. DNA analysis in both patients revealed a heterozygous base change from C to T at base c41 of *LHX3*, changing alanine into valine (A14V). Although the affected Alanine residue is highly conserved among species, its function is unknown. Among 150 healthy controls, none had this variant. The parents of the patients were healthy, although the mother of the second patient, carrying the same variant, was short (158 cm, -1.8 SDS). The fact that the parents were healthy despite the presence of this variant, makes it less probable that the variant is a pathogenic mutation.

Table 2: Clinical data of 79 patients with combined pituitary hormone deficiency

Sex		63 M / 16 F
Birth weight	kg	3.0 (0.8)
	SDS	-0.6 (1.4)
Birth length	kg	49.4 (3.0)
	SDS	-0.3 (1.4)
Gestational age (w)		38.9 (2.8)
Age at start GH treatment (y)		4.0 (3.5)
Height SDS at start of GH		-3.0 (1.3)
GH peak during Arginine test (mU/L)		5.0 (6.1)
GH peak during Clonidine test (mU/L)		4.5 (3.8)
IGF-I SDS		-4.4 (3.0)
Hypothyroidism (N) (%)		79 (100%)
Hypocortisolism (N) (%)		62 (79%)
Hypogonadotrope hypogonadism (N) (%)		34 (85%)
Hypoprolactinaemia (N) (%)		11 (15%)
Diabetes insipidus (N) (%)		4 (5%)
First-degree relatives with GHD (N) (%)		4 (5%)
Micropenis (N) (%)		24 (38%)
(Prolonged) neonatal jaundice (N) (%)		49 (62%)
Hypoglycaemia (N) (%)		34 (43%)

Data are expressed as mean (SD), SDS = standard deviation score, GH = Growth Hormone, GHD = Growth Hormone Deficiency

Another new heterozygous missense mutation in *LHX3* was found in a male patient born at term by breech delivery, with normal birth weight of 3250 g, neonatal jaundice and hypoglycaemias during the first year of life. The MRI showed a small anterior pituitary and an invisible posterior pituitary and pituitary stalk. The patient had hypothyroidism and hypocortisolism diagnosed at the age of 1 year and undetectable levels of LH, FSH and testosterone at age 13 years. DNA analysis revealed a base change of G to C at base c935 of *LHX3*, changing the hydrophilic, large and positively charged arginine into the hydrophobic, smaller, neutral proline. The R312 amino acid is located outside the LIM boxes and the homeodomain, so functional importance of this residue is unknown. R312 is conserved in mouse, rat, dog and cow. Screening of 150 healthy persons for this variant revealed one individual with the same mutation and also the patient's healthy mother (height 166 cm) had the same variant, which makes it a probably non-causal mutation.

Since the *LHX3* mutations reported to date are inherited in an autosomal recessive manner, abnormalities on the other allele could cause the phenotype by compound heterozygosity. However, screening of the promoter, exons, exon-intron boundaries and 3' UTR of the other allele of the patient revealed no abnormalities.

All patients were screened for the P89L and IVS3+1 /+2 splice site mutations in *GH1*, but no mutations were found in any of the patients. MLPA did not detect any deletion or duplication in *PROP1, HESX1, POU1F1, LHX3* or *LHX4* in any of the patients.

Discussion

We screened for mutations and deletions in *PROP1, HESX1, POU1F1, LHX3,* and *LHX4* and for the P89L and IVS3+1 /+2 splice site mutations in *GH1* in a population of 79 Dutch CPHD patients. Apart from the *POU1F1* R271W mutation previously detected in one patient[10], we did not find any deletion or known mutation. We found three new variants (one in *POU1F1* and two in *LHX3*) in heterozygosis. These variants caused a change in amino acid, but they were also found in non-affected family members or healthy controls (with a frequency below 1%), and are therefore considered rare but probably non-causal missense mutations.

Mutations in *HESX1, LHX3* and *LHX4* are known to be relatively rare. In the literature, mutations in these genes are only reported in 0 to 2.6% of CPHD patients (Table 3) and this is confirmed by our study.

POU1F1 mutations have been reported in up to 11.3% in the literature (Table 3). *POU1F1* mutations have been described in two Dutch families[12] and in one additional patient diagnosed before 1992. However, in our cohort of patients registered in the Dutch National Registry of Growth Hormone Treatment between 1992 and 2003, only one patient with a mutation was present, and therefore the overall prevalence of *POU1F1* mutations in the Dutch CPHD population is very low (1.2%).

PROP1 mutations have been reported in up to 57% of CPHD patients (Table 3). High prevalence are especially reported from populations with a large proportion of familial cases. Studies investigating sporadic cases often report a low prevalence or a total absence of *PROP1* mutations. Kim *et al*, McLennan *et al* and Rainbow *et al* reported mutation frequencies of 0% in their populations of 12, 33 and 27 sporadic CPHD patients, respectively[13-15].

The sensitivity of dHPLC has been reported to be between 96-100%[17-22] and since we analysed all exons at at least two different melting temperatures, the presence of false negative results is highly unlikely. Since a high proportion of the *PROP1* mutations reported in the literature is located in the 149delGA and 296delGA mutational hot spots in exon 2 of *PROP1*[1], we directly sequenced this exon in 40 of our CPHD patients in order to test the sensitivity of our mutation screening method.

Direct sequencing confirmed the absence of mutations in these patients. We are therefore convinced that our mutation screening method is accurate and that the low mutation frequency in our population is realistic.

Since P89L and IVS3+1 /+2 splice site mutations in *GH1* have recently been shown to cause pituitary hormone impairment in addition to GH deficiency[9], we screened for these *GH1* mutations in our CPHD patients and found they were absent.

Even though mutation screening for *PROP1, HESX1, POU1F1, LHX3*, and *LHX4* did not reveal any mutations, small deletions in these genes could theoretically cause CPHD. To exclude this, we performed Multiplex Ligation-dependent Probe Amplification (MLPA), which is a sensitive method for relative quantification of DNA. MLPA showed normal relative quantity of *PROP1, HESX1, POU1F1, LHX3,* and *LHX4*, thereby excluding deletions or duplications in all our CPHD patients.

In conclusion, we screened for mutations in *PROP1, HESX1, POU1F1, LHX3* and *LHX4*, searched for *GH1* P89L and IVS3+1 /+2 splice site mutations and performed MLPA to detect deletions in order to explain the clinical condition of 79 Dutch CPHD patients. Our search did not reveal any genetic defect in our CPHD cohort apart from one formerly known *POU1F1* mutation in one patient. Since genetic defects in these genes appeared to be rare, future research should focus on alternative explanations for CPHD, like other genes or environmental factors.

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Table 3: Mutation frequencies in *PROP1*, *HESX1*, *POU1F1*, *LHX3* and *LHX4* reported to date

	Author (ref)	Diagnosis	Sporadic/ familiar cases	N pt	N mut	% mut	origin	Mutation detection
PROP1	Present study	CPHD	Mostly sporadic (79 patients from 78 families)	79	0	0.0%	Dutch	dHPLC and Sequencing
	McLennan *et al*[14]	31 CPHD / 2 IGHD	Sporadic	33	0	0.0%	Australian	Sequencing
	Kim *et al*[13]	CPHD	Sporadic	12	0	0.0%	Korean	Sequencing
	Rainbow *et al*[15]	CPHD	Mostly sporadic (27 patients from 26 families)	27	0	0.0%	UK	Sequencing
	Turton *et al*[16]	CPHD	Sporadic			1.1%	Various	SSCP and Sequencing
	Turton *et al*[16]	CPHD	Mixed sporadic and familial	153	15	9.8%	Various	SSCP and Sequencing
	Osorio *et al*[23]	CPHD	Mostly sporadic (76 patients from 74 families)	43	5	11.6%	Brazilian	Sequencing
	Reynaud *et al*[24]	CPHD	Mixed (195 patients from 165 families)	109	20	18.3%	Various	Sequencing
	Lebl *et al*[25]	CPHD	Mostly sporadic (74 patients, of which 4 sib pairs)	74	18	24.3%	Czech	dHPLC and Sequencing
	Turton *et al*[16]	CPHD	Familial			29.5%	Various	SSCP and Sequencing
	Vallette-Kasic *et al*[26]	CPHD	Mostly sporadic (23 patients from 20 families)	23	9	39.1%	Various	Sequencing
	Lemos *et al*[27]	CPHD	Mixed (17 familial from 7 families and 29 sporadic)	46	19	41.3%	Portuguese	Sequencing
	Deladoey *et al*[28]	CPHD	Familial (73 patients from 36 families)	73	35	47.9%	?	Sequencing
	Fofanova *et al*[29]	CPHD	Mixed (7 patients from 4 families, 7 sporadic)	14	8	57.1%	Russian	Sequencing
HESX1	Present study	CPHD	Mostly sporadic (79 patients from 78 families)	79	0	0.0%	Dutch	dHPLC and Sequencing
	Kim *et al*[13]	CPHD	Sporadic	12	0	0.0%	Korean	Sequencing
	Lebl *et al*[25]	CPHD	Mostly sporadic (74 patients, of which 4 sib pairs)	74	0	0.0%	Czech	dHPLC and Sequencing
	Rainbow *et al*[15]	CPHD	Mostly sporadic (23 patients from 22 families)	23	0	0.0%	UK	Sequencing
	Reynaud *et al*[24]	CPHD	Mixed (195 patients from 165 families)	16	0	0.0%	Various	Sequencing
	McNay *et al*[30]	IGHD/CPHD	Mixed	724	3	0.4%	Various	2 different heteroduplex detection techniques

	Author (ref)	Diagnosis	Sporadic/ familiar cases	N pt	N mut	% mut	origin	Mutation detection
POU1F1	Fofanova *et al*[31]	CPHD	Mixed (7 patients from 4 families, 7 sporadic)	14	0	0.0%	Russian	Sequencing
	Kim *et al*[13]	CPHD	Sporadic	12	0	0.0%	Korean	Sequencing
	Present study	CPHD	Mostly sporadic (79 patients from 78 families)	79	1	1.2%	Dutch	dHPLC and Sequencing
	Lebl *et al*[25]	CPHD	Mostly sporadic (74 patients, of which 4 sib pairs)	74	1	1.4%	Czech	dHPLC and Sequencing
	Reynaud *et al*[24]	CPHD	Mixed (195 patients from 165 families)	17	1	5.9%	Various	Sequencing
	McLennan *et al*[14]	31 CPHD/ 2 IGHD	Sporadic	33	2	6.1%	Australian	Sequencing
	Rainbow *et al*[15]	CPHD	Mostly sporadic (27 patients from 26 fam)	27	2	7.4%	UK	Sequencing
	Turton *et al*[32]	CPHD	Mostly sporadic	80	9	11.3%	European	SSCP and Sequencing
LHX3	Present study	CPHD	Mostly sporadic (79 patients from 78 families)	79	0	0.0%	Dutch	dHPLC and Sequencing
	Kim *et al*[13]	CPHD	Sporadic	12	0	0.0%	Korean	Sequencing
	Reynaud *et al*[24]	CPHD	Mixed (195 patients from 165 families)	20	0	0.0%	Various	Sequencing
LHX4	Present study	CPHD	Mostly sporadic (79 patients from 78 families)	79	0	0.0%	Dutch	dHPLC and Sequencing
	Reynaud *et al*[24]	CPHD	Mixed (195 patients from 165 families)	39	1	2.6%	Various	Sequencing

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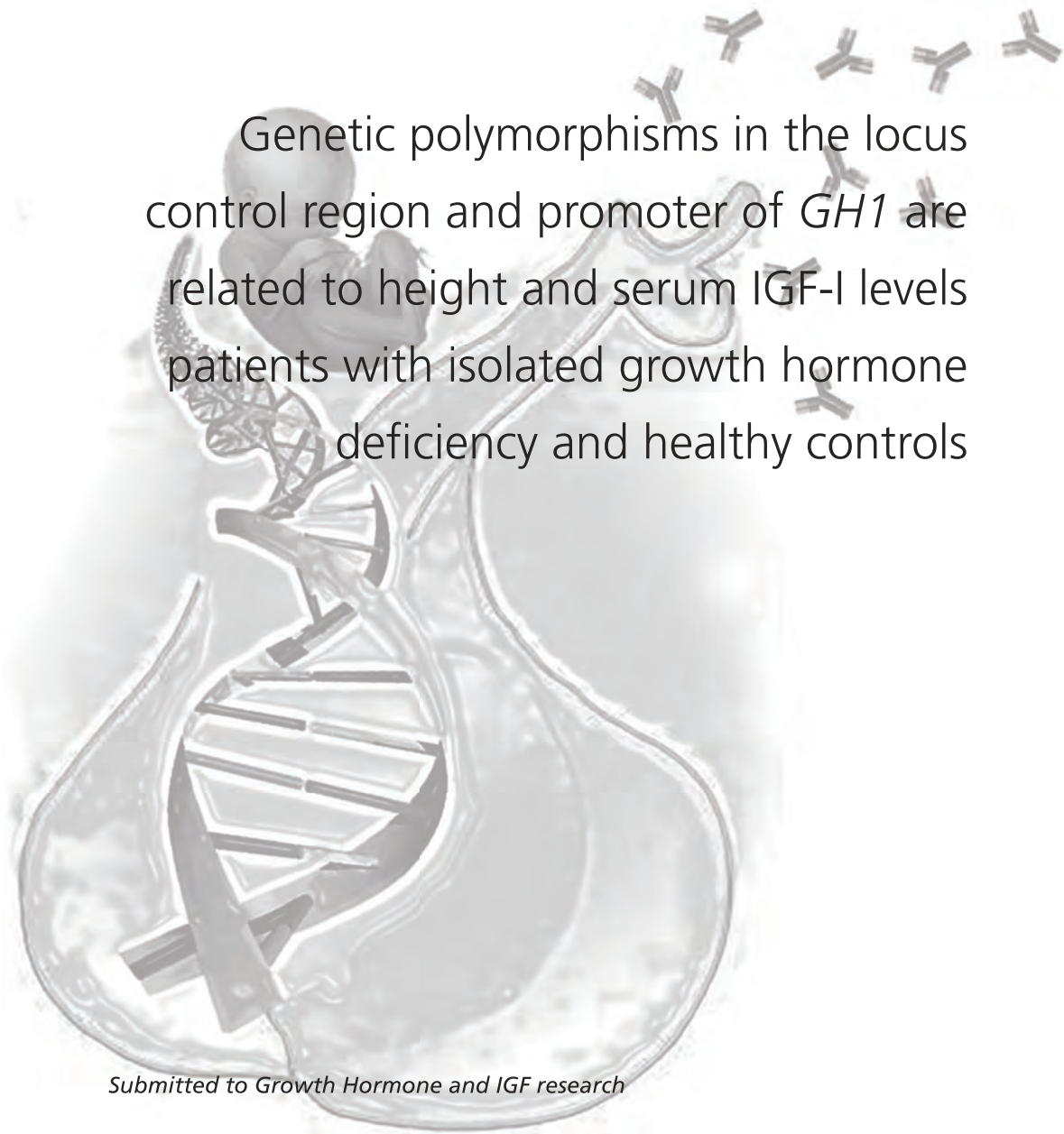
Chapter 4



Genetic polymorphisms in the locus control region and promoter of *GH1* are related to height and serum IGF-I levels patients with isolated growth hormone deficiency and healthy controls

Submitted to Growth Hormone and IGF research

Abstract

Objective: Expression of the human Growth Hormone gene (*GH1)* is regulated by a Locus Control Region (LCR) and the highly polymorphic *GH1* promoter. We analysed *GH1* LCR / promoter SNPs in patients with isolated growth hormone deficiency (IGHD) without mutations or deletions in *GH1* and *GHRHR*, and examined whether these SNPs were associated with clinical data, in order to explain phenotypic variability.

Patients, design and measurements: We directly sequenced the *GH1* LCR / promoter of 62 Dutch IGHD patients without mutations or deletions in *GH1* or *GHRHR*, participating in the Dutch HYPOPIT study, and of 72 controls with normal height. We evaluated associations of *GH1* LCR / promoter sequence variations with height, serum IGF-I levels and the first year's response to GH treatment.

Results: Promoter SNP 6 was strongly linked with SNPs 1 and 2 of the LCR. Promoter SNP 9 was significantly more frequent among patients than among controls. Promoter SNPs 6, 8, 9 and 11 were individually related with height and IGF-I levels.

Conclusion: *GH1* promoter SNPs 6, 8, 9 and 11 genotypes are associated with height and IGF-I levels in IGHD patients and controls. Although all patients had IGHD according to Dutch consensus criteria, certain genotypes were associated with relatively normal IGF-I levels.

Introduction

Human postnatal growth is determined by the interaction of various genetic and environmental factors and results from the lengthening of bones via cellular divisions, which is mainly regulated by human growth hormone (GH). GH, secreted by the somatotrope cells of the anterior pituitary gland, is a protein of 191 amino-acids (22 kD) with two disulfide bridges, which are important for its structure and bioactivity. GH synthesis and secretion are regulated by several hormones, reviewed by Goldenberg et al[1]. Growth Hormone Releasing Hormone (GHRH) is produced in the hypothalamus and stimulates GH production via binding to the GHRH receptor (GHRHR), which is located in the anterior pituitary. GH production is negatively regulated by the Somatotropin Release Inhibiting Factor, SRIF or somatostatin, and by IGF-I, the end product of GH's action.

Mutations in the genes encoding GH and GHRHR (*GH1* and *GHRHR,* respectively) can cause Isolated Growth Hormone Deficiency (IGHD) types IA, IB (OMIM #262400) and II (OMIM #173100). The frequency of *GH1* and *GHRHR* mutations in IGHD patients varies between 0 and 32% among different countries[2-7]. Thus, in the best of cases, mutations in the coding regions of *GH1* and *GHRHR* are only detected in one third of the IGHD patients.

GH1 is located in the human growth hormone cluster, in which five genes are situated on chromosome 17. Human growth hormone (*GH1*) lies at the 5' end of the cluster and is mainly expressed in pituitary somatotrophs. The remaining four genes, placental growth hormone (*GH2*) and three chorionic somatomammotropins (*CS1, CS2,* and pseudogene *CS5* or *CSHP1*), are only expressed in the placenta.

The expression of *GH1* is regulated by a locus control region (LCR), located 14.5 kb to 32 kb upstream of the gene[8] and by the *GH1* promoter. To date, three Single Nucleotide Polymorphisms (SNPs) have been reported in the LCR[9], while the *GH1* promoter is highly polymorphic[9-14].

Although in general SNPs do not necessarily cause disease, they have been associated with susceptibility to diseases[15-17] and to environmental toxins[18] by affecting splicing[19], allelic expression[20], nonsense-mediated mRNA decay[21] or transcription factor binding[22]. *GH1* promoter SNPs have been associated with height[9,10,12], bone density, bone loss, and fetal growth restriction[23,24]. In addition, some *GH1* promoter SNPs have been associated with the incidence of breast and colorectal cancer[13,25-28]. Two promoter SNPs have been studied in relation to GH and / or IGF-I levels in patients with varying degrees of GH deficiency[11,12]. However, the entire promoter region of more than 500 bp, containing at least 19 SNPs, has never been sequenced in a homogenous cohort of patients with IGHD in comparison with a control group of normal stature.

The Dutch HYPOthalamic and PITuitary gene (HYPOPIT) study is a nation-wide multi-center study investigating genetic and other causes of 'idiopathic' growth hormone deficiency in the Dutch GHD population. In order to explain the variability in our IGHD patients' phenotypes and their variable response to GH treatment, we analysed *GH1* LCR / promoter sequence variations in IGHD patients and looked for associations of these variations with height, serum IGF-I levels and height increase during the first year of GH treatment. We compared the data with those of a healthy Dutch control group. Since Hasegawa *et al.*[12] related the SNP 'P1' in *GH1* intron 4 (dbSNP 2665802) with height, GH and IGF-I levels, we also included this polymorphism in our analysis.

Patients and methods

We included 62 Dutch Caucasian patients diagnosed with IGHD based on GH <20 mU/L or GH <30 mU/L combined with IGF-I <-2 SDS, without mutations or deletions in *GH1* or *GHRHR*. Patients clinical data were available from the Dutch National Registry of Growth Hormone Treatment, where auxologic and laboratory parameters have been documented[29]. GH and IGF-I measurements were centrally performed in one laboratory with published reference values[30] for 80% of the patients. For the remaining 20%, the laboratory-specific reference values for IGF-I were used to calculate the SDS. We obtained approval from the medical ethics committees of all participating hospitals. Informed consent was obtained from all participating patients and their parents, if patients were aged less than 18 years. Seventy-two healthy caucasian Dutch young adults with height SDS between –2 and +2 agreed to participate in DNA analysis as control subjects.

Genomic DNA, extracted from peripheral blood according to standard procedures, was used for direct sequencing of *GH1* LCR / promoter using an ABI 3100 Sequencher. A 1231 bp fragment including the promoter of *GH1* was amplified using forward primer 5'-GGGAGCCCCAGCAATGC-3' and reverse primer 5'-TCTGCCTGCATTTTCGCTTCG-3' by Touchdown PCR, including denaturation for 10 min at 95°C, amplification at 68° to 61° decreasing the annealing temperature by 1°C every second cycle and 25 subsequent cycli at 61°, followed by a 7-min extension at 72°C. This fragment was sequenced by forward primers 5'-GGGAGCCCCAGCAATGC-3' and 5'-CTGTCTGGTGGGTGGAGGTTAAA-3', and reverse primers 5'-CACATTCAGAAGCCCCAAAC-3' and 5'-ACCCAACTTGTCCTCTCTTTA-3'.

Since Hasegawa *et al.*[12] related the SNP 'P1' (IVS4 +90 T/A, dbSNP 2665802) in *GH1* intron 4 with height, GH and IGF-I levels, we included this polymorphism in our

analysis. P1 genotypes were available for patients who had participated in the genetic screening of the coding regions of *GH1* and *GHRHR* as part of the HYPOPIT study. In these patients, exon 4 and intron 4 had been PCR-amplified with forward primer 5'-CCGTGAGTGGATGCCTTCTC-3' and reverse primer 5'-GTGAGTTCTCTTGGGTCAGG-3' (annealing temperature 57.7°C) and the fragment had been screened by WAVE dHPLC at 62.1 and 62.6 °C, which were shown to accurately detect the P1 variant.

We used *GH1* gene nucleotide sequence obtained from GenBank (Accession No. J03071) as control and we numbered SNPs according to Horan *et al.*[9]. We obtained the reference LCR sequence from GenBank (Accession No. AF010280) and used LCR numbering according to Jin *et al.*[31]. Of the LCR / promoter SNPs, linkage disequilibrium represented by R^2 and D',was analysed using Haploview[32].

The associations between genetic and clinical parameters were analysed by means of ANOVA and Chi-square tests using SPSS version 11.0. Response to GH treatment was defined as the increase in HSDS during the first year of GH treatment. The normality of the distribution of all analyzed parameters was assessed with the Kolmogorow-Smirnow and Shapiro-Wilk tests. When expected cell counts for comparison of genotype frequencies between patients and controls were below 5, we used the Fisher's exact test instead of the Chi-square test. Parameters that were not normally distributed were analysed using the non parametric Kruskall-Wallis test instead of ANOVA.

Results

All LCR / promoter SNPs conferred to Hardy-Weinberg equilibrium according to a Chi-square test (p>0.05) except for promoter SNP 8 (p=0.01). In contrast to Horan *et al.*[9], we found that promoter SNP 2 was not polymorphic since all patients and controls carried the same allele. There was 100% linkage disequilibrium between LCR SNP 1 and 2 and between promoter SNPs 4 and 5 in patients and controls. Gene structure, sequence variation and linkage disequilibrium between LCR and promoter SNPs in our patients is shown in Figure 1. Promoter SNP 6 was strongly linked with LCR SNP1 (D' 0.96, R^2 0.92, Figure 1) and with SNP 'P1' in intron 4 of *GH1* (D' 0.95, R^2 0.72).

Height in cases and controls, and serum IGF-I levels in patients were normally distributed. Four promoter SNPs were related with height and IGF-I levels. An overview of these SNPs, their rs numbers, alleles and their relations with clinical parameters are shown in Table 1 and Figure 2.

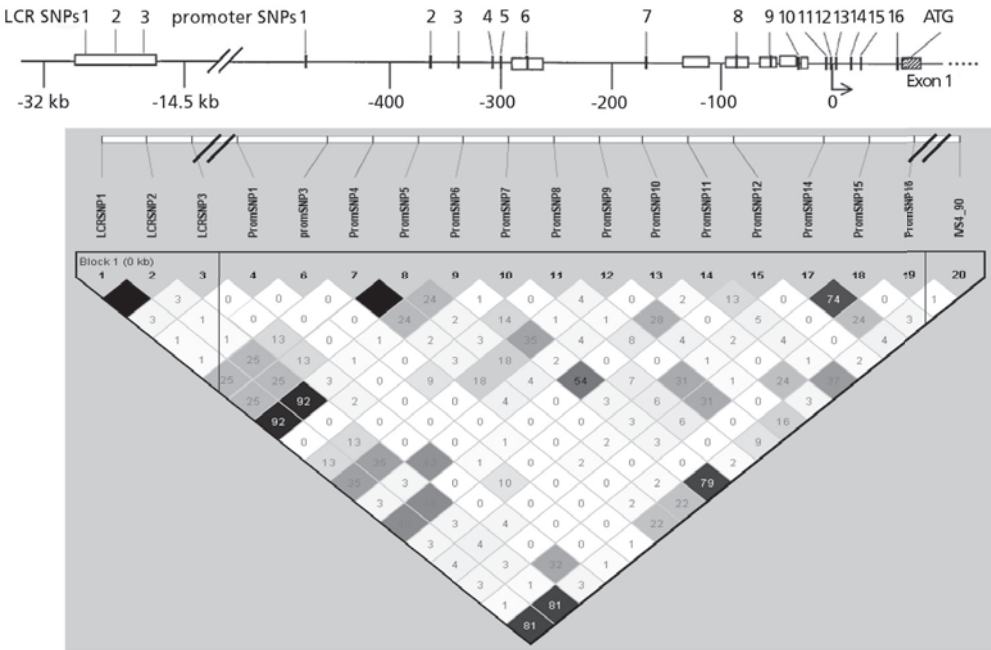


Figure 1: LD plot showing linkage disequilibrium between *GH1* LCR, promoter and intronic SNPs. Black boxes indicate SNPs that are in 100% LD ('highest possible correlation') with other SNPs. Dark and light gray boxes represent lower levels of linkage disequilibrium, represented by R squared values in the boxes. Prom = promoter, IVS4_90 = intronic SNP 'P1' IVS4 +90 T/A

Since genotype frequencies and clinical associations differed between IGHD patients and controls, we discuss the results of the IGHD patients and healthy controls separately.

IGHD patients

The study population consisted of 43 male and 19 female IGHD patients, which is in accordance with a male predominance in the overall Dutch IGHD population. Mean (±SD) age at genetic screening was 15.6±6.4 years, mean age at start of GH treatment was 5.7±2.7 years. At start of GH treatment, height SDS was –3.1±0.9 and IGF-I SDS was –3.3±2.3. There were no differences in genotype frequencies, height SDS or IGF-I SDS between males and females.

We found significant differences in height and serum IGF-I levels between carriers of the different genotypes at individual promoter SNPs 6, 8, 9 and 11 (Table 2). LCR SNP1, promoter SNP 6 and intron 4 SNP 'P1' (IVS4 +90 T/A) were highly linked

(Figure 1). However, the association between the individual intron 4 SNP and peak GH levels, HSDS and IGF-I levels formerly described by Hasegawa[12], was not present in our population.

We did not find any relation between LCR / promoter SNPs and the first year's response to GH treatment.

Table 1: SNPs located in the LCR, promoter and intron 4 of *GH1*

SNP ID[a]	Position[b]	Reference	Alleles[c]	Functional region
LCR SNP 1/2[LD]	1194/ 1144	rs4968672/ rs4968673	G/A* T/G*	PIT1
LCR SNP 3	990	-	C/T*	PIT1
Prom SNP 1	-476	-	G/A*	
Prom SNP 2	-364	-	G[e]	
Prom SNP 3	-339	-	G/-*	
Prom SNP 4/5 [LD]	-308/ -301	rs1811081/ rs2011732	G/T* G/T*	
Prom SNP 6	-278	rs2005171	G/T*	NF1
Prom SNP 7	-168	rs2727338	T/C*	
Prom SNP 8	-75	rs11568828	A/G*	PIT1
Prom SNP 9	-57	rs2005172	G/T*	VDR
Prom SNP 10	-31	rs11568827	G/-*	TATA
Prom SNP 11	-6	rs6171	A/G*	TSS
Prom SNP 12	-1	rs695	A/T*/C*	TSS
Prom SNP 13	+3	rs6175	G/C*	TSS
Prom SNP 14	+16	rs9282699	A/G*	5' UTR
Prom SNP 15	+25	-	A/C*	5' UTR
Prom SNP 16	+59	rs 6173	T/G*	5' UTR
GH1 SNP P1	IVS4+90	dbSNP 2665802	T/A*	Intron 4

TSS = transcriptional start site, PIT1 = PIT1 binding site, NF1 = NF1 binding site, VDR= VDR binding site, LCR = Locus Control Region, Prom = promoter, [a] SNP identification according to Horan *et al.*, 2003 [b] SNP position relative to *GH1* transcriptional start site [c] The most frequent genotypes in our study at the various promoter SNP sites are in accordance with the expected genotype when sequencing *GH1* instead of any of the other four *GH1* parologue genes [e]only G alleles were reported by Horan et al and our study [LD]SNPS are 100% in linkage disequilibrium with each other *minor allele

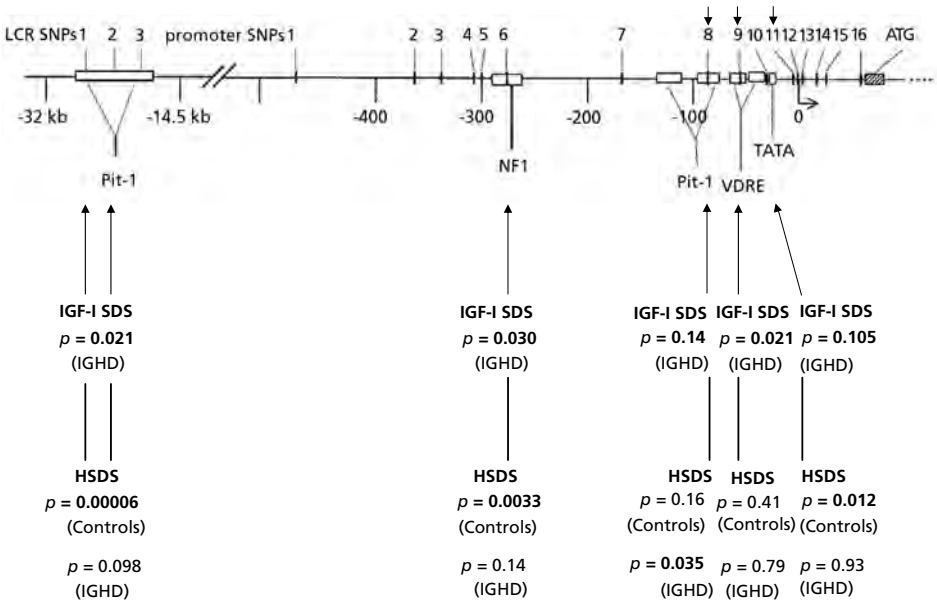


Figure 2: *GH1* promoter and LCR SNPs genotyped in this study, the functional regions in which they are located and their associations with clinical data (adapted from Horan *et al.*, 2003).

Controls

The controls (27 males and 45 females) had a mean (±SD) age at testing of 21.0±1.6 years and height SDS of –0.2±1.0. There were no differences in genotype frequencies or height SDS between males and females.

For promoter SNPs 6 and 11, height differed significantly between the carriers of various genotypes (Table 2).

IGHD patients versus controls

We compared promoter SNP genotypes between IGHD patients and controls, and found that homozygosity for the minor allele of SNP 9 was more frequent among patients than among controls (21% *vs* 7%, p=0.005). Since the minor allele at SNP 9 is associated with lower IGF-I levels in patients, this suggests this allele might contribute to impaired GH levels in IGHD patients.

The promoter SNP 12 was tri-allelic with the major allele A occurring in controls in combination with two different minor alleles (AC and AT, frequencies AA 74% / AT 19% / AC 5% / TT 1%), whereas in patients, only the AT genotype was present. Among controls, there was no difference in height SDS between individuals with the AC and those with the AT genotype.

For promoter SNP 13, the minor allele was only present in controls (all patients were homozygous for the major allele). For the remaining SNPs, genotype and allele frequencies were similar for patients and controls.

Table 2: Frequencies of individual LCR / promoter SNPs and their relations with clinical data

		IGHD patients			Controls	
		N	HSDS	IGF-I SDS	N	HSDS
Promoter SNP 6	**GG**	22 (37%)	-3.3 (0.8)	-4.0 (2.6)	19 (27%)	0.3 (0.7)
	GT	32 (53%)	-3.1 (0.9)	-3.0 (2.0)	42 (59%)	-0.6 (0.9)
	TT	6 (10%)	-2.5 (0.8)	-1.4 (1.2)	10 (14%)	-0.1 (0.8)
			p=0.14	***p***=0.03		***p***=0.003
Promoter SNP 8	**AA**	53 (88%)	-3.2 (0.9)	-3.4 (2.3)	63 (87%)	-0.3 (0.9)
	AG	7 (12%)	-2.5 (0.7)	-2.0 (1.0)	7 (10%)	-0.6 (0.8)
	GG	0	-	-	2 (3%)	0.8 (1.2)
			p=0.04	***p***=0.14		***p***=0.16
Promoter SNP 9	**GG**	25 (42%)	-3.1 (0.8)	-2.6 (1.6)	25 (35%)	-0.2 (0.9)
	GT	22 (37%)	-3.1 (1.1)	-3.0 (2.3)	42 (58%)	-0.4 (1.0)
	TT	13 (22%)**	-3.3 (0.8)	-4.8 (2.6)	5 (7%)**	0.2 (0.7)
			p=0.8	***p***=0.02		***p***=0.41
Promoter SNP 11	**AA**	24 (40%)	-3.2 (0.7)	-4.0 (2.5)	18 (25%)	0.0 (1.0)
	AG	31 (52%)	-3.1 (1.0)	-2.9 (2.0)	43 (60%)	-0.6 (0.9)
	GG	5 (8%)	-3.2 (0.7)	-2.0 (1.6)	11 (15%)	0.2 (0.9)
			p=0.9	***p***=0.10		***p***=0.012

NS = $p>0.10$. Data are shown as mean (SD), HSDS = height SDS (for patients at start of GH treatment)
** = homozygosity for the minor allele is more frequent in IGHD patients than in controls, $p=0.005$

Multiple regression

The promoter SNPs that were associated with height and/or IGF-I levels in patients or controls (SNPs 6, 8, 9 and 11) were present in 23 different combinations ('SNP 6-8-9-11 genotypes'), of which seven were present in at least 5% of the patients or controls (Table 3). Only 48 controls and 45 patients carried these seven most frequent combined genotypes; clinical data of the patients and controls with these genotypes are shown in Table 3 and Figure 3.

Table 3: Frequencies of combined genotypes of promoter SNPs 6, 8, 9 and 11 and their relations with clinical data

				IGHD patients				Controls		
	SNP 6-8-9-11 Genotype*	N[1]	%	Height SDS	Peak GH[4] Arginine (mU/L)	Peak GH[4] Clonidine (mU/L)	IGF-I[2] SDS	N[1]	%	Height[3] SDS
1	G/G A/A T/T A/A	12	27.2	-3.2 (0.8)	12.1 (9.2)	9.5 (5.1)	-5.0 (2.6)	4	8.3	0.4 (0.5)
2	G/G A/A G/T A/A	5	11.4	-3.5 (0.8)	17.6 (1.3)	15.0 (7.2)	-2.1 (2.3)	6	12.5	0.5 (0.8)
3	G/G A/A G/T A/G	0	-	-	-	-	-	6	12.5	0.0 (0.9)
4	G/T A/A G/T A/G	12	27.2	-3.2 (1.3)	11.0 (5.1)	12.0 (5.2)	-3.7 (2.6)	26	54.2	-0.6 (0.9)
5	G/T A/A G/G A/G	9	20.5	-3.2 (0.8)	9.0 (5.8)	11.5 (5.2)	-2.7 (1.2)	5	10.4	-0.5 (0.8)
6	G/T A/G G/G A/G	3	6.8	-3.0 (0.1)	6.5 (5.6)	12.0 (0.1)	-2.2 (1.7)	1	2.1	-0.4
7	T/T A/A G/G A/G	3	6.8	-3.0 (0.6)	9.3 (5.2)	10.6 (6.9)	-1.3 (0.7)	0	-	-
		44	100.0					48	100.0	

* Only genotypes present in at least 5% of the patients or controls are shown

[1] genotype frequencies between IGHD patients and controls p=0.002

[2] IGF-I SDS difference between genotypes p=0.08

[3] Height SDS difference between genotypes p=0.05

[4] Peak GH levels did not differ significantly between genotypes (tested by non-parametric Kruskall-Wallis test due to non-normal distribution of the parameters)

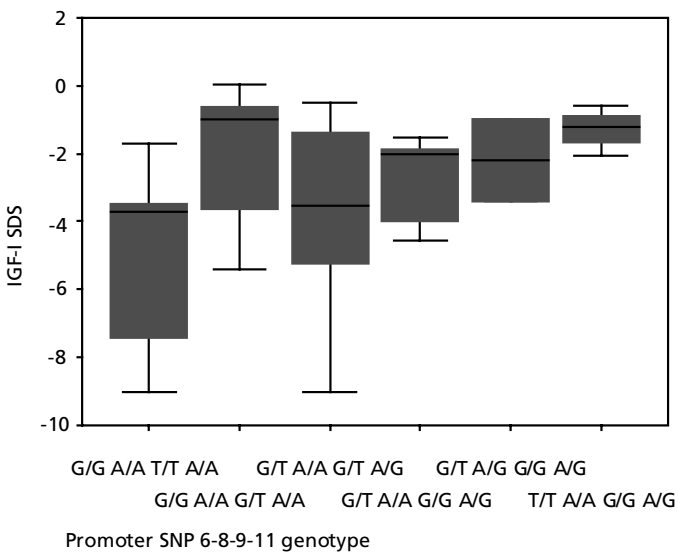


Figure 3: IGF-I SDS in IGHD patients according to combined genotypes based on promoter SNP 6, 8, 9 and 11

Backward multiple regression showed that the 'SNP 6-8-9-11 genotypes' (numbered 1 to 7 according to Table 3) explained 10.8% of variation in IGF-I SDS in IGHD patients (adjusted R^2 0.108, p=0.02). In controls, the 'SNP 6-8-9-11 genotypes' explained 15.9% of HSDS variation (R^2 0.159, p=0.003).

Discussion

We analysed *GH1* LCR / promoter SNPs in IGHD patients without mutations or deletions in *GH1* or *GHRHR*, and examined whether these SNPs were associated with height, serum IGF-I levels and response to GH treatment, in order to explain the phenotypic variability present among IGHD patients. We compared patients' data with those of a normal control group and found that homozygosity for the minor allele of SNP 9 was more frequent among patients than among controls (21% versus 7%, p=0.005). The minor allele at SNP 9 was associated with lower IGF-I levels in patients, suggesting that this allele might contribute to impaired GH production in IGHD patients. Also SNP 6, 8 and 11 were related with height and IGF-I levels.

LCR SNP1, promoter SNP 6 and intron 4 SNP 'P1' (IVS4 +90 T/A) were highly linked. However, in contrast to Hasegawa *et al.*[12], we did not find any significant association between the individual intron 4 SNP and peak GH levels, HSDS and IGF-I levels in our population. This suggests that the relation between the intron 4 SNP and growth-related parameters reported by Hasegawa, might actually be caused by its linkage with LCR / promoter SNPs.

Although patient numbers were small, we tried to combine promoter SNPs 6, 8, 9 and 11 into genotypes. Although the result of the multiple regression based on these combined genotypes should be interpreted with caution due to the reduced patient number and subsequent power reduction, in our population the combined genotypes explained 10.8% of variation in serum IGF-I levels in IGHD patients and 15.9% of height variation in controls. Although all patients had IGHD according to Dutch consensus criteria, certain genotypes were associated with a relatively mild phenotype.

The impact of individual SNPs can be explained by the fact that the regions in which they are located, are important for transcription of *GH1*. The LCR contains DNase I-hypersensitive (HS) sites located at -14.5 kb to -32 kb relative upstream of the *GH1* promoter, where the transcription factor PIT1 binds in order to regulate *GH1* transcription. Therefore, LCR SNPs may alter transcription of *GH1.* SNP 6 is located in the part of the promoter where Nuclear Factor 1 (NF1) binds[33]. Binding of NF1 to the NF1 binding site is necessary for transcription, and variation at this site may alter

expression of *GH1*. Promoter SNP 8 is located in the proximal PIT1 binding site and promoter SNP 9 is located in the region corresponding to the vitamin D receptor (VDR) response element. Disturbed binding of PIT1 and VDR to the PIT1 binding site and VDR response element, respectively, may alter transcription and thus expression of the *GH1* gene. Both SNP 8 and 9 were shown *in vitro* to interact with nucleic acid binding proteins and alternative alleles exhibit differential protein binding[9]; SNP 9 showed protein interaction only for the G allele, not the T allele[9]. This in accordance with our findings *in vivo* that patients homozygous for the T allele have very low IGF-I levels (Table 2). SNP 11 is located in the transcriptional start site (TSS) of *GH1* and therefore variation at this site may alter efficacy of transcription, leading to varying levels of *GH1* expression. Probably, the effect of the promoter SNPs is the sum of individual SNP effects and effects based on combinations with other SNPs. Variation at one individual SNP site can cause altered binding of the corresponding protein. This altered binding can in turn affect the binding of a second protein at another SNP site, but the extent to which the binding of this second protein is affected, will also depend on the variation at the second SNP site.

Associations between LCR / promoter SNPs with height and IGF-I levels are likely to be mediated by GH levels in serum, which may vary due to altered expression of *GH1*. Although the variation in IGF-I SDS between the different genotypes was largely in accordance with variation in peak GH levels obtained during GH stimulation tests, the association between genotypes and peak GH levels was not significant (Table 3). The fact that IGF-I levels are associated with LCR / promoter SNPs whereas stimulated GH levels are not, may be explained by the fact that forced GH secretion during a stimulation test does not always reliably reflect the spontaneous GH secretion pattern. Due to the fact that it reflects spontaneous daily GH secretion, serum IGF-I is considered a more reliable indicator of spontaneous GH secretion than GH peaks during stimulation tests[34-36]. Therefore, although LCR / promoter SNPs are not significantly related to peak GH levels obtained during stimulation tests, the relation of these SNPs with height and IGF-I levels is probably mediated by altered spontaneous GH secretion.

The LCR / promoter SNPs genotype frequencies and clinical associations differed between IGHD patients and healthy controls. These differences may be explained by the fact that certain genotypes could be beneficial for processes that take place in IGHD patients and not in controls, due to the altered GH/IGF-I status.

Although all controls had a height within the normal range, the lowest height SDS were found in patients heterozygous for promoter SNPs 6 and 11. This association with shorter stature was specific for the heterozygous state, since controls homozygous for the minor allele had higher HSDS. The finding that the

heterozygous state is associated with a certain phenotype and differs from the two homozygous states, has been previously reported and has been subject of research and discussion[9,37-45,46,47]. Heterozygous advantage or disadvantage of promoter SNPs may be explained by allele-dependent regulation of gene expression. A key process in allele-dependent regulation of gene expression is *trans*-regulation, which means that an enhancer from one allele acts in *trans* to activate transcription from the promoter of the second allele[48-50]. Transcription factors can bind two DNA molecules simultaneously, functioning as a protein bridge and mediating enhancer-promoter communication between two homologues[51]. *Trans*-regulation, *i.e.* the interaction of two alleles in *trans*, might explain advantages or disadvantages of heterozygosity as found in several studies [52,53] and in SNPs 6 and 11 in our study.

Recently, a map of *GH1* promoter SNPs in a Spanish adult control population with normal height was reported[10]. The investigators found 11 SNPs in the promoter region, of which two (corresponding to SNPs 8 and 14 in our study) were related to HSDS. The studied promoter SNPs accounted for 6.2% of adult height determination in the adult Spanish population. Horan *et al.* studied *GH1* locus control region and promoter SNPs in relation to height, and found that 3.3% of the variance of adult height was explained by promoter haplotypes. Our study largely analysed the same set of promoter SNPs as studied by Horan *et al.* and Esteban *et al.*, but instead of including all promoter SNPs in the combined genotype analysis, our study focussed on the SNPs 6, 8, 9 and 11, since they were individually associated with height or IGF-I levels. Our study analysed combined SNP 6-8-9-11 genotypes with frequencies above 5%. These most frequent combined genotypes were present in 73% of the patients and 68% of the controls. Although genotype numbers were small and results should be interpreted with caution, SNP 6-8-9-11 genotypes explained 10.8% of IGF-I SDS in the IGHD patients (p=0.02) and 15.9% of HSDS in controls (p=0.003). Such a strong association between LCR and promoter SNPs with height and serum IGF-I levels has not previously been described, and this may be due to the different approach and the number of SNPs included in the combined genotype analysis. By limiting the number of SNPs included in the combined genotypes to those that were individually associated with height or IGF-I, we have narrowed the window of promoter SNPs that seem clinically relevant for GH expression, height and IGF-I. However, this relation should be confirmed by SNP analysis of a larger population and functional studies should be undertaken to explain the mechanism by which the promoter SNPs interact.

In conclusion, *GH1* promoter SNP 9 was significantly more frequent among patients than among controls. The minor allele at SNP 9 was associated with lower IGF-I levels in IGHD patients, suggesting that this allele might contribute to impaired

GH production in IGHD patients. Apart from SNP 9, also SNP 6, 8 and 11 were related with height and IGF-I levels. Although all patients had IGHD according to Dutch consensus criteria, certain genotypes were associated with a relatively normal IGF-I levels.

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Chapter 5

Anti pituitary antibodies in Dutch patients with idiopathic hypopituitarism

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Abstract

Background: Despite extensive research, in the majority of patients with Isolated Growth Hormone Deficiency (IGHD) and Combined Pituitary Hormone Deficiency (CPHD), the cause of their clinical picture remains unknown. Recent articles suggest that some cases of idiopathic GHD might be explained by a silent form of autoimmune hypophysitis (AIH) based on the presence of Anti Pituitary Antibodies (APA) at high titres (>1:8).

Methods: We collected clinical data and serum from 71 patients participating in the Dutch HYPOPIT study. APA screening in 40 IGHD patients and 31 CPHD patients was performed by an indirect immunofluorescence method. APA, when present, were related to clinical and morphological pituitary findings.

Results: APA were present at high titres in 7 of 31 CPHD patients (23%) and 1 of 40 IGHD patients (2.5%). Among APA-positive CPHD patients, next to GH deficiency, all patients of pubertal age had gonadotroph deficiency, all had thyroid hormone deficiency and 50% had ACTH deficiency.

Conclusion: The high frequency of APA in our idiopathic CPHD population indicates that, in 23% of the patients diagnosed with idiopathic CPHD, the hormone deficiencies might actually be caused by a silent form of autoimmune hypophysitis. Screening for APA should therefore be considered in all patients with 'idiopathic' CPHD.

Introduction

Growth hormone deficiency (GHD) can either occur as an isolated deficiency (Isolated Growth Hormone Deficiency or IGHD), or in combination with TSH, ACTH, LH/FSH and/or prolactin (PRL) deficiency (Combined Pituitary Hormone Deficiency or CPHD).

The majority of CPHD patients present with anatomical abnormalities of the pituitary, like a hypoplastic anterior pituitary (HAP), an ectopic posterior pituitary (EPP), or a transsected or invisible pituitary stalk. These pituitary defects with consequent hormone deficiencies can be the result of genetic defects in transcription factors involved in the fetal organogenesis of the pituitary, like PROP1, HESX1, POU1F1, LHX3 and LHX4. Mutations in the genes encoding these pituitary transcription factors have been published in the literature in 0-57% for *PROP1*, 0 to 2.1% for *HESX1*, 0-11.3% for *POU1F1*, 0-1.5% for *LHX3* and 1-2.5% for *LHX4*[1-16]. In IGHD patients, up to one third of the cases is explained by mutations in the gene encoding the GH protein (*GH1*) and mutations in the gene encoding the GHRH-Receptor (*GHRHR*) are found in up to 10% of the patients[10,17-20].

Data from the HYPOthalamic and PITuitary gene (HYPOPIT) study, a Dutch multi-center study investigating the possible etiologies of CPHD and IGHD in Dutch patients, and those from other studies show that, in the majority of CPHD and IGHD patients, the cause of the deficiencies remains unknown, which is unsatisfactory for both patients and physicians.

Recently, an autoimmune involvement has been indicated as aetiology of hormone deficiencies of various endocrine axes. Lymphocytic hypophysitis, or autoimmune hypophysitis (AIH), is an uncommon autoimmune disease in which the pituitary gland is infiltrated by lymphocytes, plasma cells and macrophages[21]. AIH is known to impair pituitary hormone secretion[22-27]. Although deficiencies of all pituitary hormones have been described, the most common and earliest deficiency caused by AIH is ACTH deficiency[21], either isolated or associated with other deficiencies[22,28]. The deficiencies are considered as a direct result of the autoimmune attack on the pituitary cells[24]. GH deficiency (GHD) during AIH has also been reported, with a variable prevalence between the studies[22,28]. GHD does not seem to be frequent, although the true prevalence may be underestimated because GH function has not always been investigated in all patients. An increasing number of AIH cases is being reported, often in combination with other autoimmune diseases as part of an autoimmune polyendocrine syndrome (APS).

Autoantibodies are the hallmark of autoimmunity[29,30]. In particular when present at high titres, APA may be considered good markers of pituitary autommunity. Low

APA titers (<1:8) are also present in healthy controls and in patients with normal pituitary function, whereas APA-positive patients with high titers (>1:8) show an isolated severe GHD or pituitary imaging suggestive of lymphocytic hypophysitis or partial empty sella[26]. Thus, only when present at high titers, APA can be considered good markers of pituitary autoimmunity.

APA at high titres have been reported not only in patients with apparently isolated idiopathic GH deficiency[31] but also in adults with autoimmune endocrine diseases and additional GHD[26]. Furthermore, APA were recently reported in patients with autoimmune thyroid disease, and the presence of APA in these patients was also associated with GHD[32].

These findings suggest that part of the apparently idiopathic hormone deficiencies could actually be caused by a silent form of autoimmune hypophysitis, which can be revealed by detecting high titres of APA[31]. The aim of this study was to perform APA screening in 71 patients with idiopathic IGHD or CPHD, in order to evaluate a possible role of autoimmune involvement in these patients.

Patients and methods

Two hundred forty four Dutch CPHD and IGHD patients participated in the HYPOPIT study, a Dutch multi-center study investigating genetic causes of GHD, in which all but two Dutch university hospitals and two regional hospitals participated. Seventy-one patients (31 with CPHD and 40 with IGHD) were available for APA screening because stored sera and detailed clinical data were available.

APA were detected by an indirect immunofluorescence method on cryostat sections of young baboon pituitary gland as previously described[26]. In particular, fluorescein isothiocyanate (FITC)-conjugated goat antihuman IgG sera were used to detect the presence of APA. Only specimens with titres of at least 1:8 were considered positive, based on earlier findings[26] showing that lower titres (<1/8) are also present in patients with pituitary adenomas and healthy controls, and titres below 1/8 should be considered an non-specific epiphenomenon. The sera were tested, by two different investigators in a blinded manor, without the investigators performing the immunofluorescence knowing the clinical diagnosis of the respective patients. Antibodies against single pituitary hormone-producing cells were determined by a four-layer double-immunofluorescence technique as previously described[33,34].

We used SPSS version 11.0 (T-test, Chi-square, ANOVA and Pearson's correlation) to analyse associations between APA-related data and clinical parameters of the patients.

Results

Clinical data of the 31 CPHD and 40 IGHD patients screened for APA are shown in Table 1. Eight patients had antibody titres above 1:8 and were therefore considered APA-positive. Clinical data of the APA-positive patients are shown in Table 2.

Table 1: Clinical data of our research population

		CPHD	IGHD
Number of patients		31	40
Sex F/M		11 / 20	11 / 29
Age at start GH		3.7 (3.3)	6.2 (2.8)
HSDS at start GH		-2.8 (1.3)	-3.1 (1.2)
GH peak (Arg) mU/L		2.6 (2.4)	9.2 (6.6)
GH peak (Clon) mU/L		3.8 (2.4)	10.1 (6.4)
IGF-I SDS		-3.4 (2.4)	-3.2 (2.4)
Age at AB testing (y)		17.8 (8.0)	15.5 (6.1)
Known mutations (gene)		0	5 (*GH1*)
MRI	Normal	7 (23%)	20 (50%)
	Classic triad	11 (35%)	1 (2.5%)
	HAP and/or EPP	8 (26%)	12 (30%)
	Empty sella	3 (10%)	1 (2.5%)
	Other	1 (3%)	1 (2.5%)
	Not available	1 (3%)	5 (12.5%)

Arg = Arginine test, Clon = clonidine test, AB = antibody, HAP = Hypoplastic Anterior Pituitary, EPP = Ectopic Posterior Pituitary, classic triad = HAP, EPP and invisible pituitary stalk, SOD = Septo-Optic Dysplasia

APA positivity was mainly related to CPHD, especially to thyroid hormone deficiency and gonadotroph hormone deficiency in addition to GHD (Table 3). All 3 APA-positive patients who had reached pubertal age, required hormone suppletion to induce puberty, whereas, among 42 APA-negative patients of pubertal age, only 13 required induction of puberty (100% *vs* 31%, p=0.039). All APA-positive and -negative patients had GH deficiency, since this was one of the inclusion criteria of our study.

In 5 of 8 APA positive patients, enough serum was available to perform antibody characterisation after initial screening and titration of the antibodies. Results of the characterisation are shown in Table 2.

Table 2: Clinical data of APA-positive patients

Pt	APA titre (cells targeted by APA)	Diagnosis	Hormonal deficiencies (age start suppletion)	MRI	Phenotypic abnormalities	Peak GH (mU/L)	IGF-I SDS	HSDS start GH
1	>1:8 (GH and LH/FSH prod cells)	CPHD	GH (2.2 y), Thyr (0.1 y), Cort (0.1 y), prepubertal age, PRL normal	Triad	DF, IAF	5.6	-1.8	-2.0
2	>1:8 (TSH and LH/FSH prod cells)	CPHD	GH (0.8 y), Thyr (0.4 y), Cort (0.4 y), prepubertal age, PRL normal	Triad	MP	13.0 (age 0.5 y)	-0.4	-2.1
3	>1:16 (GH, TSH, LH/FSH and PRL prod cells)	CPHD	GH (1.1 y), Thyr (1.1 y), Cort (1.1 y), prepubertal age, PRL normal	Triad	MP	25.2 (age 0.1 y)	-9.0	-2.7
4	>1:16 (TSH, LH/FSH and PRLprod cells)	CPHD	GH (5.1 y), Thyr (12.4 y), Gon (18.8 y), PRL normal	Small pituitary	-	4.0	-	-3.9
5	>1:8	CPHD	GH (4.4 y), Thyr (2.9 y), Cort (4.2 y), Gon (15.4 y), PRL normal	Normal	SMMCI, MP	2.8	-	-4.9
6	>1:8	CPHD	GH (4.3 y), Thyr (3.7 y), prepubertal age, PRL normal	Normal	FB, DF, IAF	3.6	-6.2	-2.5
7	>1:16 (GH, TSH and PRL prod cells)	IGHD	GH (2.4 y), prepubertal age, PRL normal	NA	-	5.6	-4.7	-2.7
8	>1:8	CPHD	GH (4.3 y), Thyr (5.7 y), Gon (15.4 y), PRL normal	NA (CT normal)	DF, IAF	0.01	-	-4.0

FB= frontal bossing, DF= Doll's face, IAF = increased abdominal fat, MP= micropenis, SMMCI = single median maxillary central incisor, NA = not available

Table 3: Clinical data of APA-positive vs. APA-negative patients

		APA pos	N	APA neg	N	*p*
Age at start GH		3.1 (1.7)	8	5.4 (3.3)	63	0.06
HSDS at start GH		-3.1 (1.1)	8	-3.0 (1.3)	63	0.77
GH peak (Arg)		3.2 (2.1)	5	7.7 (6.6)	41	0.14
GH peak (Clon)		4.5 (1.5)	2	8.6 (6.3)	35	0.37
IGF-I SDS		-4.4 (3.4)	5	-3.2 (2.3)	54	0.28
Thyroid suppletion	**no**	13%	1	49%	31	
	yes	87%	7	51%	32	0.065
Corticosteroid suppletion	**no**	50%	4	70%	44	
	yes	50%	4	30%	19	0.42
Induction of puberty	**no**	0%	0	69%	29	
	yes	100%	3	31%	13	0.039
	prepubertal		5		21	
Prolactin levels	**normal**	100%	8	83%	34	
	low	0%	0	7%	3	0.45
	high	0%	0	10%	4	
	NA				22	
Pituitary MRI	**Classic triad**	50%	3	12%	7	0.046
	Other anomalies	17%	1	45%	26	
	Normal	33%	2	43%	25	

Data are shown as mean (SD) NA = not available. GH = growth hormone, HSDS = height SDS, Arg = arginine test, clon = clonidine test, SDS = standard deviation score

APA prevalence was higher in CPHD patients than in IGHD patients: of 31 CPHD patients screened for APA, 7 had APA (23%). However, of 40 IGHD patients screened for APA, only 1 was positive for these antibodies (2.5%, p=0.018). In total, 88% of APA-positive patients had CPHD (7 out of 8 patients), whereas only 38% (24 of 63) of the APA-negative patients had CPHD (p=0.018).

In one of the eight APA-positive patients, a mutation in *GH1* (P89L) was detected prior to APA screening. In the 63 APA-negative patients, 4 patients had mutations in *GH1* (p=0.44).

We related MRI findings of the pituitary with presence of APA and found that the classic triad of a hypoplastic anterior pituitary (HAP), ectopic posterior pituitary (EPP) and invisible pituitary stalk was more frequent in APA-positive patients than in APA-negative patients (50% *vs.* 12%, p=0.046). We did not find any association between the presence of an empty sella and APA-positivity.

Discussion

Our study shows antibodies against pituitary cells in 23% of the patients with idiopathic CPHD and in 2.5% of the patients with idiopathic IGHD. In patients with CPHD, APA positive patients had, next to GHD, mostly thyroid and gonadotroph hormone deficiency. Four of eight patients received corticoid hormone suppletion.
Our results indicate that in 23% of patients diagnosed with 'idiopathic' CPHD had antibodies against pituitary cells, which indicates that they had a silent form of autoimmune hypophysitis (AIH). There are different mechanisms by which autoimmunity can cause tissue damage. On one hand, macrophages can be activated by T-helper cells, causing damage to the target tissues. On the other hand, T-cells can help self-reactive B cells which can initiate autoantibody responses, thereby also causing damage to the target tissue.

Various authors have studied the role of different types of pituitary antibodies in patients with several disorders, often with controversial results[35-54].

De Bellis *et al.* (2006) were the first to provide substancial evidence for the relationship between GHD and the presence of APA at high titres (>1:8)[31]. They found APA against GH-producing cells at high titers in 27% of children with apparently idiopathic GHD. They did not find any APA in patients with secondary forms of GHD caused by lesions or abnormalities of the pituitary or hypothalamus.

APA are considered markers of pituitary autoimmunity. Since they do not have a direct pathogenic role, it was not surprising that there was no strict correlation between the pituitary hormone impairment and the pituitary cells targeted by APA in our population.

Cases in which a certain pituitary hormone axis is deficient but the corresponding antibody is not detectable, could be explained by the long-term duration of the pituitary impairment, with subsequent disappearance of APA or presence of APA at low titers (<1:8). APA are then no longer detectable, while the deficiency does persist. In our patients, 50% of the APA positive patients had ACTH deficiency, without presence of APA targeting ACTH secreting cells. Since ACTH deficiency is often the earliest deficiency to occur in AIH[21], the absence of APA might be explained by this mechanism.

On the other hand, the presence of APA in patients in which the pituitary hormone levels are (still) normal, suggests a subclinical form of autoimmune hypophysitis that could evolve towards a clinical pituitary hormone impairment. Also, antibodies against PRL secreting-cells, have been frequently observed in different forms of pituitary autoimmune involvement without clinical and biochemical modificaton of prolactin secretion[34].

Although some cells targeted by the APA did not correspond to the pituitary hormone deficiency, there was a clear relation between the overall clinical picture of our patients, and the presence or absence of APA. APA were related to CPHD as opposed to IGHD, especially to thyroid hormone deficiency and gonadotroph deficiency in addition to GHD. Furthermore, the classic triad of a hypoplastic anterior pituitary, ectopic posterior pituitary and invisible pituitary stalk was slightly more frequent in APA-positive patients than in APA-negative patients, but small sample size makes it hard to draw conclusions from this finding.

We were surprised to find APA against GH, TSH and PRL producing cells in one IGHD patient who also had a *GH1* P89L mutation. The two older sisters of this patient both had the same *GH1* P89L mutation but did not have APA. Mullis *et al* previously suggested that in patients with certain *GH1* mutations, macrophages killing the mutant somatrotropes could also kill pituitary cells located nearby, a process called bystander killing[55,56]. The fact that, in this *GH1* P89L family, the child with the most recent onset of GH deficiency had APA whereas his older sisters with longer duration of GH deficiency were APA-negative, might suggest involvement of autoimmunity in this process, although the exact mechanism is unknown.

In summary, we found APA in 23% of the CPHD patients and in 2.5% of the idiopathic IGHD patients. Although screening of a larger CPHD population is indicated to confirm the clinical relevance of Anti Pituitary Antibodies, their frequent presence at high titers in our patients with idiopathic CPHD suggests autoimmune involvement in quite a large number of these patients. Therefore, screening for APA should be considered in all patients with 'idiopathic' CPHD.

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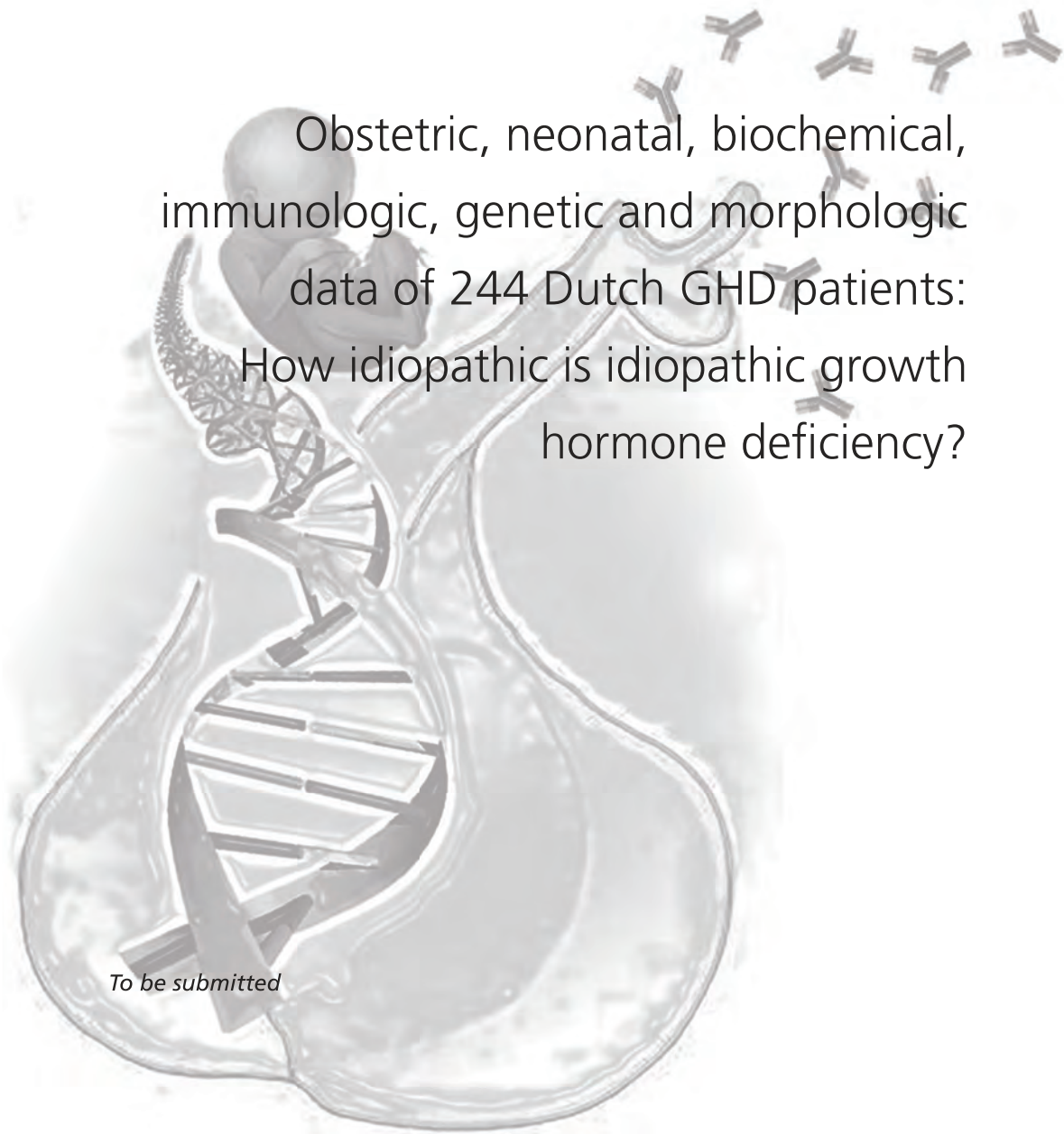
Chapter 6



Obstetric, neonatal, biochemical, immunologic, genetic and morphologic data of 244 Dutch GHD patients: How idiopathic is idiopathic growth hormone deficiency?

To be submitted

Abstract

Background: In the majority of patients with hypopituitarism, the cause of their clinical picture is unknown. With regard to the aetiology of hypopituitarism, three major hypotheses exist: the (embryo-)genetic, birth trauma and the autoimmune hypothesis.

Method: We analysed obstetric, neonatal, biochemical, immunologic, genetic and morphologic data of 244 patients with Isolated Growth Hormone Deficiency (IGHD) or Combined Pituitary Hormone Deficiency (CPHD) in order to assess the number of cases that could be explained by the three hypotheses.

Results: Clinical data of 19% of the IGHD patients and 12% of the CPHD patients pointed towards an (embryo-)genetic cause. In 17% of the IGHD patients and 32% of the CPHD patients, clinical data suggested that birth trauma could have caused their deficiencies. 2.5% of IGHD patients and 23% of CPHD tested for Anti Pituitary Antibodies (APA) were APA positive, suggesting an autoimmune process was involved in their condition.

Conclusion: Although in individual patients, different pathogenic mechanisms may play a role, causing an overlap between the three groups, clinical data pointing towards an (embryo-)genetic, birth trauma-related or autoimmune-related explanation for hormonal deficiencies were present in 36% of the patients with 'idiopathic' IGHD and 67% of patients with 'idiopathic' CPHD. Our data suggest that the aetiology of IGHD and CPHD is multifactorial, with an important proportion of underlying birth traumas.

Introduction

Growth hormone deficiency (GHD) can either occur as an isolated deficiency (Isolated Growth Hormone Deficiency or IGHD), or in combination with deficiencies of either TSH, ACTH, LH/FSH, or prolactin (Combined Pituitary Hormone Deficiency or CPHD). Mutations in the gene encoding the GH protein (*GH1*) and the GHRH-Receptor (*GHRHR*) explain only a minority of IGHD cases in the Netherlands. In CPHD patients, the disorder can be the result of genetic defects in transcription factors involved in the fetal organogenesis of the pituitary, like *PROP1*, *HESX1*, *POU1F1*, *LHX3* and *LHX4*. However, in the majority of IGHD and CPHD patients, the cause of their condition is unknown, which is unsatisfactory for both patients and physicians.

There are several hypotheses concerning the aetiology of IGHD and CPHD, their association with pituitary anomalies and the relation with clinical parameters[1-21]. Hypotheses related to the aetiology of idiopathic hypopituitarism can be divided in two major groups: the (embryo-)genetic hypothesis, according to which the prenatal organogenesis of the pituitary is assumed to have failed. The other hypothesis is the birth trauma hypothesis, in which a traumatic (breech) delivery is thought to have caused (partial) rupture of the pituitary stalk, sometimes with subsequent regeneration of the neuropituitary at the base of the pituitary stalk, causing an ectopic posterior pituitary (EPP). Recently, anti-pituitary antibodies (APA) were found in patients with idiopathic IGHD and CPHD[22,23]. As a result, the autoimmune hypothesis was added to the (embryo-)genetic and birth trauma hypotheses.

To our knowledge, a detailed study of clinical data of a large population of well-documented IGHD and CPHD patients has never been performed in order to find arguments to accept or reject the various hypotheses. We analysed obstetric, neonatal, biochemical, immunologic, genetic and morphologic data of 244 patients with idiopathic IGHD and CPHD in order to assess in how many cases, clinical data were present pointing towards an (embryo-)genetic, birth trauma-related or autoimmune cause.

Patients and methods

Patients with idiopathic IGHD or CPHD from six university and two regional hospitals in the Netherlands, who participated in the Dutch Hypothalamic and Pituitary Gene study, were included in the analysis. Patients with GH deficiency of known cause, such as a brain tumour, brain surgery, brain radiation, and patients with known syndromes were excluded from the study.

Patients' obstetric, neonatal, biochemical and morphologic data were collected from the Dutch National Registry of Growth Hormone Treatment, where clinical and laboratory parameters of 2500 GH-treated Dutch patients have been registered. Mutation screening of *GH1* and *GHRHR* had previously been performed in 108 IGHD patients and of *PROP1, HESX1, POU1F1, LHX3* and *LHX4* in 78 CPHD patients, using dHPLC and DNA sequencing. 71 patients (40 IGHD and 31 CPHD) had been previously screened for APA by the immunofluorescence method. Of 39 patients, no genetic or immunologic results were available, but detailed biochemical, ostetric, neonatal and morphologic data were available for analysis.

We used Descriptive statistics (T-test, Chi-square, ANOVA and Pearson's correlation of SPSS version 11.0) to analyse relations between obstetric, neonatal, biochemical, immunologic, genetic and morphologic data of the patients. Clinical findings were grouped by aetiologic hypothesis they represented: the presence of mutations known to cause IGHD or CPHD, the presence of congenital facial midline defects (suggestive of disturbed craniofacial development), and the presence of affected first-degree relatives (FDR) were classified as indicators of an (embryo-)genetic aetiology. Since the presence of a micropenis at birth is suggestive of prenatal hormone deficiencies, we analysed associations between micropenis and other clinical parameters, and we described them in the Embryogenesis section. However, we did not consider it an indicator of an (embryo-)genetic aetiology. Birth trauma was suspected in case the delivery was complicated: a history of vaginal breech delivery, a history of perinatal hypoxia or a combination of both, was classified as indicator of a birth trauma related cause of hormone deficiencies. Patients were said to have a history of perinatal hypoxia when in the patients history an annotation was present of asphyxia, hypoxia, intubation, resuscitation, artificial respiration, apnea, respiratory arrest, respiratory problems, bronchopulmonary hypo- or dysplasia, or respiratory insufficiency.

The presence of APA was classified as indicator of an autoimmune process. Neonatal jaundice and hypoglycaemia could be explained by either a traumatic birth or a congenital defect, so these phenomena were excluded from the analysis.

Results

We included 129 IGHD patients from 121 families. Eight families had two affected children and one family had three affected children. We included 115 CPHD patients from 113 families. Patients' clinical data are shown in Table 1.

MRI was available for 97 IGHD and 99 CPHD patients. Pituitary anomalies in relation to hormone deficiencies of the CPHD patients are shown in Table 2. The

classic triad of a hypoplastic anterior pituitary (HAP), ectopic posterior pituitary (EPP) and invisible or interrupted stalk (IPS) was present in 3.1% of IGHD patients vs. 19.2% of CPHD patients (p=0.0004).

Table 1: Auxological characteristics of 244* IGHD and CPHD patients.

		N	Mean	SD
Birth weight SDS	IGHD	83	-0.9	1.3
	CPHD	67	-0.6	1.5
Birth length SDS	IGHD	109	-0.3	1.2
	CPHD	95	-0.3	1.3
Gestational age (weeks)	IGHD	117	39.0	2.6
	CPHD	105	39.2	2.6
CA-BA (years)	IGHD	128	1.6	1.3
	CPHD	115	1.5	2.0
Arginine test peak GH (mU/L)	IGHD	105	9.4	6.6
	CPHD	62	5.0	6.5
Clonidine test peak GH (mU/L)	IGHD	82	11.3	6.4
	CPHD	37	4.6	3.7
IGF-I SDS	IGHD	111	-3.5	2.5
	CPHD	77	-4.4	2.8
Age at start GH (years)	IGHD	128	6.3	3.5
	CPHD	113	5.5	7.0
HSDS at start GH	IGHD	128	-3.2	1.2
	CPHD	111	-3.2	1.5

CA-BA = delay in boneage * Due to missing data, patient numbers do not always add up to 244, SDS = standard deviation score

CPHD patients with pituitary anomalies had a higher frequency of thyroid hormone deficiency (99% *vs.* 85%, p=0.06), hypocortisolism (86% *vs.* 31%, p=0.00001) and hypogonadotrope hypogonadism (88% *vs.* 63%, p= 0.07) than patients with a normal pituitary on MRI (Table 2).

An overview of the proportion of the different aetiologic hypotheses among the patients is given in Figure 1.

(Embryo-)genetic hypothesis

Eleven IGHD patients (9%) had a mutation in *GH1*, whereas only one patient with CPHD had a mutation in *POU1F1*. Three IGHD patients and nine CPHD patients

had midline defects, with varying degrees of pituitary abnormalities and variable hormone deficiencies.

Table 2: Pituitary morphology and hormonal deficiencies of 99 CPHD patients

		Normal pituitary	Classic triad	Other pituitary anomalies[1]	$P_{norm\text{-}abn}$	$p_{triad\text{-}other}$
Thyr	**y**	11	18	67	0.06	NS
	n	2	1	0		
Cort	**y**	4	18	56	0.00001	NS
	n	9	1	11		
Gon	**y**	5	7	35	0.07	NS
	n	3	2	4		
PRL	**N**	4	13	43	NS	NS
	L	2	3	8		
	H	2	2	4		
DI	**y**	2	1	4	NS	NS
	n	11	18	62		

Thyr= hypothyroidism, Cort= hypocortisolism, Gon= hypogonadotrope hypogonadism, PRL= prolactin: N= normal, L = low, H = high, DI= diabetes insipidus, y = yes, n = no, 1Other pituitary anomalies= any combination of hypoplastic or aplastic AP, hypoplastic, aplastic or ectopic PP, thin, thick, interrupted or invisible PS and (p)ES, not being the classic triad $p_{norm\text{-}abn}$ = significance level of the difference between patients with normal *vs.* abnormal pituitary, $p_{triad\text{-}other}$ = significance level of the difference between patients with the classic triad *vs.* otherwise abnormal pituitary

Of 103 IGHD patients with a known family history, 21 (20%) had an affected FDR. Of 82 CPHD patients, 6 (7%) had an affected FDR (p=0.002). Eleven patients had an affected FDR but did not have any mutation. The presence of an affected FDR was associated with a lower frequency of macroscopic pituitary anomalies on MRI; in IGHD patients this is explained by the fact that 11 of the patients with affected FDR had mutations in *GH1*, which usually do not cause pituitary abnormalities. Among CPHD patients, 2 of 5 patients with affected FDR had an abnormal neuropituitary vs. 57 of 66 CPHD patients without affected FDR (p=0.031).

Since the presence of a micropenis at birth is suggestive of prenatal hormone deficiencies, we evaluated the presence of micropenis in our patients. 'Micropenis' or 'very small penis' was reported in 14% of the male IGHD patients *vs.* 37% of the male CPHD patients (p=0.0005). IGHD patients with micropenis at birth were significantly more likely to have *GH1* mutations than patients without micropenis (25% *vs.* 4%, p=0.030), with a corresponding more severe phenotype. However, when the patients with *GH1* mutations were left out of the analysis, the presence of a micropenis at birth was not associated with a more severe phenotype.

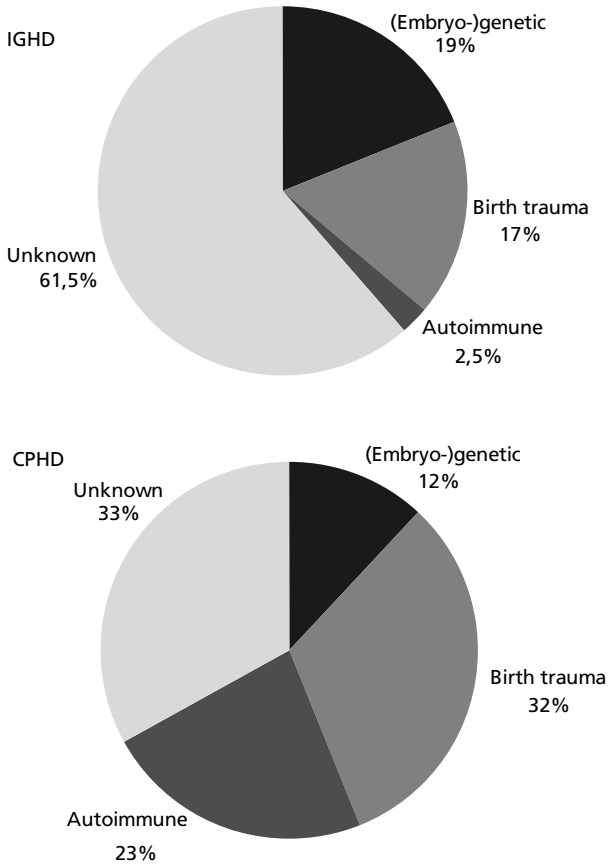


Figure 1: Possible explanation for the clinical picture of 129 IGHD and 115 CPHD patients participating in the HYPOPIT study. (Embryo-)genetic= embryonic or genetic.

Among the CPHD patients with pituitary anomalies, micropenis was especially associated with the classic triad: 71% of 14 patients with the classic triad had a micropenis, *vs.* 34% of 47 patients with other pituitary abnormalities (p=0.0013). The presence of a micropenis seemed to be associated with an uncomplicated delivery: of 29 CPHD patients with a micropenis at birth, 14 (48%) had a delivery complicated by breech presentation or perinatal hypoxia, whereas of 50 CPHD patients with a normal phallus at birth, 33 had a complicated delivery (48% *vs.* 66%, p=0.12).

Micropenis was associated with adrenocorticotroph deficiency: 28 of 29 CPHD patients with micropenis (97%) needed corticosteroid suppletion, whereas of CPHD patients without micropenis, only 66% needed corticosteroid suppletion (p=0.002). CPHD patients born with a micropenis started GH replacement at an earlier age (3.2

y vs. 6.8 y, p=0.015), and also required thyroid hormone (2.6 y *vs.* 5.4 y, p=0.022) and corticosteroid hormone (4.1 y *vs.* 7.4 y, NS) suppletion at an earlier age than patients who were born without a micropenis.

Birth trauma hypothesis

Delivery details were available for 95 IGHD and 98 CPHD patients (Figure 2). Figure 3 gives an overview of the clinical differences between patients born by head delivery, vaginal breech delivery and caesarean section (CS) because of breech presentation. Of the 60 breech presenting patients who were born by vaginal delivery, 45 had CPHD versus 15 IGHD (p=0.0002), whereas of 7 breech-presenting patients who were delivered by CS, only 3 had CPHD (75% *vs.* 43%, p=0.2).

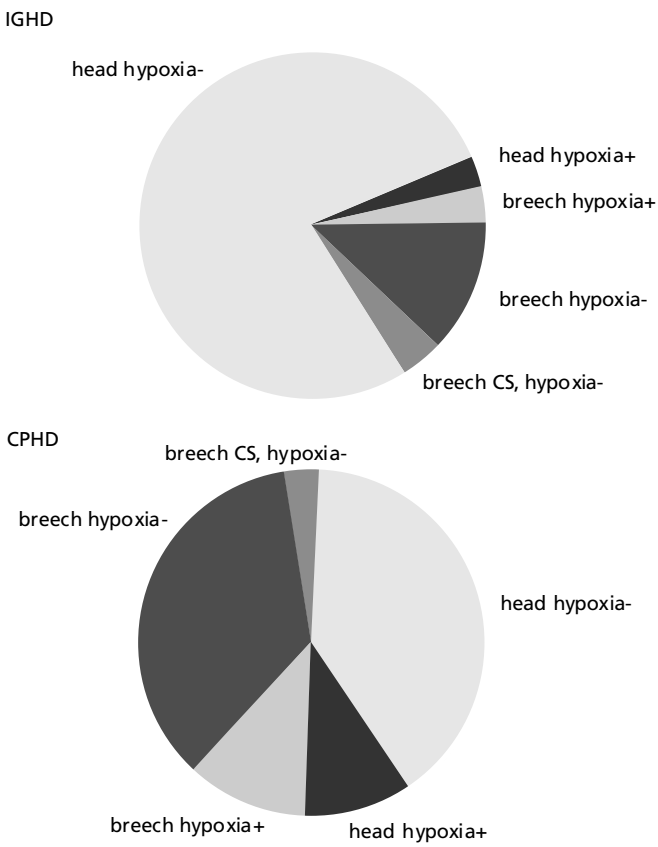


Figure 2: Birth data of 129 IGHD and 115 CPHD patients, CS = caesarean section

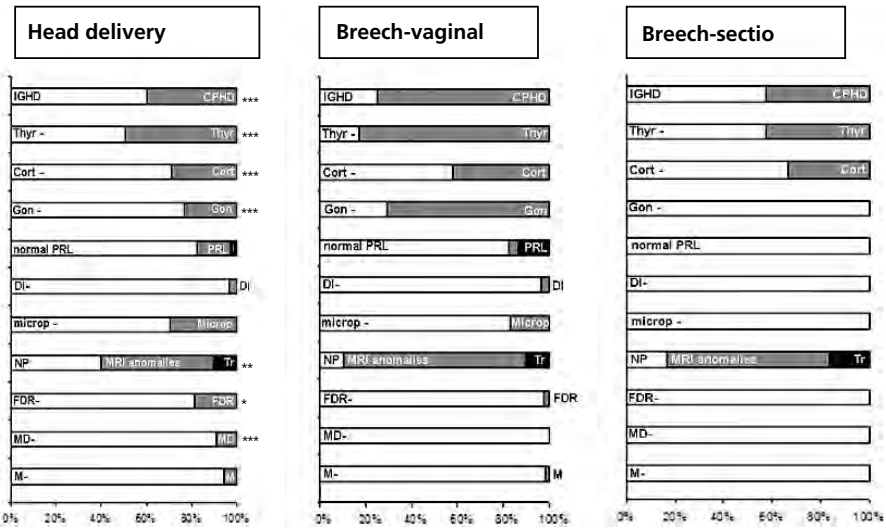


Figure 3: Birth data of 129 IGHD and 115 CPHD patients related to clinical data

Thyr (-) = (no) thyroid hormone suppletion, Cort (-) = (no) adrenocortical hormone suppletion, Gon (-) = (no) induction of puberty, PRL = prolactin (white bar = normal PRL, grey bar = low PRL; black bar = high PRL), DI (-) = (no) diabetes insipidus, microp (-) = (no) micropenis, NP = normal pituitary on MRI, Tr = classic triad, MRI anomalies = MRI anomalies other than the classic triad, FDR (-) = (no) affected first-degree relatives, MD (-) = (no) midline defect, M (-) = (no) mutations, *** $p<0.001$; ** $p<0.01$ * $p<0.05$ (shown for the differences between patients born by head delivery and those born by vaginal breech delivery)

In IGHD patients, we did not find any association between pituitary image and the mode of delivery. In CPHD patients, however, anomalies of the posterior pituitary (PP) were more frequent in patients born by vaginal breech delivery. Of 39 patients born by vaginal breech delivery, 36 (91%) had an abnormal posterior pituitary (26 had an ectopic, 3 a small and 7 an invisible PP), whereas of 42 head-presenting CPHD patients, 32 (76%) had an abnormal PP (25 had an ectopic, 2 a small and 5 an invisible PP, $p<0.05$). Of 33 breech-presenting patients born by vaginal delivery, 27 had an abnormal pituitary stalk, whereas of 5 breech presenting patients delivered by CS, only 1 had an abnormal pituitary stalk (82% *vs.* 20%, $p=0.12$).

Breech delivery was associated with hypogonadotrope hypogonadism: 29 of 31 patients (94%) born by vaginal breech delivery required induction of puberty, versus 16 of 24 patients (67%) born by head delivery ($p=0.015$). Four of our CPHD patients had hyperprolactinaemia and deficiencies of all anterior pituitary cell lineages. All four patients were born by vaginal breech delivery.

Among 129 IGHD patients, 8 (6%) suffered from perinatal hypoxia, whereas among 115 CPHD patients, 25 (22%) suffered perinatal hypoxia. In the total group

of IGHD and CPHD patients with available obstetric data, breech presentation and hypoxia were clearly related: of 28 patients with perinatal hypoxia, 15 (54%) were born by breech delivery. Of 164 patients without perinatal hypoxia, only 45 (27%) were born by vaginal breech delivery (p=0.009) Of 7 breech presenting patients born by CS, none suffered perinatal hypoxia (Figure 2).

Perinatal hypoxia was associated with corticotroph deficiency. Of 26 (both head- and breech delivered) CPHD patients who suffered perinatal hypoxia, 25 needed corticosteroid suppletion (96%), whereas of patients who did not suffer perinatal hypoxia, only 70% needed corticosteroid suppletion (p=0.007). Also when breech- and head-presenting patients were analysed separately, perinatal hypoxia was associated with corticotroph deficiency: all of the twelve patients born by vaginal breech delivery with perinatal hypoxia had corticotroph deficiency, versus 23 of 33 patients born by vaginal breech delivery without perinatal hypoxia (p=0.031).

Autoimmune hypothesis

APA prevalence was higher in CPHD patients than in IGHD patients: of 31 CPHD patients screened for APA, 7 had APA against various pituitary cells, whereas of 40 IGHD patients screened for APA, only one patient, who also had a *GH1* mutation, was positive for these antibodies (23% *vs.* 2.5%, p=0.018). We related MRI findings of the pituitary with APA positivity and found that the classic triad of a hypoplastic anterior pituitary (HAP), ectopic posterior pituitary (EPP) and invisible pituitary stalk was significantly more frequent in APA-positive patients than in APA-negative patients (50 *vs* 12%, p=0.046).

Discussion

We analysed obstetric, neonatal, clinical, immunologic, biochemical, genetic and morphologic data of 244 patients with idiopathic IGHD or CPHD in order to assess the number of cases that could be explained by the three major hypotheses concerning the aetiology of hypopituitarism.

(Embryo-)genetic hypothesis

According to the (embryo-)genetic hypothesis – currently the most widely accepted theory to explain hypopituitarism – a genetic defect or a defect during embryonic pituitary organogenesis is responsible for abnormalities of the hypothalamo-hypopituitary axis. This theory is supported by the fact that other cerebral developmental abnormalities are often observed in association with the pituitary abnormalities, and by the existence of familial cases of GH deficiency[24-26].

In children with midline defects, the hormonal deficiencies might be caused by a disturbed embryonic craniofacial development, which could also affect pituitary development. The presence of FDR with GHD is highly suggestive of a genetic component in GHD and therefore, next to the presence of genetic defects, these features were classified as indicators of an (embryo-)genetic cause of GHD.

Although it can not be considered purely indicative of an embryogenetic cause of GHD, the presence of a micropenis at birth is thought to be caused by inadequate testosterone levels during fetal life, caused by gonadotropin deficiency prior to delivery[27,28]. This implies that it is not the result of a complicated delivery. In our population, patients with a micropenis were indeed more often born by an easy, non-complicated head delivery than patients with a normal phallus length at birth.

Birth trauma hypothesis

According to the birth trauma hypothesis, vaginal breech deliveries may lead to transsection of the pituitary stalk[13,29-31], resulting in a partial or total loss of the connection between hypothalamus and pituitary. The hypothalamus contains neurotransmitters and polypeptides that inhibit prolactin release. Damage to the pituitary stalk has been shown to result in increased prolactin release and sometimes in initiation of lactation[32,33]. We were therefore interested in patients with high PRL levels and deficiencies of all other hormonal axes, which might indicate complete disconnection between pituitary and hypothalamus. Four of our CPHD patients fulfilled these criteria and they were all born by vaginal breech delivery (2 with, and 2 without perinatal hypoxia).

The percentage of breech deliveries in the normal Dutch population is 8.2%, with a variation among hospitals of 4.2–12.4%[34], whereas in our CPHD population 36% was born by breech delivery. It is uncertain whether breech delivery is the consequence of abnormal intra-uterine development or rather the cause of pituitary damage. The idea that vaginal breech delivery is the cause of CPHD, is supported by the fact that our breech-presenting patients born by vaginal delivery had a higher frequency of CPHD than the breech-presenting patients born by caesarean section (CS). Of the 60 breech presenting patients who were born by vaginal delivery, 75% had CPHD, whereas of the breech-presenting patients who were delivered by CS, only 43% had CPHD. Although patient numbers were small and data should be interpreted with caution, this difference of 32% may indicate that 32% of the CPHD cases is caused by the vaginal breech delivery. These findings are in accordance with the findings of Maghnie *et al.*[35], who found that their patients with the classic triad, who were born by vaginal breech delivery (68%) all had CPHD, whereas caesarean or head delivery in such patients was followed by IGHD only.

In the Netherlands, vaginal delivery in case of breech presentation was a common practice, until the publication the results of the Term Breech Trial[36]. Before 2000, roughly 25% of all term breeches were delivered by planned CS, 25% by emergency CS during trial of labour and 50% were delivered vaginally. Within 2 months following publication of the results of the Term Breech trial, the total CS rate for term breeches increased from 50% to 80%, due to an increase of the planned elective CS to 50%[37]. However, the Netherlands still have a low frequency of CS when compared to other countries[38,39]. Due to small sample size, we cannot prove a direct causal relation between vaginal breech delivery and the occurrence of CPHD. However, based on our data we strongly suspect that the high frequency of vaginal breech deliveries in our population is the cause, rather than the consequence of hypopituitarism.

In our population, 6% of the IGHD patients had perinatal hypoxia versus 22% of the CPHD patients, whereas prevalences of perinatal hypoxia range from 0.5 to 5% in the normal population[40-43]. Perinatal hypoxia is a prolonged anoxic state during or after delivery that can be due to compression of the umbilical cord or respiratory problems that limit oxygen intake. Perinatal hypoxia of any origin may result in hypoxic lesions in the brain, including damage of hypothalamus and pituitary. Inamo *et al.*[44] described the relation between disturbed blood supply to the pituitary and disturbed pituitary anatomy. Taking into acount the severe neurologic damage that has been described in children who suffered perinatal hypoxia, we believe that part of the hormonal deficiencies might well be the consequence of perinatal hypoxia.

Autoimmune hypothesis

The autoimmune hypothesis was recently added to the embryogenetic and birth trauma hypotheses when APA were found in patients with idiopathic IGHD and CPHD[22]. APA were present in 23% of our CPHD patients and in 2.5% of our IGHD patients tested for these antibodies.

Possible explanations for the hormonal deficiencies were found in half of the IGHD and CPHD patients. Among IGHD patients, 19% had findings suggestive of a disturbed embryogenesis and 17% had findings pointing towards a birth trauma-related cause, whereas the remaining 64% of the cases was truly idiopathic. Among CPHD patients, 12% had findings suggestive of a disturbed embryogenesis. Since 75% of the breech presenting patients born by vaginal delivery had CPHD, versus 43% of the breech-presenting patients who were delivered by CS, the difference of 32% of the CPHD cases may be caused by the vaginal breech delivery. Of the patients tested, 23% had APA suggesting an autoimmune pathogenesis. Since the patients tested for APA were randomly selected from the total study population, this APA

frequency is reprentative for all CPHD patients. The remaining 33% of the CPHD cases was truly idiopathic.

In summary, clinical data pointing towards an (embryo-)genetic, birth trauma-related or autoimmune-related explanation for hormonal deficiencies were present in 36% of the patients with 'idiopathic' IGHD and 67% of patients with 'idiopathic' CPHD. Although the literature suggests that pituitary antibody formation does not occur secondary to pituitary damage but rather as a primary autoimmune process[22], pituitary antibodies might be present in patients with certain genetic variations or in those born by vaginal breech delivery. Therefore, in individual patients, different pathogenic mechanisms might overlap, resulting in lower percentages than 36 and 67%, respectively. Concluding, our data suggest that the aetiology of IGHD and CPHD is multifactorial, with an important proportion of underlying birth traumas. Although breech presentation could be the consequence of abnormal intra-uterine development, our data suggest that vaginal breech delivery is rather the cause of CPHD, because breech-presenting patients born by vaginal delivery have CPHD more often than breech-presenting patients born by caesarean section. However, due to small sample size, we cannot prove a direct causal relation between vaginal breech delivery and the occurrence of CPHD. Before we can recommend caesarean section in all breech-presenting deliveries, it is necessary to perform a prospective study of the occurrence of hypopituitarism in a large population of children born by vaginal breech delivery, in comparison with breech-presenting patients delivered by caesarean section and those born by uncomplicated head delivery.

Acknowledgements

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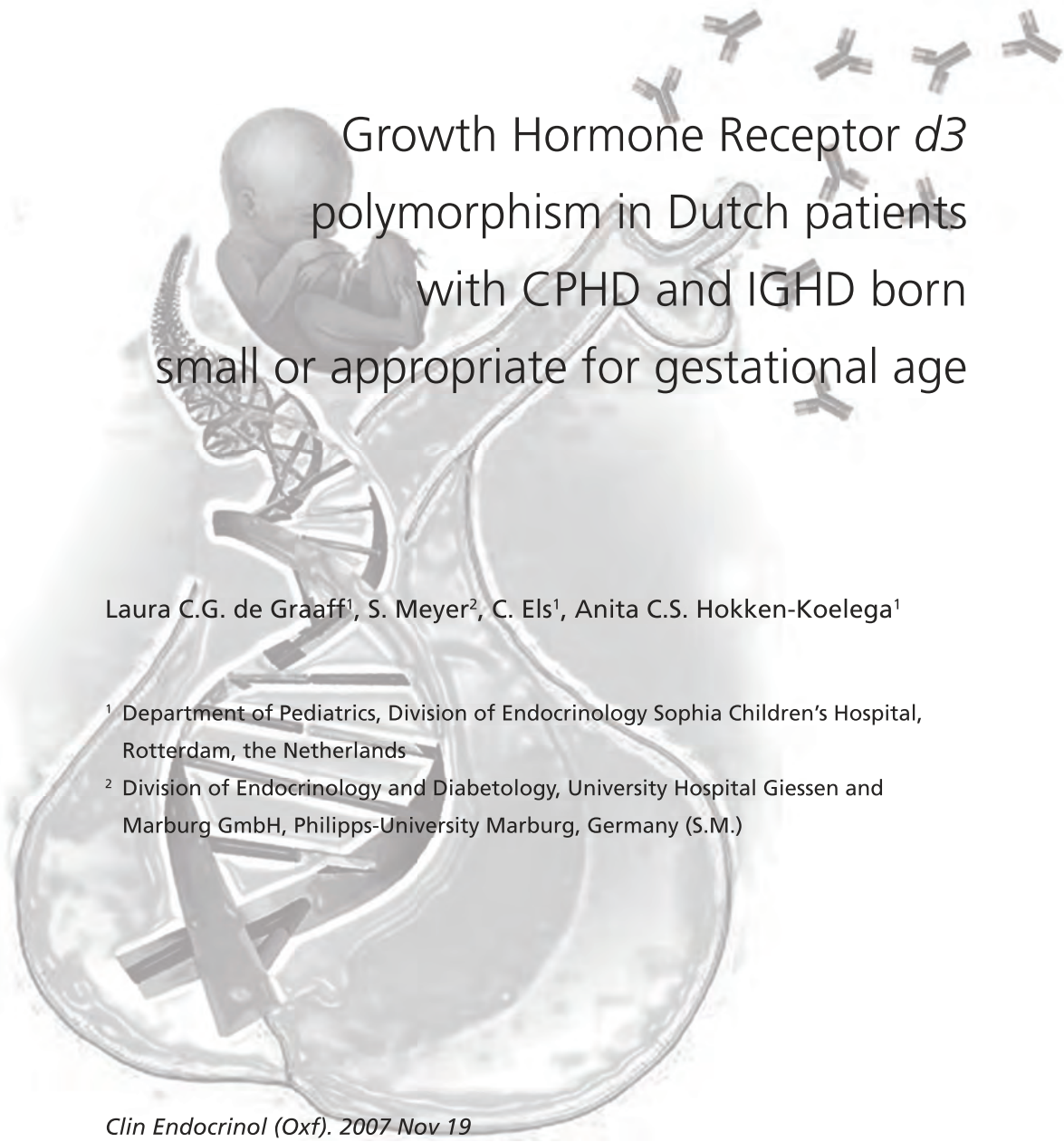
Chapter 7

Growth Hormone Receptor *d3* polymorphism in Dutch patients with CPHD and IGHD born small or appropriate for gestational age



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Abstract

Objective: Growth hormone (GH) acts through the GH receptor (GHR). The *GHR* gene contains a genetic polymorphism caused by a deletion of exon 3 (*d3*), with high frequency in the normal population. There is a continuing controversy whether the presence or absence of the exon 3 deletion (*d3+ vs. d3-)* affects the effect of GH in human growth.

Design, patients and measurements: For 144 patients with idiopathic Isolated Growth Hormone Deficiency (IGHD, n=77) or Combined Pituitary Hormone Deficiency (CPHD, n=72), amplification of the region around exon 3 of the *GHR* gene was performed. Clinical data and response to GH treatment were compared between *GHR d3+* and *d3-* IGHD and CPHD patients born either Small for Gestational Age (SGA) or Appropriate for Gestational Age (AGA).

Results: IGHD patients born SGA had a significantly higher *d3+* frequency (82%) than IGHD patients born AGA (34%). Within the group of IGHD patients born SGA, *d3-* patients showed a slightly better spontaneous catch up growth before start of GH treatment than *d3+* patients (1.1±1.1 SD *vs.* 0.6±1.1 SDS, p=0.040). There was no difference in patients first year's response to GH treatment between *GHR d3+* and *d3-* patients.

Conclusions: In IGHD and CPHD patients, response to GH treatment was independent of *GHR* genotype. *GHR d3* was significantly more frequent among IGHD patients born SGA. Since we are the third to report an association between birth size and *GHR d3* status, it is conceivable that the *GHR d3* might affect prenatal growth in IGHD patients by a yet unknown mechanism.

Introduction

Growth Hormone Deficiency (GHD) can occur either as isolated GH deficiency (IGHD), or in combination with other pituitary hormone deficiencies (Combined Pituitary Hormone Deficiency or CPHD). The response to GH treatment is variable. Variables associated with adult height of GH-treated patients with GHD, are duration of GH treatment, height SDS at the start of treatment, bone age delay at start of GH treatment, height at onset of puberty, midparental height, growth velocity during first year of GH treatment, age at start of GH treatment and peak GH levels during stimulation tests[1-4]. These variables, however, explain only part of the wide variation in response to GH treatment in children with GHD.

GH acts at the target cell through the GH receptor (GHR)[5]. After binding to GHR, GH induces activation of the JAK/ STAT pathway, leading to an increased expression of *IGF-I* and other GH-dependent genes. The human GH receptor (GHR) gene is located at the 5p13-p12 chromosomal region. It contains nine coding exons (exons 2–10): exon 2 codes for the signal peptide, exons 3–7 encode the extracellular domain, exon 8 encodes the transmembrane domain, and exons 9 and 10 the cytoplasmic domain[6]. The GHR is a 620-amino-acid single-transmembrane protein. The *GHR* gene contains a genetic polymorphism caused by a deletion of exon 3 (*GHR d3*)[7]. This polymorphism is present in heterozygosis in 40% of the healthy population, and in homozygosis in 15%[8]. It results in the loss of amino acid residues 7–28 and the amino-acid substitution A6D at the N-terminal part of the extracellular receptor domain[7]. *In vitro*, it does not appear to alter binding of GH to the GHR[9,10]. However, it has been speculated that AA 6-28 may play a role in the conformational changes during transactivation of the GHR dimer by GH[11]. On the other hand, effects on gene transcription, RNA splicing, protein stability and glycosylation as well as an effect on transport of the GHR protein to the cell membrane might be possible.

There is a continuing controversy whether the *d3* polymorphism affects the effect of GH in human growth and whether this could explain the variable response to GH treatment[8,11-15]. Various authors have studied the influence of the *GHR* exon 3 deletion in patients with either GHD[13-15] or SGA[8,11,12] with controversial results. To our knowledge, the combination of GHD and SGA has never been investigated. The aim of this study was to relate birth weight and birth length, height SDS, serum GH and IGF-I levels, spontaneous growth and response to GH treatment to *GHR* genotype in 144 patients with IGHD and CPHD born either AGA or SGA

Patients and methods

Patients

We included 144 GH deficient patients with IGHD and CPHD, based on inclusion criteria of Height SDS <-2 SDS at start of GH treatment, GH peak during provocation tests <20 mU/L and IGF-I SDS or IGFBP-3 SDS <-2. Exclusion criteria were: GH deficiency of known cause, such as a brain tumour, brain surgery, brain radiation, diabetes mellitus, syndromes or chromosomal disorders. IGHD patients with mutations in *GH1* and *GHRHR* were excluded, as well as CPHD patients with mutations in *PROP1, HESX1, POU1F1, LHX3* or *LHX4*. All patients received GH treatment at replacement dose. All hormone deficiencies additional to GHD were appropriately suppleted. We obtained approval from the medical ethics committees of all participating hospitals. Informed consent was obtained from all participating patients and their parents, if patients were aged less than 18 years.

Methods

Genomic DNA was extracted from samples of peripheral venous blood according to standard procedures. A 38-cycle amplification of the region around exon 3 of the GHR gene was performed using specific primers G1, G2 and G3 (GenBank accession no. AF155912) that amplify a fragment of 935 bp for the full length *(fl)* allele and 532 bp for the *d3* allele. To obtain maximum genotyping certainty, three PCRs were performed for each patient: two PCRs using primer pairs G1-G2 and G1-G3 and a triplex PCR using primers G1, G2 and G3. Samples were analysed by gel electroforesis using a 1% agarose gel stained with ethidium bromide.

Statistical analysis

Patients' clinical data were collected from the Dutch National Registry of Growth Hormone Treatment[16]. SGA was defined as BL-SDS and/or BW-SDS <-2 below the mean weight for gestational age according to intrauterine growth charts of Usher and McClean[17]. Patients were grouped as IGHD-SGA, IGHD-AGA, CPHD-SGA or CPHD-AGA. The growth response during GH treatment was calculated as the increase in height SDS (ΔHSDS) during the first year of treatment[18]. Height SDS, peak GH levels during provocation tests, IGF-I SDS, birth weight (BW), birth length (BL) and ΔHSDS were compared between IGHD and CPHD patients born SGA or AGA, according to *GHR* exon 3 genotype. Patients heterozygous and homozygous for the *d3* allele (*fl/d3* and *d3/d3*, respectively) were grouped together *(d3+)* for comparison with patients with fl/fl genotype *(d3-).*

Backward multiple regression analysis was used to assess predictors for BW and BL. Independent variables were gestational age in weeks, sex (1 = male, 2 = female)

and the presence of one or more *d3* alleles (*fl/fl* = 0, *d3/d3* or *fl/d3* = 1). Genetic and clinical data were analysed using Chi-square and ANOVA. If expected cell counts for comparison of *d3*+ frequencies between different subgroups of patients were below 5, we used the Fisher's exact test instead of the Chi-square test. The normality of the distribution of analyzed parameters was tested with the Kolmogorow-Smirnow and Shapiro–Wilk tests. Parameters that were not normally distributed were analysed using the non parametric Kruskall-Wallis test instead of ANOVA. Statistics were performed using the computer statistical package SPSS (version 11.0.1; SPSS Inc., Chicago, IL). The level of significance was determined at $p<0.05$.

Results

The overall percentages of the three *GHR* genotypes in the total group of 144 IGHD and CPHD patients, were *fl/fl* 43%, *fl/d3* 47% and *d3/d3* 9%. All growth-related parameters were normally distributed, except GH levels during GH stimulation test and increase in height SDS (ΔHSDS) during the first year of treatment.

BW in kg was associated with gestational age in weeks (Odds ratio (B) = 0.2, standard error (SE) for B 0.02, 95% CI for B 0.13 to 0.18; $p<0.001$), sex (B = -0.3, SE(B) 0.091, 95% CI –0.5 to –0.1; $p=0.002$) and presence of one or more *d3* alleles (B = -0.3, SE(B) 0.09, 95% CI –0.4 to –0.1; $p=0.002$). This model explained 48% of the variance of BW.

BL in cm was associated with gestational age in weeks (B = 0.74, SE(B) 0.1, 95% CI 0.54-0.94; $p<0.001$) and sex (B = -1.2, SE(B) 0.5, 95% CI –2.1 to –0.3; $p=0.01$), but only weakly with the presence of one or more *d3* alleles (B = -0.8, SE(B) 0.4, 95% CI –1.7 to 0.1; $p=0.08$). This model explained 38% of the variance of BL.

Among IGHD patients, *d3*+ frequency was significantly higher in patients born SGA than in patients born AGA (82% *vs.* 35%, $p=0.006$, Figure 1). Within the IGHD SGA groups, there was no difference in *d3*+ frequency between the patients who had only BL-SDS below -2 and those who had both BL-SDS and BW-SDS below –2. IGHD patients born SGA had higher GH levels during provocation tests than IGHD patients born AGA (12.9±5.5 vs. 7.5±4.2 mU/L, $p=0.003$).

Among CPHD patients, *d3*+ frequency was not significantly different between patients born AGA and SGA. The frequency of the *d3/d3* genotype appeared to be higher in SGA patients (14.3% *vs.* 7.9%), but this was not statistically significant ($p=0.79$). Among patients born AGA, *d3*+ frequency was higher in CPHD patients than in IGHD patients (60.5% vs. 34.5%, $p=0.035$), which was due to a higher frequency of the *d3/fl* genotype (52.6% vs. 24.1%, $p=0.060$). Among SGA patients, *d3*+ frequency was comparable for patients with IGHD and CPHD.

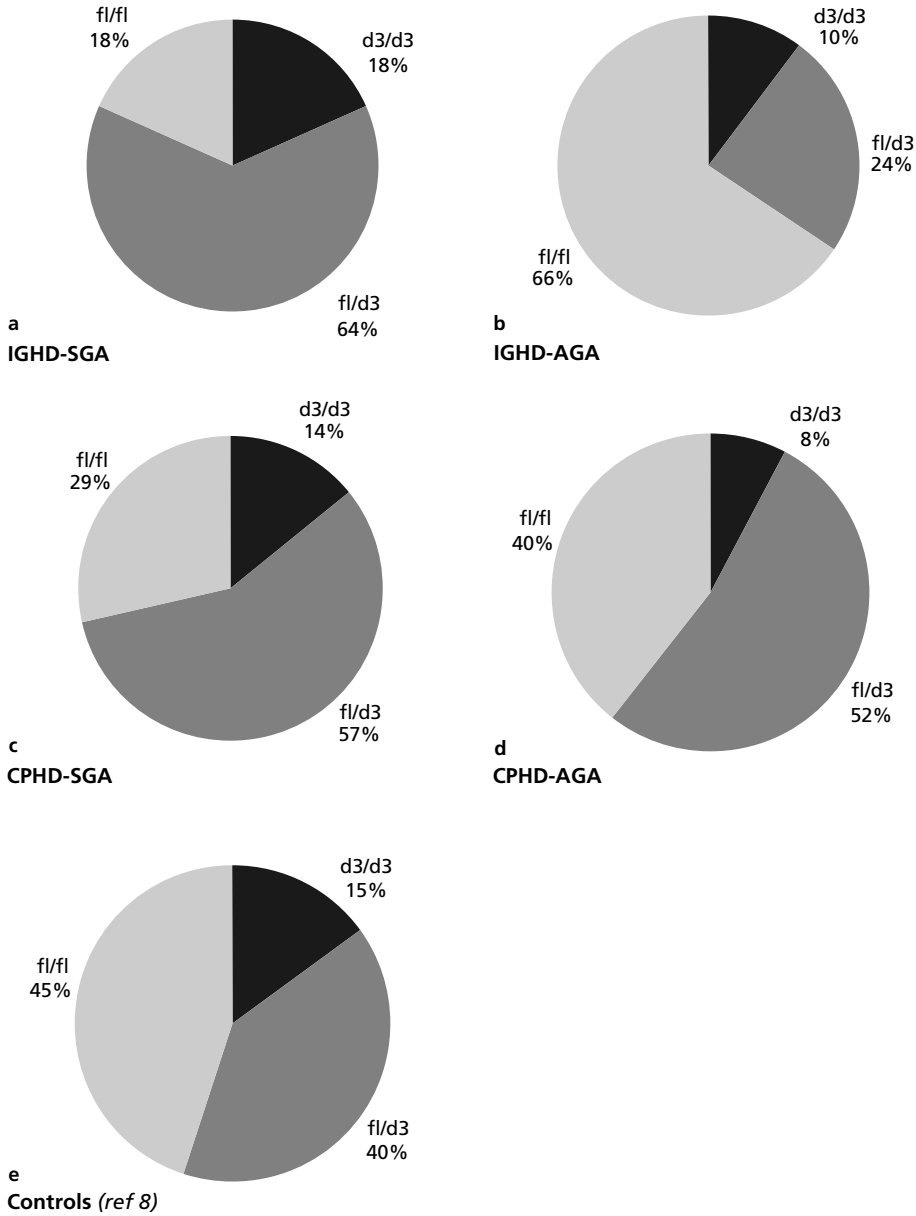

Figure 1: *GHR* genotype frequencies among IGHD and CPHD patients born SGA and AGA a) IGHD-SGA (n=11) b)IGHD-AGA (n=19) c)CPHD-SGA (n=7) d) CPHD-AGA (n=38) e) healthy controls as reported by Binder, 2006

Table 1: Clinical data of *d3+* and *d3-* IGHD and CPHD patients born AGA or SGA

	IGHD				CPHD						
	AGA		SGA		AGA		SGA				
	29		11		38		7				
	d3-	***d3+***	***d3-***	***d3+***	***d3-***	***d3+***	***d3-***	***d3+***	***p AGA vs. SGA***	***p d3- vs d3+***	***p IGHD vs CPHD***
Number	19	10	2	9	15	23	2	5	NS	NS	NS
Gestational age	38.8(2.9)	39.0(3.5)	39.0(0)	39.2(1.6)	40.4(1.2)	40.1(1.6)	38.3(3.9)	40.0(1.2)	NS	NS	p=0.012
BL-SDS	-0.7(1.0)	-0.4(0.9)	-3.0(1.5)	-2.5(0.8)	-0.3(1.1)	-0.7(1.0)	-2.8(1.0)	-2.6(0.6)	p<0.001	NS	NS
BW-SDS	0.0(1.1)	0.2(0.7)	-1.6(0.9)	-1.9(1.0)	0.0(1.2)	-0.6(0.8)	-1.2(1.5)	-2.3(0.8)	p<0.001	p<0.010[2]	NS
HSDS at start GH	-3.3(0.8)	-3.4(1.0)	-2.4(0.1)	-3.1(0.6)	-2.9(1.2)	-2.9(1.3)	-4.1(0.2)	-3.0(0.3)	NS	NS	NS
GH levels (mU/L)[4]	8.3(4.3)	6.3(4.0)	7.1(2.7)	14.3(5.1)	3.1(3.0)	4.4(3.6)	3.5(2.1)	4.7 (3.0)	p=0.048[3,4]	NS	p<0.001[4]
IGF-I SDS	-3.2(1.8)	-3.2(2.9)	-1.1(0.5)	-3.1(1.5)	-4.6(2.8)	-3.6(2.6)	-9.0[1]	-5.1(3.6)	NS	NS	p=0.062
Δ $HSDS_{birth\text{-}start\ GH}$	-2.6(0.9)	-3.0(1.3)	0.6(1.4)	-0.6(0.8)	-2.7(1.3)	-2.2(1.2)	-1.3(0.8)	-0.5(0.3)	$p<10^{-8}$	p=0.08	NS
Age at start GH	6.1(3.2)	4.7(2.8)	8.8(3.1)	4.4(2.7)	5.7(4.5)	4.2(4.8)	2.0(1.9)	6.4(3.4)	NS	NS	NS
Δ HSDS 1st yr GH[4]	0.9(0.8)	1.2(0.8)	1.0[1]	1.1(0.8)	1.8(1.5)	1.4(1.3)	NA	1.2(0.4)	NS	NS	NS

[1]only one patient tested, NS: p>0.10 [2]NS when SGA and AGA patients were analysed separately [3] only among IGHD patients, BL-SDS = birth length SDS, BW-SDS = birth weight SDS, $\Delta HSDS_{birth\text{-}start\ GH}$ = spontaneous increase in HSDS from birth to start GH, ΔHSDS 1st yr GH = increase in HSDS during the first year of GH treatment, [4]tested by non-parametric Kruskall-Wallis test due to non-normal distribution of the parameters

Clinical data of IGHD and CPHD patients born SGA or AGA are shown in Table 1, according to *GHR* genotype. We did not find any association between *GHR* genotype and birth weight or birth length within the SGA group.

Apart from birth size, SGA and AGA patients had comparable phenotypes.

Among IGHD patients born AGA, *d3*+ patients had lower GH levels than *d3*- patients, although the rest of the phenotype was not significantly different. We did not find any other significant phenotypic differences between patients with and without the *d3* allele in any of the other groups.

Within the group of IGHD patients born SGA, *d3*- patients showed better spontaneous catch up growth from birth until start of GH treatment than *d3*+ patients. Once GH treatment was started, we did not find any association between first year's response to GH treatment and *GHR* genotype in any of the groups.

Discussion

Although the genotypic distribution among the total group of IGHD and CPHD patients born either SGA or AGA was comparable with the frequencies reported by other authors, the frequency varied with hormonal deficiencies (IGHD *vs.* CPHD) and birth size (SGA *vs.* AGA). Among IGHD patients, *d3*+ frequency was significantly higher in patients born SGA than in patients born AGA. Among patients born AGA, *d3*+ frequency was significantly higher in CPHD patients than in IGHD patients.

The phenotype of patients born SGA was similar to the phenotype of patients born AGA, apart from birth size and the difference in GH level found between IGHD patients born SGA *vs.* AGA. Although patient numbers were relatively small, IGHD patients born SGA had a significantly higher *d3*+ frequency than patients born AGA. Within the group of IGHD patients born SGA, *d3*- patients showed better catch up growth from birth until start of GH treatment than *d3*+. However, the number of SGA patients was small, which makes it difficult to draw conclusions from this finding.

Various authors have investigated the response to GH in *d3*+ and *d3*- patients with GHD[13-15] and SGA[8,11,12], but their results were controversial. Our study made clear that, among patients with GHD, the *GHR d3* allele was associated with SGA and with a slightly lower spontaneous catch up growth. However, growth response to GH treatment was independent of *GHR* genotype.

Although *d3*+ frequency was higher in children born SGA, within the SGA group we did not find any association between *GHR* genotype and birth weight or birth

length. Tauber *et al.* reported an association between *d3*/*d3* genotype and a lower birth weight in short SGA children[19]. They did not find this association in healthy controls and concluded that the difference was unlikely due to the *d3* polymorphism. Their conclusion represents the common belief that, although IGF-I levels are known to be associated with fetal size, GH does not play any role in prenatal intra-uterine growth.

Also, Jensen *et al.*[20] reported decreased fetal growth in healthy subjects carrying the *d3* allele. They also showed that, in healthy subjects born SGA, presence of *d3* was associated with a decreased growth velocity in the third trimester of pregnancy. This suggests that GH does not only act postnatally but is also important for prenatal growth. It is conceivable that the *d3* variant is being activated by non-22K pituitary GH in utero, which would explain the association between *GHR* genotype and SGA or AGA status in IGHD babies.

Further support for a prenatal role of GH and GHR comes from the finding that, although hypophysectomised mouse, rat, pig and rabbit fetuses display almost normal intrauterine growth[21], birth size of humans with Laron-type dwarfism lacking GHR is significantly reduced. Also, the mean birth length of patients with idiopathic GHD is significantly lower than those of control age-matched neonates; between 12% and 20% are born SGA[22,23].

In rodents, GHR is expressed both in non-pregnant uteri and placental tissues[24-26], but in the bovine, GHR is only expressed in the pregnant uterus and not in the non-pregnant uterus[27]. These findings suggest that GHR is important for placental metabolism. During pregnancy, placental GH (GH-V) and human chorionic somatomammotropin (hCS) act together in the mother to enhance the availability of glucose and amino acids to the fetus[28]. Like pituitary GH, GH-V and hCS act through the GHR and therefore variations in the GHR might cause variations in fetal growth. Although GH, GH-V and hCS have comparable affinities for GHR and GHR*d3*[9,10], the lack of AA 6-28 might have a yet unknown impact on the effect of GH, GH-V and hCS on placental function and prenatal growth, for example by altering signal transduction.

In conclusion, although IGHD and CPHD patients born SGA do not clinically differ from patients born AGA, in our study IGHD patients born SGA had a significantly higher *d3*+ frequency than IGHD patients born AGA. Since we are the third to report an association between birth size and *GHR d3* status, it is conceivable that the *GHR d3* might affect prenatal growth in IGHD patients by a yet unknown mechanism.

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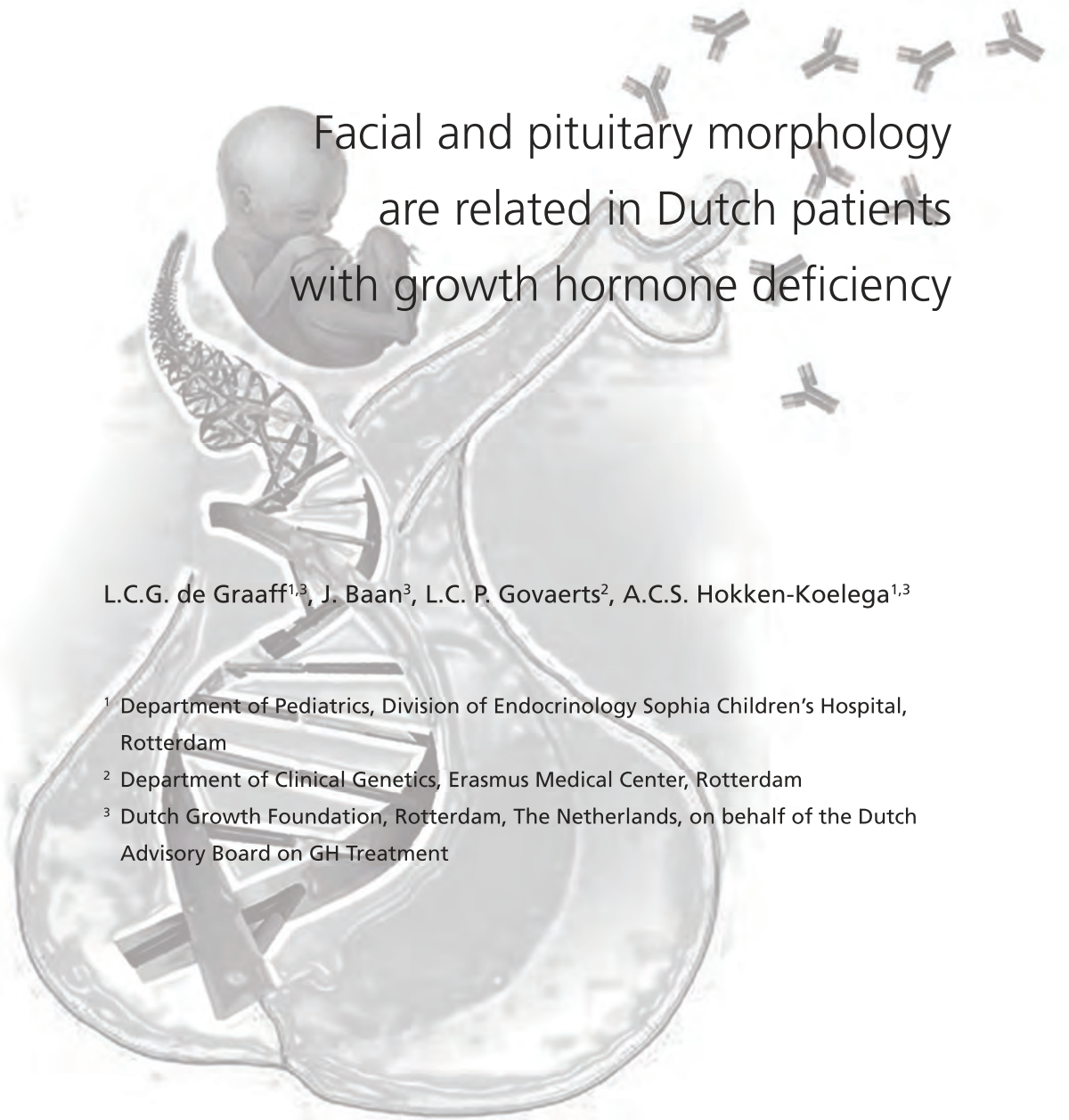
Chapter 8

Facial and pituitary morphology are related in Dutch patients with growth hormone deficiency



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Abstract

Objective: Classical growth hormone deficiency (GHD) is associated with typical phenotypic features. We have analysed standardised photographs of 137 Caucasian patients with GHD, in order to examine the relations between auxological, biochemical, pituitary and facial morphometric features.

Design, patients and measurements: We analysed pictures of 137 patients: 73 (55 M / 18 F) with Isolated GHD and 64 (48 M / 16 F) with Combined Pituitary Hormone Deficiency. Of each patient, standardized frontal and lateral digital pictures were taken and analysed using Adobe Photoshop 5.0.

Results: Canthal Index (CI), the relative distance between the eyes, was related to pituitary morphology. Patients with an Ectopic Posterior Pituitary (EPP) had significantly higher CI values than patients without EPP. We found CI>39 to be a good cut-off value to select children with highest probability of having EPP. The combination of CI>39 with the presence of hormonal deficiencies additional to GHD strongly predicted EPP: 93% of the patients with a CI>39 and additional hormonal deficiencies had EPP, in contrast to 77% of the patients with additional hormonal deficiencies but a CI <39, and 29% of the patients with none of these criteria (p=0.0001).

Conclusions: Canthal Index, measured on digital pictures, is associated with ectopia of the posterior pituitary and this might be caused by an altered midline development, affecting both the pituitary and the facial structures of GHD patients

Introduction

Classical Growth hormone deficiency (GHD) is associated with typical phenotypic features, like frontal bossing and a flattened nasal bridge causing a childish appearance, also called a 'doll's face'. Furthermore, hypertelorism, cleft lip and palate, single median maxillary central incisor (SMMCI) and other phenotypic variations have been described in patients with GHD[1].

Pre- and postnatal development of the craniofacial structures is extremely complex[2]. Congenital defects of the brain are often accompanied by minor or major facial anomalies[3-6].

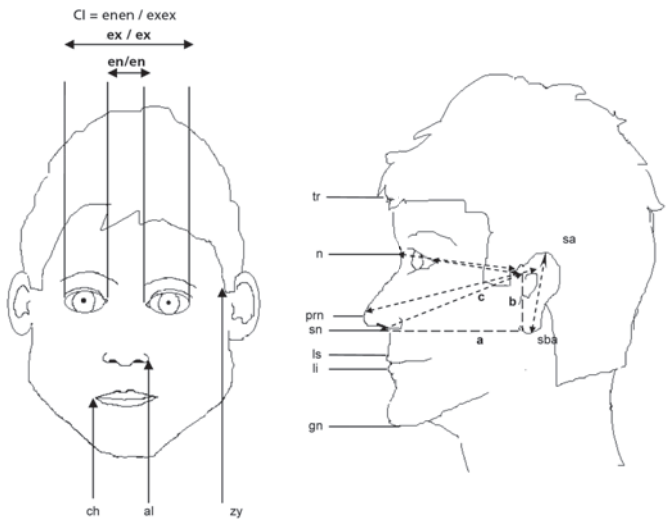
The Dutch HYPOthalamic and PITuitary gene (HYPOPIT) study is a multi-center study investigating genetic and other causes of 'idiopathic' growth hormone deficiency in the Dutch GHD population. We have analysed standardised photographs of 137 patients with Isolated GHD and Combined Pituitary Hormone Deficiency (CPHD), in order to investigate relations between auxological, chemical and morphometric data.

Patients and methods

85 IGHD patients and 62 CPHD patients, who were treated in the eight participating hospitals and who fulfilled the Dutch consensus criteria for GHD of either GH <20 mU/L or GH <30 mU/L combined with IGF-I <-2 SDS, agreed to participate in the study. All patients received GH treatment. We collected clinical data of the patients from the Dutch National Registry of Growth Hormone Treatment. We excluded patients with GH deficiency of known cause, such as brain tumour, brain surgery, brain radiation, diabetes mellitus, syndromes or chromosomal disorders. All IGHD and CPHD patients had been screened for *GH1, GHRHR* and *PROP1, POU1F1, HESX1, LHX3* or *LHX4*, respectively, and those with mutations were excluded. Finally, we included 137 patients. We obtained approval from the medical ethics committees of all participating hospitals. Informed consent was obtained from all participants and their parents if they were less than 18 years old. MRI data were available for 95 patients.

Skull X-rays of the patients were not available, but pictures were taken under standardised conditions in order to obtain comparable pictures of reliable quality. Pictures were taken using a Canon® 4.0 Megapixel digital camera. Pictures of 4 patients were excluded because of suboptimal quality due to movement artefacts. Adobe Photoshop version 5.0 was used for morphometric analysis. Facial

measurements of all pictures were performed by an independent observer (JB). The landmarks we used were those described by Farkas[7]. Horizontal and vertical lines were drawn and distance between the lines were measured (Figure 1, Table 1). Horizontal measurements were taken from frontal pictures; vertical and diagonal measurements were taken from lateral pictures, and all measurements were adjusted to a relative internal scale, in order to prevent bias that can occur by flexion or rotation of the neck. Canthal Index, the relative distance between the eyes, was defined as inner canthal distance divided by outer canthal distance, multiplied by 100 (Table 1). The squared background plate was used to position pictures parallel to the lower extreme of the computer screen, according to the method used by Bishara[8]. The mean of two corresponding measurements of the same patient was taken together from right and left lateral photos, and used as a single measurement.



	Frontal view		Lateral view
ex	exocanthion	tr	trichion
en	endocanthion	n	nasale
al	alare	sn	subnasale
ch	cheilion	ls	labrale superius
p	pupil*	li	labrale inferius
zy	zygion	gn	gnathion
		sa	superaurale
		sba	subaurale
		prn	pronasale

Figure 1: Anthropometric landmarks for frontal and lateral facial photographs.
Solid lines and arrows represent vertical and horizontal measurements. Dashed arrows indicate diagonal measurements and dashed lines are shown to illustrate the diagonal measurement method (in this example sn-zy is calculated by taking the root of c^2, which is the sum of the squares a^2 and b^2). *not shown in figure.

Table 1: Distances between landmark lines and their physical meaning.

Measurement	Synonym
Horizontal[1]	
En-en / ex-ex * 100	Canthal Index
En-en	Inner canthal distance
Ex-ex	Outer canthal distance
Zy-zy	Total facial width
En-en / zy-zy	Relative inner canthal distance
Ex-ex / zy-zy	Relative outer canthal distance
P-p / zy-zy	Relative interpupillary distance
Ex-en / zy-zy	Relative eye width
Al-al / zy-zy	Relative nose width
Ch-ch / zy-zy	Relative mouth width
Vertical[2]	
Tr-gn[3]	Total facial height
Tr-n / tr-gn	Relative forehead height
N-sn / tr-gn	Relative nose length
Sn-ls / tr-gn	Relative philtrum length
Li-gn / tr-gn	Relative chin length
Diagonal	
Ex-zy / zy-zy	Relative upper facial depth 1
N-zy / zy-zy	Relative upper facial depth 2
Prn-zy / zy-zy	Relative nose depth
Sn-zy / zy-zy	Relative midfacial depth
Sa-sba / tr-ls	Relative ear height

[1]All vertical and diagonal measurements are divided by total upper facial width (zy-zy), [2]All vertical measurements were divided by total facial height (tr-gn), [3]Total facial height is calculated as the sum of the distance from medial hairline to upper lip and from lower lip to chin. The distance between upper and lower lip was not included in total facial height, to prevent errors that can occur when patients do not keep their jaws totally closed.

We used SPSS 11.0 to assess the correlation of photographic measurements with growth- related parameters (GH levels, IGF-I SDS, IGF-BP3 SDS and Ht SDS at start GH) and T-tests or crosstabs with Chi-square analysis to assess differences between patients with and without CI levels above the cut-off value, and to assess differences between patients with and without EPP.

Results

We analysed pictures of 137 patients; 73 (53%, 55 M / 18 F) had IGHD and 64 (47%, 48 M / 16 F) had CPHD. All 73 IGHD patients fulfilled Dutch consensus criteria for IGHD of either GH < 20 mU/L or GH < 30 mU/L combined with IGF-I <-2 SDS. However, nineteen patients had GH levels higher than 10 mU/L and IGF-I levels >-2 SDS. These patients could have normal variant short stature instead of IGHD and are considered as a separate group.

Patients were divided into male and female children, adolescent and adult groups (Table 2). The male predominance in our patients was in accordance with that in the Dutch GHD population. Since the groups of females were too small to obtain sufficient power, we only report results from the total group of 137 patients (if measurements were neither age- nor sex- related), and separate results from males (if measurements were sex-related).

Table 2: Canthal Index by age and gender groups

	N	Mean	SD	Minimum	Maximum	*p*
Girls <12 y	10	37.9	2.0	34.8	41.6	0.90
Boys <12 y	35	37.7	2.7	31.4	43.0	
Girls 12-18 y	11	37.1	2.5	32.6	41.7	0.38
Boys 12-18 y	30	38.0	2.7	31.5	43.4	
Women >18 y	14	36.2	3.6	30.0	42.2	0.73
Men >18 y	25	36.6	2.9	30.2	41.8	

p= level of significance for differences in CI between males and females

The relative nose length, philtrum length and chin length were positively related with age. The horizontal facial measurements were not related with age. CI was not correlated with any growth parameter (GH levels, IGF-I SDS, IGF-BP3 SDS or Ht SDS) but it was significantly related with MRI findings: patients with an ectopic posterior pituitary (EPP) had significantly higher CI values (CI 38.2±2.7) than patients who had the posterior pituitary (PP) in the normal position (36.9±2.9, $p<0.05$, Figure 2). CI was not related to any other MRI finding. CI values were normally distributed. CI was neither age-related ($r=-0.06$, $p=0.52$) nor significantly different between males and females (Table 2).

We found CI>39 to be the best cut-off value to select children with highest probability of having EPP (85% of patients with CI>39 had EPP **vs.** 56% of children

with CI <39, p=0.011, Table 3). The presence of hormonal deficiencies additional to GHD, next to a CI>39 increased the predictive value. Among the patients for whom MRI was available, 13 of 14 patients with a CI>39 and additional hormonal deficiencies had EPP (93%), in contrast to 27 of 35 with additional hormonal deficiencies but a CI <39 (77%), and 6 of 21 with none of these criteria (29%, p=0.0001). Among patients with CI>39, those who had deficiencies additional to GHD, all had two or more additional deficiencies.

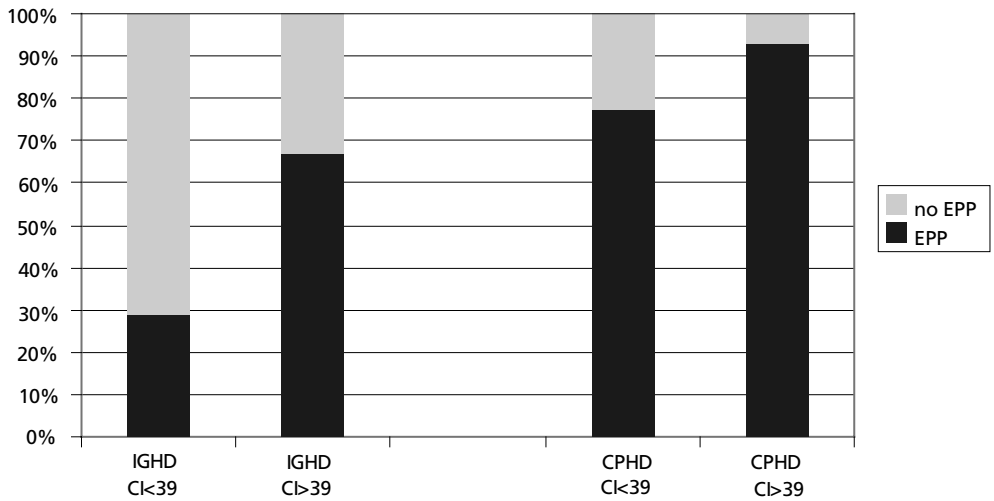


Figure 2: Percentage of ectopic posterior pituitary among IGHD vs. CPHD patients with canthal index below and above 39.

IGHD = Isolated Growth Hormone Deficiency, CPHD = Combined pituitary hormone deficiency, EPP =Ectopic Posterior Pituitary

EPP occurred in combination with normal anterior pituitary (AP) in 16 patients, with a small AP in 31 patients and with an invisible AP in 2 patients. CI was only associated with EPP; abnormalities of the anterior pituitary or pituitary stalk were not predicted by CI.

Patients with EPP had significantly lower GH levels and lower IGF-I SDS than patients without EPP (p<0.05). Patients with EPP required more thyroid hormone suppletion (74% *vs.* 32%), more corticosteroid suppletion (62% *vs.* 3%) and more induction of puberty (72% *vs.* 9%) than patients without EPP (all p values $<10^{-6}$). There was no difference in frequency of hypoprolactinamia or diabetes insipidus between patients with and without EPP.

Of the nineteen patients with possible normal variant short stature, two had pituitary abnormalities on MRI: both had a hypoplastic AP, one combined with

EPP and one combined with an invisible PP. The percentage of MRI abnormalities between IGHD patients and patients with possible normal variant short stature was not statistically significant (p=0.16)

Table 3: Clinical data of patients with Canthal Index above and below 39

		CI<39	CI>39
Sex		65 M/28 F	25 M/7 F
Diagnosis		44 CPHD / 49 IGHD	15 CPHD / 17 IGHD
Age at date photo		15.5 (7.6)	16.7 (11.0)
Age at start GH (y)		5.5 (3.6)	6.3 (8.0)
HSDS at start of GH		-3.0 (1.1)	-3.1 (1.3)
GH peak levels (mU/L)[1]		8.3 (6.7)	9.2 (7.1)
IGF-I SDS		-3.7 (2.7)	-3.4 (2.4)
Number of deficiencies additional to GHD			
(N) (%)	1	5/44 (11%)	0/15
	2	18/44 (41%)	8/15 (53%)
	3	20/44 (45%)	7/15 (47%)
	4	1/44 (2%)	0/15
Hypothyroidism (N) (%)		51/87 (59%)	16/30 (53%)
Hypocortisolism (N) (%)		31/87 (36%)	12/30 (40%)
Hypogonadotrope hypogonadism (N) (%)		20/52[2] (39%)	9/17[2] (53%)
Hypoprolactinaemia (N) (%)		8/58[3] (14%)	1/21[3] (5%)
Diabetes insipidus (N) (%)		1/87 (1%)	1/30 (3%)
MRI (N) (%)[4]	Stalk	29 (49%)normal	8 (35%) normal
		4 (7%) thin	4 (17%) thin
		26 (44%) invisible / interrupted	11 (47%) invisible / interrupted
	AP	30 (48%) normal	11 (39%) normal
		31 (49%) small	16 (57%) small
		2 (3%) absent	1 (4%) absent
	PP	26 (44%) normal	4 (15%) normal
		33 (56%) ectopic	22 (85%) ectopic

[1]Peak GH levels during Arginine or Clonidine test, [2]percentage based on number of patients that had reached pubertal age, [3]percentage based on number of patients in whom prolactin levels were available, AP = anterior pituitary, PP = posterior pituitary, p=level of significance, [4]some MRI reports did not explicitly report on stalk, AP and PP abnormalities separately but only described the parts that were abnormal. Therefore MRI numbers do not always add up to N=95, the total number of MRI reports available. Apart from the difference in frequency of EPP (p= 0.011), there were no other significant differences between the patients with CI < 39 and those with CI > 39.

Discussion

We report the results of facial morphometry based on standardized frontal and lateral photographs, and their relation with clinical data. A canthal index greater than 39 was associated with ectopia of the posterior pituitary.

Canthal Index (CI), the relative distance between the eyes, is useful for off-clinic analysis of photographs[9] and is defined as inner canthal distance / outer canthal distance x 100[10].

Although morphologic data specific for Dutch controls were not available, hypertelorism is generally defined as CI higher than 42, and hypotelorism as CI lower than 38[10]. However, our study shows that more subtle variations within the normal range can be clinically relevant.

Patients with an ectopic posterior pituitary (EPP) had significantly higher CI values than patients who had a normally positioned PP, and this could not be explained by a difference in age or sex distribution between both groups. GH therapy has been shown to affect craniofacial growth[11,12] but duration and dose of GH therapy was comparable for patients with or without a CI>39, which means that the differences in CI can not be explained by differences in duration and dose of GH treatment. All patients were treated with GH, so we can not conclude about any relation between CI and EPP in patients who were not treated with GH.

Our impression from the clinic is that GH treatment reduces the relative distance between the eyes in GHD patients. In our study, one in every four patients had relatively high CI despite GH treatment. We suspect that the relation between CI and EPP might be even stronger when pictures are taken prior to GH treatment. To test this, we plan to take pre-treatment digital pictures of newly diagnosed GHD patients in the future.

The combination of CI>39 and the presence of hormonal deficiencies additional to GHD strongly predicted ectopia of the PP. Ninety-three percent of patients with a CI>39 and hormonal deficiencies additional to GHD had EPP, in contrast to 77% of those with additional hormonal deficiencies but a CI<39, and 29% of those with none of these criteria. Thus, measurement of CI might be useful in the clinical setting. However, we recommend only photogrammetric measurements of the face to obtain CI. Facial morphometry based on standardized frontal and lateral photographs is an accurate method[8]. Since direct measurement from the patients face are dependent on patient behavior and the need for the patient to keep still for long periods, direct measurements are subject to high intra- and inter observer variability, especially when children are being examined[13,14]. This variability does not apply to photogrammetric measurements.

Pituitary abnormalities have been associated with midline facial abnormalities. The absence of the pituitary gland was reported in relation with nasal pyriform aperture stenosis[15], whereas duplication of the pituitary was accompanied by cleft palate in 19 of 31 cases (61%)[16]. Absent pituitary gland was described in a patient with depressed nasal bridge and a high arched palate, abnormalities of eyes and eyelids, and a prominent and high forehead.[6]

The relation between the development of the facial midline and the development of the brain has been subject of investigation[2,17,18]. Well-known genes like Sonic Hedgehog (*Shh*) and *Pax6* are involved in development of the midline facial structures[19-22]. *Shh* deficiency states are associated with variable degrees of midline defects and in extreme cases with cyclopia. *Pax6* deficient mice are anosmic due to absence of olfactory epithelium. Both *Shh* and *Pax6* are also important for pituitary proliferation. Furthermore, the role of *Hox* and *Gli* genes in facial morphogenesis and in development of the brain and pituitary is subject of ongoing study[23-29]. Our findings are in line with the associations found by others. However, the exact genetic and developmental relationship between pituitary and facial morphology remains to be established.

To our knowledge, we are the first to investigate the relation between morphometric and clinical data in such detail and this is the first study in support of a common developmental cause for facial and pituitary morphology variations. Ectopia of the posterior pituitary is associated with an increased Canthal Index and this might be caused by an altered midline development, affecting both the pituitary and the facial structures of GHD patients.

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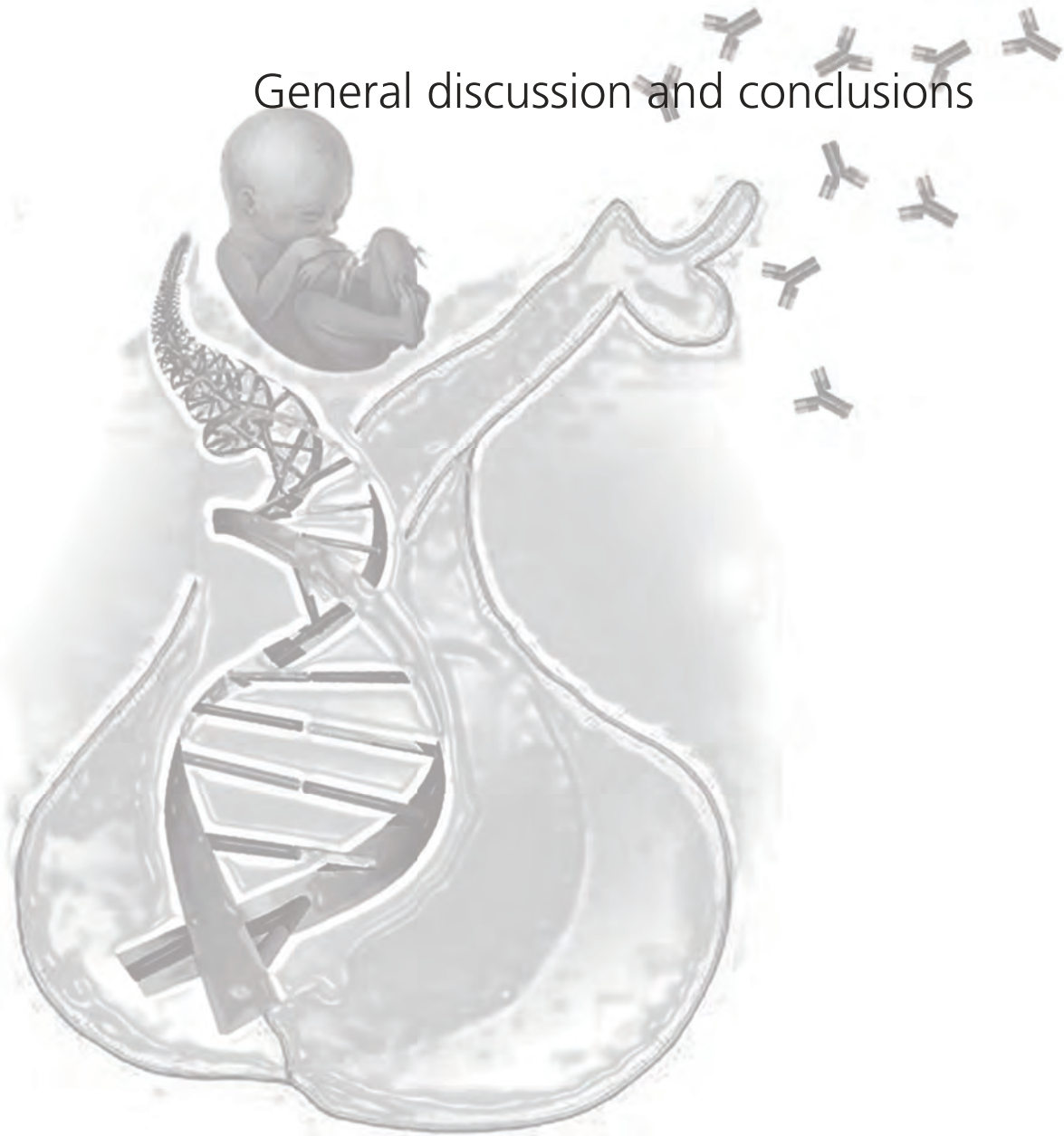
Chapter 9

General discussion and conclusions



This doctoral dissertation describes in detail various studies undertaken to clarify the aetiology of IGHD and CPHD. The studies were mostly initiated in 2003 and concluded in 2007. We studied a nation-wide cohort of patients with IGHD and CPHD from the Netherlands. The following discussion seeks a relationship between the various findings as such and in the context of literature data. The questions and hypotheses at the start of the studies, as listed in the Introduction (Chapter 1) are addressed, and new hypotheses and recommendations for future research are given.

9.1 Screening for mutations in coding regions and splice sites of *GH1* and *GHRHR* in IGHD patients

Five different mutations in *GH1*, which were previously reported in the literature to cause IGHD, were identified in 9% of the Dutch IGHD patients (6.1% of the examined families). We did not find any mutation in *GHRHR.* Even though mutation screening revealed only a small number of mutations, deletions in the same genes could also cause IGHD. To analyse this, we performed Multiplex Ligation-dependent Probe Amplification (MLPA), which excluded deletions and duplications in all our patients within the resolution of MLPA.

Mutation frequencies reported by other studies vary between 0 and 32%[1-5] and therefore the mutation frequency found in our study, although low, is as could be expected from the literature.

Despite the fact that some of the patients with *GH1* P89L, R183H and IVS3+1 G>A/C/T mutations had rather mild growth retardation, the pre-treatment clinical condition of the total group of patients with mutations was significantly more severe than that of patients without mutations.

In conclusion, *GH1* and *GHRHR* mutations are relatively rare in Dutch Caucasian IGHD patients, which suggests the involvement of other genetic determinants in the aetiology of IGHD. The overall phenotype of patients with *GH1* mutations is more severe than that of patients without *GH1* sequence changes. IGF-I <-4.0 and peak GH levels <5.7 mU/l are strong predictors of *GH1* mutations in the studied Dutch population. Independently of height, patients fulfilling these criteria had a *GH1* mutation frequency of 40%.

9.2 Screening for mutations in coding regions and splice sites of *PROP1*, *HESX1*, *POU1F1*, *LHX3* and *LHX4* and for *GH1* P89L and IVS3+1 /+2 splice site mutations in CPHD patients

In our CPHD patients, no deletions, duplications or mutations were present in *PROP1, HESX1, POU1F1, LHX3,* or *LHX4.* Mutations in *HESX1, LHX3* and *LHX4* are known to be relatively rare. In the literature, mutations in these genes are only found in up to 1.5% of the patients[6-10] and this low prevalence is confirmed by our study. *POU1F1* mutations have been reported in up to 11.3% in the literature[6,7,9-13] and *POU1F1* mutations have been found in three Dutch families before 1992[14,15]. However, in the population of patients registered in the Dutch National Registry of Growth Hormone Treatment in Children between 1992 and 2003, only one patient had a mutation (R271W) in *POU1F1* and therefore the overall prevalence of *POU1F1* mutations is low. *PROP1* mutations have been reported in up to 57% of CPHD patients[6,7,9,10,12, 16-20]. High mutation frequencies have been reported in the past, especially from populations with a large proportion of familial cases. However, recent studies investigating sporadic cases often report low prevalences or a total absence of *PROP1* mutations. Kim *et al*, McLennan *et al* and Rainbow *et al* reported mutation frequencies of 0% in their Korean, Australian and Brittish sporadic CPHD populations[6,9,12,19] and our findings are in line with these results.

During our study, Mullis *et al*[21] reported CPHD in six patients with mutations in *GH1,* a gene that has traditionally been associated with IGHD. They suggested that this phenomenon could be explained by the process of bystander killing, in which pituitary cells located close to the mutant somatotrope cells are killed by the same macrophages that kill the mutant somatotropes[21,22]. We wondered whether these mutations in *GH1* (P89L and IVS3+1 /+2) could account for the clinical condition of some of the Dutch CPHD patients. Our study showed that none of these *GH1* mutations was present in any of the CPHD patients.

In conclusion, we did not find any *PROP1*, *HESX1*, *POU1F1*, *LHX3*, *LHX4*, *GH1* P89L or *GH1* IVS3+1 /+2 mutation among 78 Dutch patients with CPHD. Since sporadic CPHD often remains unexplained when screening for mutations in these genes, future research should focus on alternative explanations for CPHD.

9.3 Clinical impact of polymorphisms in the locus control region and the promoter of the *GH1* gene

The analysis of *GH1* LCR / promoter SNPs in IGHD patients and normal controls revealed that homozygosity for the minor allele of one SNP, promoter SNP 9, was more frequent among patients than among controls (21% versus 7%, $p = 0.005$). The minor allele at SNP 9 was associated with lower IGF-I levels in patients, suggesting that this allele might be partly responsible for the impaired GH production in IGHD patients. Promoter SNPs 6, 8 and 11 were also related with height and IGF-I levels. We did not find any relation between LCR / promoter SNPs and the first year's response to GH treatment.

The impact of individual SNPs might be explained by their occurrence in combination with other SNPs and by the fact that they are located in regions which are important for *GH1* transcription. Since the SNPs are located in binding sites for transcription factors NF1 (promoter SNP 6), PIT1 (LCR SNP 1 and promoter SNP 8), the VDR response element (promoter SNP 9) and the transcriptional start site (promoter SNP 11), they may alter transcription and thus expression of the *GH1* gene. *In vitro*, promoter SNP 9 has formerly been shown to interact with nucleic acid binding proteins and alternative alleles exhibit differential protein binding; SNP 9 showed protein interaction only for the G allele, not the T allele[9]. This is in accordance with our *in vivo* findings that patients homozygous for the T allele had very low IGF-I levels.

Other studies have reported that *GH1* LCR and promoter SNP explained up to 6.2% of adult height variation[23,24]. In our study, combined genotypes based on promoter SNPs 6, 8, 9 and 11 explained 10.8% of IGF-I SDS in the IGHD patients and 15.9% of Height SDS in controls. However, due to small patient numbers, these data should be interpreted with caution and the results should be confirmed by SNP analysis of a larger population.

In conclusion, promoter SNPs 6, 8, 9 and 11 were related with height and IGF-I levels. Although all patients had IGHD according to Dutch consensus criteria, certain genotypes were associated with a relatively mild phenotype.

9.4 Prevalence and clinical significance of Anti Pituitary Antibodies (APA)

Our study showed antibodies against pituitary cells in a high percentage (23%) of the patients with idiopathic CPHD and in 2.5% of the patients with idiopathic IGHD.

APA at high titres are highly suggestive of an autoimmune-related cause of

hypopituitarism, as they have been reported in patients with apparently isolated idiopathic GH deficiency[25], in patients with autoimmune endocrine diseases and additional GHD[26] and in patients with autoimmune thyroid disease and additional GHD[27]. Recently, in another study, APA selectively immunostaining gonadotrophs were found in patients with hypogonadotropic hypogonadism, whereas in a patient with hypogonadotropic hypogonadism and GH deficiency, antibodies were found targeting gonadotrophs and GH-secreting cells. Among CPHD patients, antibodies were found against GH-, ACTH-, and TSH-secreting cells in four patients with panhypopituitarism[28]. In contrast, APA at high titres were absent in controls and in patients with secondary forms of hypopituitarism.

In conclusion, we found APA in 23% of the CPHD patients and 2.5% of the idiopathic IGHD patients. The high frequency of Anti Pituitary Antibodies at high titres in patients with idiopathic CPHD suggests autoimmune involvement in quite a large number of these patients. Therefore, we recommend to search for APA in all patients with idiopathic CPHD.

9.5 Evaluation of possible causes of IGHD and CPHD

Our study shows that possible explanations for the hormonal deficiencies were present in half of the IGHD and CPHD patients.

The (embryo-)genetic hypothesis, hypothesizing that a defect in embryogenesis or a genetic defect is responsible for abnormalities of the hypothalamo-hypopituitary axis, is currently the most widely accepted theory to explain hypopituitarism. It could explain 19% of the IGHD cases and 12% of the CPHD cases in our study. This hypothesis is supported by studies reporting that other developmental abnormalities are often observed in association with the pituitary abnormalities and by the existence of familial cases of GH deficiency[29-31].

The birth trauma hypothesis, hypothesizing that birth trauma (in particular vaginal breech delivery) is the cause of hypopituitarism, was found as an explanation for 17% of our IGHD patients and 32% of our CPHD patients. Our findings are supported by the findings of Maghnie *et al*, who found that their patients with the classic triad on pituitary MRI, who were born by vaginal breech delivery (68%) all had CPHD, whereas caesarean section (CS) or normal delivery in such patients was followed by IGHD only[32]. Also in our study, of the 60 breech presenting patients who were born by vaginal delivery, 75% had CPHD, whereas of the breech-presenting patients who were delivered by caesarean section, only 43% had CPHD. Although patient numbers were small and data should be interpreted with caution, this difference of

32% suggests that 32% of the CPHD cases is caused by the vaginal breech delivery.

The autoimmune hypothesis states that an autoimmune process of the pituitary is involved in the aetiology of hypopituitarism. We found Anti Pituitary Antibodies, markers of pituitary autoimmunity, in an additional 2.5% of the IGHD patients and 23% of the CPHD patients.

Together, the three pathogenic mechanisms applied to 36% of the IGHD patients and 67% of the CPHD patients. However, in individual patients, different pathogenic mechanisms may play a role, causing an overlap between the three mechanisms. Although the literature suggests that pituitary antibody formation does not occur secondary to pituitary damage but rather as a primary autoimmune process[25], pituitary antibodies might be present in patients with certain genetic variations. Therefore the actual percentage of cases that is explained by the three hypotheses, may be lower than 36% in IGHD and 67% in CPHD.

In conclusion, although there may be overlap between the three pathogenic mechanisms, clinical data pointing towards an (embryo-)genetic, birth trauma-related or autoimmune-related explanation for hormone deficiencies were present in half of the patients with 'idiopathic' IGHD and CPHD. We believe that the aetiology of IGHD and CPHD is multifactorial, both within the individual patient and within the groups of patients, with an important proportion of underlying birth traumas. Although breech presentation might be the consequence of abnormal intra-uterine development, our data suggest that vaginal breech delivery is rather a cause than a consequence of CPHD.

9.6 Impact of *d3* polymorphism of the growth hormone receptor on the growth in response to GH treatment, spontaneous growth and birth size

Our study did not show any association between *GHR* genotype and first year's response to GH treatment. When we classified patients by birth weight and birth length, the IGHD patients born small for gestational age (SGA) carrying the *GHR d3-* genotype showed better spontaneous catch up growth from birth until start of GH treatment than *d3+* patients. However, since the number of SGA patients was small, it was difficult to draw definitive conclusions from this finding.

A more remarkable finding was that IGHD patients born SGA had a significantly higher *d3+* frequency than IGHD patients born AGA. In contrast, among CPHD patients, those born AGA or SGA had similar *d3+* frequency.

Although various investigators have studied the response to GH in *d3+* and *d3-*

patients with GHD[33-35] and SGA[36-38], their results were controversial. Our study has made clear that, among patients with GHD, the *GHR d3* allele was associated with SGA and with a slightly lower spontaneous postnatal catch up growth.

In spite of the studies reporting that GH does not play any role in prenatal intra-uterine growth, our study suggests that a variation in *GHR* might result in decreased fetal growth. A prenatal role for GH is supported by some studies reporting decreased fetal growth in healthy subjects carrying the *GHR d3* allele[39], by other studies reporting reduced birth size of humans lacking either a functional GHR or humans with GHD[40, 41] and by studies reporting that *GHR* is only expressed in the pregnant uterus and not in the non-pregnant uterus[42]. All these findings suggest that the growth hormone receptor is important for placental metabolism and thus for prenatal growth.

In conclusion, IGHD patients born SGA had a significantly higher *d3+* frequency than IGHD patients born AGA. Since we are the third to report an association between birth size and *GHR d3* status, it is conceivable that the *GHR d3* might affect prenatal growth in IGHD patients by a yet unknown mechanism.

9.7 Relation between facial and pituitary morphology

Our study is the first to investigate the relation between facial and pituitary morphology by showing that ectopia of the posterior pituitary is associated with an increased Canthal Index (CI, the ratio of inner and outer canthal distance multiplied by 100), which represents the relative distance between the eyes.

In the literature, pituitary abnormalities have been associated with midline facial abnormalities. The absence of the pituitary gland was reported in relation with nasal pyriform aperture stenosis[43], whereas duplication of the pituitary was accompanied by cleft palate in 19 of 31 cases (61%)[44]. An absent pituitary gland was described in a patient with depressed nasal bridge and a high arched palate, abnormalities of eyes and eyelids and a prominent and high forehead[45]. Although the exact relation between pituitary and facial morphology remains to be established, there is a strong suggestion for a genetic and developmental relationship between the brain and the facial midline[46-48]. Genes like Sonic Hedgehog (*SHH*), *GLI2* and *Pax6* are involved in development of the midline facial structures and are also important for pituitary proliferation[49-54]. Furthermore, the role of *Hox* genes in facial morphogenesis and in development of the brain and pituitary is subject of ongoing study[55-60].

We found that the presence of an Ectopic Posterior Pituitary (EPP) was strongly predicted by the combination of CI>39 and the presence of hormonal deficiencies

additional to GHD: 93% of the patients with CI>39 and one or more hormonal deficiencies additional to GHD had EPP, in contrast to 77% of the patients with only additional hormonal deficiencies to GHD and 29% of the patients with none of these criteria. Our *in vivo* findings are in line with *in vitro* associations described by others[47-61].

In conclusion, to our knowledge, we were the first to investigate the relation between morphometric and clinical data in such detail. Our study is in support of a common developmental cause for facial and pituitary morphology variations. The combination of CI>39 with the presence of hormonal deficiencies additional to GHD strongly predicted EPP and our data suggest that an altered midline development affects both the pituitary and the facial structures of GHD patients.

9.8 Conclusions, practical implications and directions for future research

The study described in this thesis was set up in order to provide an overall picture of genetic defects in a nation-wide cohort of Dutch childhood onset idiopathic IGHD and CPHD patients, and to relate these defects with clinical parameters. However, genetic defects were only present in a very small minority of the IGHD and CPHD patients. In contrast, our data suggest a multifactorial aetiology of hypopituitarism, with a relatively large proportion of underlying birth traumas and auto-immune processes (Figure 1).

Before the start of the study, we did not know the frequency of mutations in *GH1* and *GHRHR* or *PROP1, HESX1, POU1F1, LHX3* and *LHX4* in Dutch patients with IGHD or CPHD. We expected to find *GH1* and *GHRHR* mutations in about 30% of the IGHD patients and mutations in *PROP1, HESX1, POU1F1, LHX3* or *LHX4* in about 25% of the CPHD patients, based on the literature available at start of the study. However, our study showed that the mutation frequency is significantly lower in this population of Dutch patients.

Occasionally, associations have been reported between mutations in *POU1F1* or *HESX1* and (apparent) IGHD[8, 13] Also mutations in *PROP1* can initially cause IGHD, with additional pituitary hormone deficiencies developing over time. Therefore, future research should evaluate *PROP1, HESX1* and *POU1F1* mutations in a large IGHD population. Since *PROP1, HESX1, POU1F1, LHX3* and *LHX4* mutations are rare in CPHD patients, we do not expect to find high mutation frequencies in these genes in IGHD patients. However, *PROP1, HESX1* and *POU1F1* mutation screening of IGHD patients is necessary to further delineate the aetiology of IGHD and CPHD.

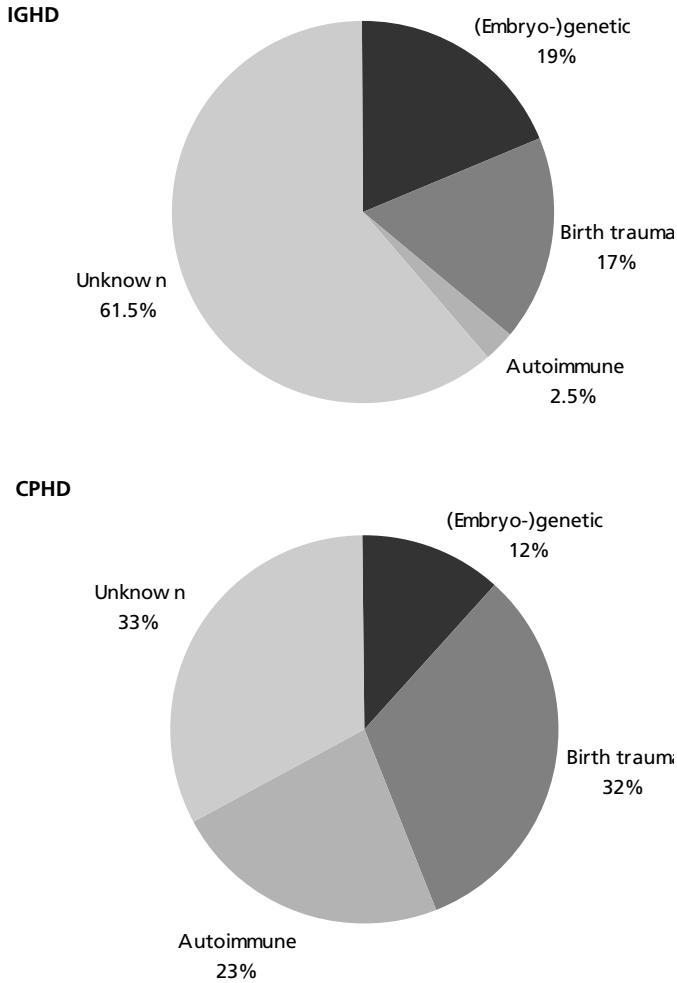


Figure 1: Possible causes for IGHD and CPHD in Dutch patients clarified by the HYPOPIT study

Since mutation frequencies reported from different countries are highly variable, the differences might be caused by the different genetic backgrounds of patients in the reporting countries. Population genetics studies have shown that the genetic background of different regions within Europe varies widely due to human migration in the Paleolithic and Neolithic periods. Semino *et al* suggested the existence of three geographic / genetic clusters within Europe[61]. Chikhi *et al* and Dupanloup *et al* also described an east-west gradient of Neolithic admixture across Europe[62,63]. These clusters might explain the differences in mutation frequencies reported for different countries. Therefore, in the future, mutation frequencies among patients of similar

phenotype should be compared between countries.
Before the start of the study, we did not know whether minor genetic variations outside the coding regions of the IGHD related genes were related with phenotype. Our study showed that *GH1* promoter SNPs 6, 8, 9 and 11 were related with height and IGF-I levels, indicating that variation at these sites adds to the phenotypic variability of IGHD patients. However, the power of the analysis was rather small and therefore the results should be confirmed by SNP analysis of a larger population. Functional studies should be undertaken to explain the functional impact of the promoter SNPs.

Genetic screening of regulating regions and promoters of *GHRHR, PROP1, HESX1, POU1F1, LHX3* and *LHX4* should also be performed in the future, in order to assess whether mutations and polymorphisms in these regions could explain (part of) the phenotype of IGHD and CPHD patients.

Since genetic variation in the studied genes explained only a small part of the clinical condition of our patients, we started the search for other possible causes of CPHD and IGHD.

We wondered whether autoimmune processes could account for the clinical picture of our patients and our data show that 23% of the CPHD cases and 2.5% of IGHD cases might have been caused by a silent form of auto-immune hypophysitis. Therefore, we recommend to screen for anti pituitary antibodies in all patients with idiopathic CPHD.

Although the literature suggests that pituitary antibody formation does not occur secondary to pituitary damage but rather as a primary autoimmune process[25], mechanic or genetic alterations of the pituitary might theoretically trigger an immune reaction, inducing the formation of pituitary antibodies. Future research should therefore also study the mechanisms provoking the formation of pituitary antibodies, as well as their clinical and functional relevance.

We combined the genetic, immunologic, obstetric and additional clinical data of our CPHD patients and we found a possible explanation for the clinical condition of 67% of them (Figure 1). Based on the literature, we had expected to find mutations in *PROP1, HESX1, POU1F1, LHX3* and *LHX4* in about 25% of the cases. Although 12% of the patients had affected first-degree relatives or congenital facial midline defects, suggesting either a genetic or otherwise developmental defect during pituitary organogenesis, we were surprised to find that only one patient in our cohort had a mutation (R271W in *POU1F1*). In contrast, we found immunologic and obstetric factors as an explanation in 55% of the cases. 23% of CPHD patients

had APA suggesting an autoimmune-related cause for their hormonal deficiencies. Obstetric data suggested that birth trauma caused CPHD in 32% of the patients. Our findings suggest a non-genetic cause for a relatively high percentage of patients with CPHD and future research should therefore focus on alternative explanations for these patients.

Especially vaginal breech delivery was associated with CPHD, since CPHD was more frequent among the breech-presenting patients born by vaginal delivery than among breech-presenting patients born by caesarean section. However, we cannot prove a direct causal relation between vaginal breech delivery and the occurrence of CPHD. Before we can recommend caesarean section in all breech-presenting deliveries, it is necessary to perform a prospective study in order to assess the occurrence of hypopituitarism in a large population of children born by vaginal breech delivery in comparison with breech-presenting patients delivered by caesarean section and those born by uncomplicated head delivery.

We also combined genetic, immunologic, obstetric and additional clinical data of all IGHD patients. Nine percent of the IGHD patients had *GH1* mutations known to cause IGHD and none had mutations in *GHRHR*. Non-genetic factors explained only an additional 27%. Of the patients without mutations in the studied genes 10% had findings suggesting an (embryo-)genetic cause, like affected first-degree relatives or facial midline defects. 17% of the IGHD patients had a history of a breech delivery or otherwise complicated delivery, which may have contributed to their condition (Figure 1).

In the total group of IGHD patients, only 36% was therefore explained by one of the three major aetiologic hypotheses, whereas the majority of the IGHD cases remained unexplained. We suggest that genetic variations in the regulating factors and pathways of the GH-IGF-I axis might be responsible for IGHD in some of these patients. Future genetic research should focus on genes involved in these regulating mechanisms and pathways.

Since our knowledge of the genome in general, and of genetic causes for human disease in particular, are rapidly developing, our hopes are focused on finding more genetic causes for IGHD and CPHD. In the search for possible additional genetic defects causing IGHD and CPHD, a promising tool might be genome-wide association (GWA) studies. GWA studies have been shown to provide a new and powerful approach to investigate the effects of inherited genetic variation on the risk of human disease. Next to the screening of candidate genes, future research should therefore include GWA studies in a large and well-defined population like the HYPOPIT population, in order to explain patients' phenotype, to provide individualised care and to eliminate the unsatisfactory diagnosis of 'idiopathic' hypopituitarism.

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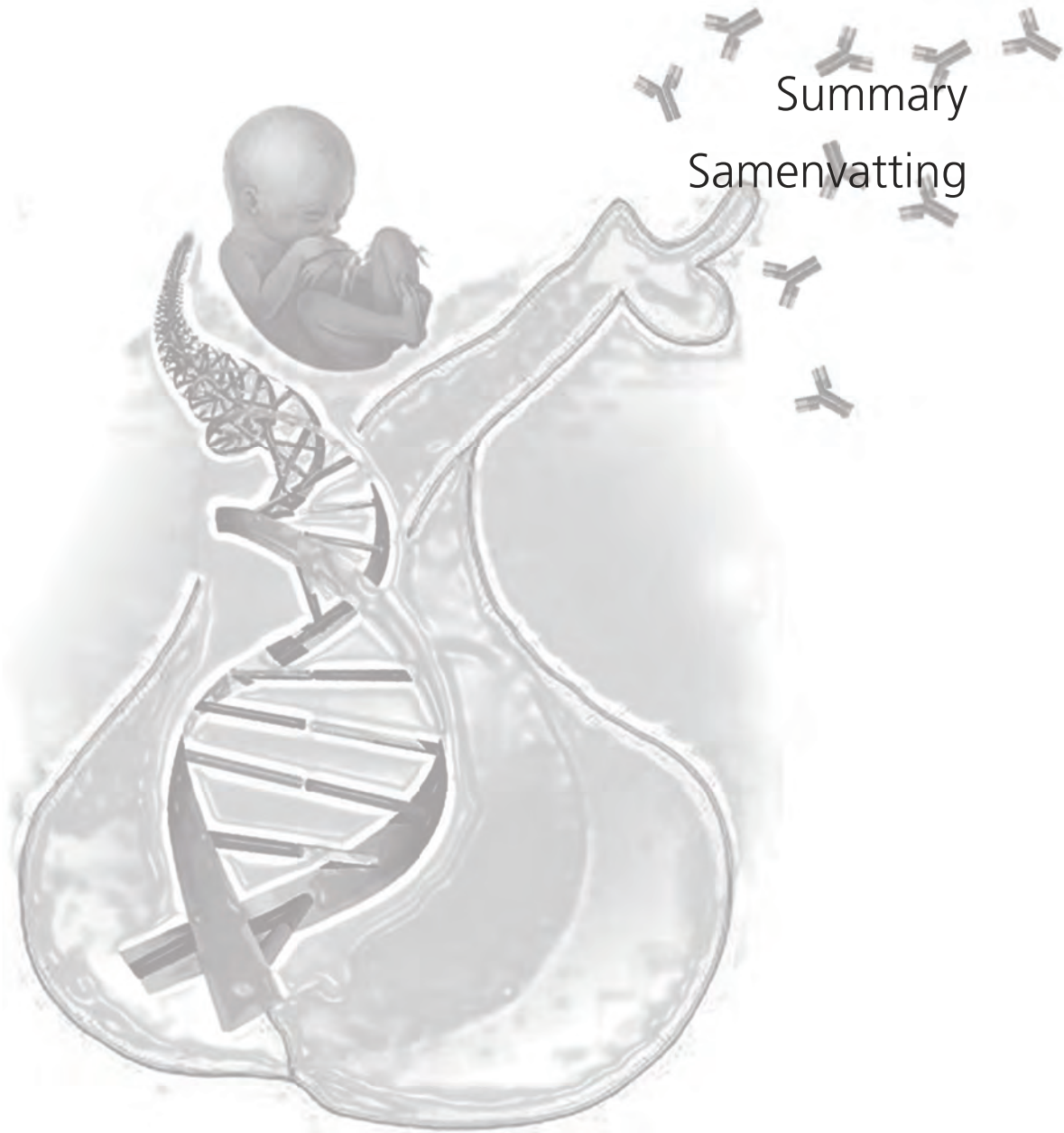
Chapter 10



Summary

Samenvatting

Summary

Hypopituitarism, the deficiency of one or more pituitary hormones, causes stunted growth and severe health problems. Understanding the etiology of pituitary hormone deficiencies is important for anticipation of clinical problems, for genetic counselling and for possible prevention. This doctoral thesis describes the results of studies performed in patients with isolated growth hormone deficiency (IGHD) and combined pituitary hormone deficiencies (CPHD), in order to explore the genetic and non-genetic causes of pituitary hormone deficiencies and the phenotypic variability among patients.

Chapter 1, introduction, describes the normal embryology and physiology of the pituitary. It briefly describes the clinical condition of IGHD and CPHD, the proteins (transcription factors, hormones and their receptors) involved in these disorders and the role of mutations and polymorphisms in the genes encoding these proteins. It presents the considerations, questions and hypotheses explored in this doctoral thesis.

Chapter 2 describes the study performed to provide an overall picture of *GH1* and *GHRHR* mutations and deletions in Dutch IGHD patients and the relation of these defects with clinical parameters. Genetic analysis was performed of exons and exon-intron boundaries of *GH1* and *GHRHR* in 89 Caucasian IGHD patients from 82 families, by using dHPLC, DNA sequencing and MLPA. In addition, functional studies were performed on novel identified *GH1* exonic variants. Five different heterozygous *GH1* mutations were present in five out of 82 participating families (6.1%), whereas no mutations in *GHRHR* were found. Patients with IGF-I SDS <-4.0 and peak GH levels <5.7 mU/L had a mutation frequency of 40%, in contrast to 6.8% in patients with only one criterion, and 0.0% in patients with none of these criteria (p=0.00007). Five new *GH1* and two *GHRHR* variants were also identified; two of them (*GH1* F92L and D153H) caused a marked reduction of GH secretion *in vitro*.
In conclusion, *GH1* and *GHRHR* mutations are rare in Caucasian Dutch IGHD patients, which suggests the involvement of other genetic determinants in the aetiology of IGHD. The overall phenotype of patients with *GH1* mutations is more severe than that of patients without *GH1* sequence changes. IGF-I SDS <-4.0 and peak GH levels <5.7 mU/l are strong predictors of *GH1* mutations in the studied IGHD population.

Chapter 3 presents the results of the DNA analysis of CPHD patients. We screened 79 CPHD patients from 78 families for mutations and deletions in exons and exon-intron

boundaries of *PROP1, HESX1, POU1F1, LHX3,* and *LHX4*, by using MLPA, dHPLC and DNA sequencing. Since *GH1* P89L and IVS3+1 /+2 splice site mutations have been shown to cause pituitary hormone impairment in addition to GH deficiency, we also screened for these mutations. We did not find any mutation or deletion in *PROP1, HESX1, LHX3* or *LHX4*. *GH1* P89L and *GH1* IVS3+1 /+2 mutations were also absent. One patient in our cohort was formerly known to have a *POU1F1* mutation. We found three new missense mutations in *LHX3* and *POU1F1*, which were considered rare non-causal variants since they were also present in non-affected family members or healthy controls with a frequency below 1%.
In conclusion, thorough screening for mutations and deletions in *PROP1, HESX1, POU1F1, LHX3, LHX4,* as well as screening for *GH1* P89L or *GH1* IVS3+1 /+2 mutations, did not reveal any genetic defect in our CPHD cohort, apart from one formerly known *POU1F1* mutation in one patient. Future research should focus on alternative explanations for CPHD, like other genes or environmental factors.

Chapter 4 shows the study of the highly polymorphic promoter and locus control region of *GH1*, which regulate the expression of the *GH1* gene. We analysed *GH1* LCR / promoter SNPs in patients with isolated growth hormone deficiency (IGHD) without mutations or deletions in *GH1* and *GHRHR*, and looked for associations of these SNPs with clinical data. We directly sequenced the *GH1* LCR / promoter of 62 Dutch IGHD patients without mutations or deletions in *GH1* or *GHRHR*, participating in the Dutch HYPOPIT study, and of 72 controls with normal height. We evaluated associations of *GH1* LCR / promoter SNPs with height, serum IGF-I levels and the first year's response to GH treatment. Promoter SNP 6 was strongly linked with SNPs 1 and 2 of the LCR. Promoter SNPs 6, 8, 9 and 11 were related with height and IGF-I levels.
Concluding, *GH1* promoter SNPs 6, 8, 9 and 11 are associated with height and IGF-I levels in IGHD patients and controls. Although all patients had IGHD according to Dutch consensus criteria, some genotypes were associated with a relatively mild phenotype.

Chapter 5 gives an overview of the immunologic studies performed in patients with IGHD and CPHD. Despite extensive research, in the majority of patients with these disorders, the cause of their clinical picture remained unknown. Based on recent articles suggesting that some cases of idiopathic GHD might be explained by a silent form of autoimmune hypophysitis (AIH) based on the presence of Anti Pituitary Antibodies (APA) at high titres (>1:8), we collected clinical data and serum from 71 patients participating in the Dutch HYPOPIT study. APA screening in 40 IGHD

patients and 31 CPHD patients was performed by an indirect immunofluorescence method. APA, when present, were related to clinical and morphological pituitary findings. APA were present at high titres in 7 of 31 CPHD patients (23%) and 1 of 40 IGHD patients (2.5%).
Conclusion The high frequency of APA in our idiopathic CPHD population indicates that, in 23% of the patients diagnosed with idiopathic CPHD, the hormone deficiencies might actually be caused by a silent form of autoimmune hypophysitis. We therefore recommend screening for APA in all patients with 'idiopathic' CPHD.

Chapter 6 gives an overview of the causes of hypopituitarism present in our study population according to three major hypotheses: the (embryo-)genetic, birth trauma and the autoimmune hypothesis. We summarize obstetric, neonatal, biochemical, immunologic, genetic and morphologic data of 244 patients with IGHD and CPHD in order to assess the number of cases that could be explained by the three hypotheses. Clinical data of 19% of the IGHD patients and 12% of the CPHD patients pointed towards an (embryo-)genetic cause. In 17% of the IGHD patients and 32% of the CPHD patients, clinical data suggested that birth trauma could have caused their deficiencies. 2.5% of IGHD patients and 23% of CPHD tested for Anti Pituitary Antibodies (APA) were APA positive, suggesting an autoimmune process was involved in their condition.
In conclusion, clinical data pointing towards an (embryo-)genetic, birth trauma-related or autoimmune-related explanation for hormonal deficiencies were present in 36% of the patients with 'idiopathic' IGHD and 67% of patients with 'idiopathic' CPHD. Our data suggest that the aetiology of IGHD and CPHD is multifactorial, with an important proportion of underlying birth traumas.

Chapter 7 describes the study of a common polymorphism in GHR, the Growth Hormone Receptor, through which GH exerts its action. The *GHR* gene contains a genetic polymorphism caused by a deletion of exon 3 (*d3*), with high frequency in the normal population.There is a continuing controversy whether the presence or absence of the exon 3 deletion (*d3+ vs. d3-)* affects the effect of GH in human growth. For 144 patients with idiopathic IGHD (n=77) or CPHD (n=72), amplification of the region around exon 3 of the *GHR* gene was performed. Clinical data and response to GH treatment were compared between *GHR d3+* and *d3-* IGHD and CPHD patients born either Small for Gestational Age (SGA) or Appropriate for Gestational Age (AGA). IGHD patients born SGA had a significantly higher *d3+* frequency (82%) than IGHD patients born AGA (34%). Within the group of IGHD patients born SGA, *d3-* patients showed a slightly better spontaneous catch up growth before start of

GH treatment than *d3*+ patients. There was no difference in patients first year's response to GH treatment between *GHR d3*+ and *d3*- patients.
Conclusion In IGHD and CPHD patients, response to GH treatment was independent of *GHR* genotype. *GHR d3* was significantly more frequent among IGHD patients born SGA. Since we are the third to report an association between birth size and *GHR d3* status, it is conceivable that the *GHR d3* might affect prenatal growth in IGHD patients by a yet unknown mechanism.

Chapter 8 describes the analysis of standardised photographs of 137 Caucasian patients with GHD, which we studied in order to examine the relations between auxological, biochemical, pituitary and facial morphometric features. We analysed pictures of 137 patients: 73 (55 M / 18 F) with IGHD and 64 (48 M / 16 F) with CPHD. Of each patient, standardized frontal and lateral digital pictures were taken and analysed using Adobe Photoshop 5.0. Canthal Index (CI), the relative distance between the eyes, was related to pituitary morphology. Patients with an Ectopic Posterior Pituitary (EPP) had significantly higher CI values than patients without EPP. We found CI >39 to be a good cut-off value to select children with highest probability of having EPP. The combination of CI>39 with the presence of hormonal deficiencies additional to GHD strongly predicted EPP: 93% of the patients with a CI>39 and additional hormonal deficiencies had EPP, in contrast to 77% of the patients with additional hormonal deficiencies but a CI <39, and 29% of the patients with none of these criteria (p=0.0001).
In conclusion, Canthal Index, the relative distance between the eyes, measured on digital pictures, is associated with ectopia of the posterior pituitary and this might be caused by an altered midline development, affecting both the pituitary and the facial structures of GHD patients

Chapter 9 discusses the results of our studies in the context of the most recent literature. Hypotheses are presented for the mechanisms involved in hypopituitarism. The chapter ends with final conclusions, clinical implications and recommendations for future research.

Samenvatting

Een tekort aan één of meer hypofyse hormonen kan groeiachterstand en ernstige gezondheidsproblemen veroorzaken. Om erfelijkheidsadviezen te kunnen geven, om voorbereid te zijn op hormoontekorten en om zo mogelijk in de toekomst het ontstaan van hormoontekorten te voorkomen, is het belangrijk om de oorzaken van de aandoening te begrijpen. Dit proefschrift beschrijft de studies die zijn uitgevoerd in een grote groep patiënten met hormoontekorten, om genetische en niet-genetische oorzaken te onderzoeken en om te verklaren waarom sommige kinderen wel en andere niet goed op groeihormoonbehandeling reageren.

Hoofdstuk 1, de inleiding, beschrijft de normale ontwikkeling en functie van de hypofyse. Het beschrijft de ziektebeelden IGHD (geïsoleerd GH tekort) en CPHD (uitval van meerdere hypofyse hormonen). Het beschrijft welke eiwitten (hormonen, hormoonreceptoren en transcriptiefactoren) betrokken zijn bij deze aandoeningen en welke afwijkingen kunnen voorkomen in de genen die de 'streepjescode' vormen voor de aanmaak van deze eiwitten.

Hoofdstuk 2 beschrijft het genetisch onderzoek dat verricht is bij de IGHD patiënten. In 89 Nederlandse IGHD patiënten afkomstig uit 82 families zijn de exonen en de exon-intron overgangsgebieden van de genen *GH1* en *GHRHR* door middel van drie verschillende technieken (dHPLC, sequencing en MLPA) onderzocht op afwijkingen. Bij 9 patiënten uit 5 families zijn mutaties gevonden, waarvan bekend is dat ze IGHD veroorzaken. Deze mutaties werden gevonden bij de patiënten met het meest ernstige ziektebeeld. De bloedspiegel van IGF-I, een GH afhankelijke groeifactor, en de bloedspiegels van GH zelf, waren belangrijke voorspellers voor het vinden van een mutatie. Daarnaast zijn 5 nieuwe afwijkingen gevonden die nog niet eerder beschreven zijn in de literatuur. Twee van deze afwijkingen verhinderden normale GH produktie toen ze werden ingebracht in een celsysteem. Dit suggereert dat deze nieuwe mutaties ook in het menselijk lichaam GH produktie kunnen verminderen, mogelijk met IGHD tot gevolg.
Concluderend zijn mutaties zeldzaam bij de Nederlandse IGHD patiënten, en worden ze met name gevonden bij de patiënten met het meest ernstige ziektebeeld. Het feit dat er zo weinig mutaties gevonden zijn, betekent dat er andere oorzaken moeten zijn voor het hormoontekort.

Hoofdstuk 3 beschrijft de genetische analyse van de CPHD patiënten. We onderzochten 79 patiënten uit 78 families op mutaties in *PROP1, HESX1, POU1F1, LHX3*

en *LHX4*, door middel van MLPA, dHPLC and DNA sequencing technieken. Onlangs is aangetoond dat twee mutaties in *GH1* (P89L and IVS3+1 /+2) verrassenderwijs ook CPHD kunnen veroorzaken. Daarom hebben we de patiënten ook onderzocht op die mutaties. Hoewel bij één patiënt in onze groep in het verleden al een mutatie in *POU1F1* was vastgesteld, vonden we geen mutaties in de rest van de groep. Wel vonden we drie nieuwe afwijkingen waarvan het effect nog onbekend is. Deze afwijkingen waren echter ook aanwezig bij gezonde ouders van de patiënten, of in een gezonde controle groep, wat inhoudt dat deze mutaties niet noodzakelijkerwijs CPHD veroorzaken.
Conclusie: aangezien bij de Nederlandse CPHD patiënten mutaties in *PROP1, HESX1, POU1F1, LHX3, LHX4* of *GH1* zeer zeldzaam zijn, moet in de toekomst onderzoek gedaan worden naar andere genetische en niet-genetische oorzaken.

Hoofdstuk 4 beschrijft het onderzoek naar de promoter en locus control region, de regulerende gebieden van het *GH1* gen. We hebben deze gebieden in kaart gebracht met de direct sequencing techniek in patiënten bij wie geen mutaties waren gevonden in *GH1* of *GHRHR.* In de LCR en promoter komen vele polymorfismen voor (in onze studie genummerd van 1 tot 16), waarvan we de frequentie van voorkomen hebben vergeleken tussen 62 IGHD patiënten en een gezonde controlegroep van 72 personen. We hebben verbanden gezocht tussen de aanwezigheid van deze polymorfismen en de lengte, IGF-I bloedspiegels en de reactie op GH behandeling van IGHD patiënten. De promoter polymorfismen 6, 8, 9 en 11 toonden een relatie met lengte en IGF-I bloedspiegels.
Conclusie: De polymorfismen 6, 8, 9 en 11 in de promoter van *GH1* zijn gerelateerd aan lengte en IGF-I bloedspiegels. Bepaalde combinaties van deze polymorfismen gaan gepaard met een relatief mild ziektebeeld.

Hoofdstuk 5 beschrijft het immunologische onderzoek verricht bij de Nederlandse IGHD en CPHD patiënten. Aangezien veel patiënten geen duidelijk aanwijsbare oorzaak hebben voor hun ziektebeeld, hebben we onderzocht of er bij deze patiënten antistoffen tegen de hypofyse in het bloed aanwezig waren. Met behulp van de direkte immunofluorescentie methode is gezocht naar deze antistoffen, die aanwezig bleken te zijn bij 23% van de CPHD patiënten en 2.5% van de IGHD patiënten. **Conclusie:** Antistoffen tegen de hypofyse zijn aanwezig bij een kwart van de CPHD patiënten en de hormoontekorten bij deze patiënten worden daarom mogelijk (mede) veroorzaakt door een auto-immuunziekte. We adviseren om alle patiënten met idiopathisch CPHD te testen op deze antistoffen.

Hoofdstuk 6 geeft een overzicht van alle genetische en niet-genetische afwijkingen die gevonden zijn bij 244 IGHD en CPHD patiënten, die mogelijk hun hormoontekorten kunnen verklaren. We hebben de patiënten naar drie hypotheses ingedeeld. De (embryo-)genetische hypothese stelt dat genetische afwijkingen of afwijkingen tijdens de zwangerschap de oorzaak zijn van de hormoontekorten. De geboortetrauma theorie stelt dat kinderen tijdens een moeizame (stuit-)bevalling schade oplopen aan de hypofyse, met hormoontekorten als gevolg. De auto-immuunhypothese stelt dat het eigen lichaam d.m.v. antistoffen schade aan de hypofyse aanricht. 19% van de IGHD patiënten en 12% van de CPHD patiënten hadden aanwijzingen voor een (embryo-)genetische oorzaak. 17% van de IGHD patiënten en 32% van de CPHD patiënten had aanwijzingen voor een geboortetrauma als oorzaak van de hormoontekorten. 2.5% van de IGHD patiënten en 23% van de CPHD patiënten had antistoffen tegen de hypofyse in het bloed, hetgeen suggereert dat een autoimmuun proces (mede) verantwoordelijk was voor de hormoontekorten.
Concluderend was er bij 36% van de IGHD patiënten en 67% van de CPHD patiënten een mogelijke (embryo-)genetische, geboortegerelateerde of auto-immuungerelateerde oorzaak aan te wijzen voor de hormoontekorten. Dit wijst erop dat hypopituïtarisme meerdere oorzaken kan hebben, met moeizame stuitbevallingen als belangrijke onderliggende factor.

Hoofdstuk 7 beschrijft de studie naar het '*d3*' polymorfisme in de GH receptor. Een groot deel van de gezonde bevolking mist een deel (exon 3) van het *GHR* gen, waardoor er een te kort GHR eiwit wordt gemaakt. In de literatuur worden wisselende berichten gepubliceerd over het effect van deze zogenaamde 'exon 3 deletie' ook wel kortweg '*d3*' genoemd. Wij hebben de *GHR d3* variant onderzocht bij 77 IGHD patiënten en 72 CPHD patiënten. Aangezien GH werkt via binding aan de GHR, hebben wij in onze groep de effectiviteit van GH behandeling onderzocht bij patiënten met en zonder *d3* variant. Wij vonden dat het al dan niet hebben van de *d3* variant niet van invloed was op de groei van de patiënten tijdens het eerste jaar van de GH behandeling. Wel vonden we dat IGHD patiënten, die bij geboorte te klein waren voor de zwangerschapsduur (Small for Gestational Age, SGA), vaker de *d3* variant hadden dan kinderen met normaal geboortegewicht en -lengte. Dit suggereert dat de *GHR d3* variant van invloed is op de groei van het kind in de baarmoeder ('prenatale groei'), terwijl tot op heden werd aangenomen dat GH alleen belangrijk is voor de groei na de geboorte ('postnatale groei').
Conclusie: De bevinding uit onze studie en uit twee recente andere studies suggereren dat GH niet alleen de postnatale, maar ook de prenatale groei beïnvloedt via een nog onbekend mechanisme.

Hoofdstuk 8 beschrijft de analyse van gestandaardiseerde digitale foto's van 137 patiënten: 73 (55 mannen / 18 vrouwen) met IGHD en 64 (48 mannen / 16 vrouwen) met CPHD. We hebben digitale gezichtsfoto's bestudeerd en gezichtsmetingen verricht om een relatie te zoeken tussen uiterlijk enerzijds, en groei, bloedspiegels van hypofyse hormonen en hypofyse afwijkingen anderzijds.
We vonden dat bij patiënten, bij wie op de MRI scan van de hersenen een afwijkende ligging van de hypofyse achterkwab te zien was, de ogen verder uit elkaar stonden. De Canthal Index (CI) is een maat voor de relatieve afstand tussen de ogen. Een CI boven de afkapwaarde van 39 verhoogde de kans op het vinden van de afwijkende ligging van de hypofyse achterkwab. Aangezien bepaalde genen (*SHH, GLI2* en *pax6)* zowel belangrijk zijn voor de aanleg van de hypofyse als voor de aanleg van het gezicht, zouden mutaties in deze genen een rol kunnen spelen bij hypopituïtarisme.
Conclusie: De Canthal Index is groter bij patiënten met een afwijkende ligging van de hypofyse achterkwab, hetgeen suggereert dat hypopituïtarisme mogelijk het gevolg is van een afwijkende embryonale ontwikkeling van de midlijn van de hersenen en het aangezicht.

Hoofdstuk 9, de discussie, bespreekt de resultaten van de onderzoeken in de context van recente onderzoeksliteratuur. Verschillen en overeenkomsten worden besproken en geïnterpreteerd, en hypotheses worden geopperd om diverse aspecten van hypopituïtarisme te verklaren. Het hoofdstuk eindigt met conclusies, aanbevelingen voor de dagelijkse klinische praktijk en aanbevelingen voor toekomstig wetenschappelijk onderzoek.

Kort samengevat blijkt uit onze studie dat er relatief weinig genetische afwijkingen zijn gevonden in de genen waarvan tot op heden bekend is dat ze betrokken zijn bij hypopituïtarisme. Daarentegen zijn moeizame (stuit-)bevallingen en auto-immuun processen mogelijk mede verantwoordelijk voor de hormoontekorten van een groot deel van de CPHD patiënten. Toekomstig onderzoek zou zich daarom moeten richten op deze niet-genetische oorzaken en nog onbekende genetische oorzaken.

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De ‘pelgrimstocht’ ten einde

Tegen het einde van dit promotie onderzoek ondernam ik, samen met een goede vriendin, een fietstocht van de Pyreneeën naar het pelgrimsoord Santiago de Compostela. Hoewel ons tochtje van 1100 km niets was vergeleken met de ‘echte’ pelgrims die uit Nederland komen lopen of fietsen, heb ik toch een idee gekregen van het pelgrim zijn. Toen ik in de hitte tussen de dorre graanvelden van Castilla fietste, links en rechts veldmuisjes zag wegschieten langs de kant van de weg, en mijn gedachten wat rond dwaalden, zag ik plots een opvallende gelijkenis tussen AIO zijn en pelgrim zijn.

Pieken en dalen Van beide trajecten was het begin het zwaarst, vol pieken en dalen (we begonnen met een alternatieve route door de Pyreneeën) en met onverwachte donder- en hagelbuien. Ik had nog weinig conditie en ervaring. Op de zwaarste momenten vroeg ik mij af wat hier ook al weer zo leuk aan was. Terwijl het juist door dat afzien in beide gevallen later allemaal makkelijker zou lijken.

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Curriculum Vitae

Laura de Graaff was born on october 16th, 1975 in De Lier. In 1987 she started secondary school (VWO) at the Interconfessioneel Westland College (IWC) in Naaldwijk, where she met her boyfriend Erno Herder in 1990. She passed her secondary school exam in 1993 and additionally studied VWO physics and chemistry for one year, in order to be admitted to the medical training.

From september 1994 to december 2000 she received her medical training at the University of Leiden. She passed her first year successfully, and during her medical training she participated in several scientific and educational projects. She taught physiology to medical students at the University of Leiden from 1996 to 1998. In 1997, she did an internship at the university hospital of Guadalajara, Spain. In 1998, she participated in a pilot study investigating the prevalence and clinical significance of organ-specific antibodies in Diabetes Mellitus Type 1 (DM1) in part of the DM1 population of the Leiden University Medical Center (LUMC). She extended the analysis to the entire DM1 population after the completion of her medical training. Also in 1998, she taught microscopic anatomy to medical students at the University of Leiden and she participated in nocturnal blood sampling studies at the Endocrinology department of LUMC. From 1998 to 2000 she did her internships and she finalised her medical training with a research project investigating body proportions before and during growth hormone therapy in children with chronic renal failure, under supervision of Prof. Dr. Hokken-Koelega, which lead to a publication in Pediatric Nephrology.

From january 2001 to january 2003 she worked as a medical physician at the department of internal medicine of the Reinier de Graaf hospital in Delft.

From january 2003 to december 2007 she performed her PhD research under supervision of Prof. Dr. Hokken-Koelega at the Dutch Growth Foundation and the Pediatric Endocrinology department of the Erasmus Medical Center- Sophia Children's Hospital in Rotterdam, which has resulted in the present thesis. As part of the PhD training, she worked at the research laboratoy of the Paediatric Endocrinology department of the Hospital Infantil Universitario "Niño Jesús" in Madrid (head of the laboratory: Prof. Dr. Jesús Argente) from may 2003 to october 2005 and she continued at the genetic laboratory of the Internal Medicine department (head of the laboratory: Prof. Dr. André Uitterlinden) until december 2007.

In january 2008 she started her clinical residency at the department of Internal medicine at the Reinier de Graaf hospital in Delft.