CHARGE syndrome

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CHARGE syndrome: molecular diagnosis, clinical aspects and its overlap with Kallmann syndrome

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CHARGE syndrome: molecular diagnosis, clinical aspects and its overlap with Kallmann syndrome

1. Bij het voorspellen van de pathogeniteit van een missense variant in het *CHD7* gen is het zinvol om informatie uit verschillende bronnen, o.a. predictieprogamma's, structuurmodellen van het gemuteerde eiwit en segregatie van de variant in de familie, met elkaar te combineren (dit proefschrift).
2. MLPA analyse is aan te bevelen bij alle patiënten bij wie geen *CHD7* mutatie is gevonden bij sequentieanalyse, maar die wel worden verdacht van CHARGE syndroom (dit proefschrift).
3. Genetisch identieke muizen met een heterozygote *Chd7* mutatie laten een grote fenotypische variabiliteit zien, hetgeen erop wijst dat de variabiliteit van CHARGE syndroom eerder het gevolg is van variaties in de foetale micro-omgeving of stochastische gebeurtenissen dan van modifier genen (dit proefschrift).
4. Met behulp van een reuktest kan voorspeld worden of een kind met CHARGE syndroom al dan niet spontaan in de puberteit zal komen (dit proefschrift).
5. Als bij een patiënt met Kallmann syndroom een *CHD7* mutatie gevonden wordt, dan zullen bij uitvoerige klinische evaluatie vrijwel altijd andere kenmerken van CHARGE syndroom worden gevonden (dit proefschrift).
6. CHARGE syndroom is voornamelijk een klinische diagnose, maar *CHD7* analyse draagt bij aan het stellen van de diagnose in patiënten met een mild fenotype (dit proefschrift).
7. De verbeterde genetische diagnostiek en de daaraan gekoppelde toename in prenatale screeningsmogelijkheden zou nooit mogen leiden tot een verminderde maatschappelijke acceptatie van mensen met een erfelijke ziekte.
8. Patiëntenzorg en onderzoek zijn soms lastig te combineren; in dat geval verdient het aanbeveling om in een onderzoeksgroep onder te duiken.
9. "Yesterday is history. Tomorrow is a mystery. Today is a gift. That's why we call it the present." Babatunde Olatunji.
10. "Remember that not getting what you want is sometimes a wonderful stroke of luck." Dalai Lama.
11. "Never mistake knowledge for wisdom. One helps you make a living; the other helps you make a life." Sandra Carey.
12. "The H of CHARGE stands for humorogenius." Noam Raphael, 17-year-old with CHARGE syndrome.

Jorieke van Kammen-Bergman

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CHAPTER 1

Introduction

In this thesis we discuss the clinical effects of mutations in the *CHD7* gene in the context of two rare heritable syndromes: CHARGE syndrome and Kallmann syndrome. CHARGE syndrome is a multiple congenital anomaly syndrome, predominantly caused by mutations in the *CHD7* gene.[1,2] A subset of patients has hypogonadotropic hypogonadism (delayed or absent puberty) and anosmia (an inability to smell).[3-5] This combination of features is also seen in Kallmann syndrome,[6] which is genetically heterogeneous: mutations in six different genes account for less than 30% of patients.[7] Because of the clinical overlap between CHARGE and Kallmann syndromes, the *CHD7* gene was thought to play a role in Kallmann syndrome as well.

1.1 CHD7

Discovery of the *CHD7* gene

The *CHD7* gene was identified as the causative gene in CHARGE syndrome in 2004.[2] Because CHARGE syndrome was expected to be a microdeletion syndrome at that time, two patients with CHARGE syndrome were analysed with array-based comparative genomic hybridisation (array CGH) in order to detect copy number alterations. In one patient, a *de novo* microdeletion of 4.8 Mb was identified on chromosomal band 8q12. Previously, a patient with CHARGE syndrome was reported with an apparently balanced translocation between chromosomes 6 and 8.[8] Array CGH analysis in this patient identified a complex microdeletion that partially overlapped with the deletion of the first patient. The shortest region of deletion overlap encompassed 2.3 Mb and contained nine genes. Seventeen additional patients with CHARGE syndrome were screened for deletions in this region, but no copy number changes were found.[2] Next, all the genes in the region were sequenced and ten heterozygous mutations were found in the *CHD7* gene. Optimisation of the sequence analysis technique led to six additional *CHD7* mutations being found in the cohort of 17 patients, indicating that *CHD7* was the major gene involved in CHARGE syndrome.[4]

The *CHD7* gene and its expression

The *CHD7* gene consists of 37 coding exons and one non-coding exon. Expression of *CHD7* is tissue- and developmental stage-dependent and correlates with the congenital anomalies found in CHARGE syndrome. In human embryos, *CHD7* is ubiquitously expressed during early development.[9] At later stages, *CHD7* is expressed in undifferentiated neuroepithelium and in the neural crest containing mesenchyme of the pharyngeal arches. *CHD7* expression then appears in the otic vesicle, the limb bud mesenchyme, dorsal root ganglia, and cranial nerves, and subsequently in the neural retina, semicircular canals, pituitary gland, nasal epithelia, and olfactory bulb and nerves.[9] In human adults, *CHD7* was detected at equal levels in different tissues (retina, cornea, brain, skeletal muscle, heart, kidney and lung).[2]

The CHD7 protein and function

The *CHD7* gene encodes for the CHD7 protein, a highly conserved protein of 2997 amino acids.

CHD7 is a member of the chromodomain helicase DNA binding protein family that consists of nine members.[10,11] CHD7 belongs to CHD subfamily III, which is characterised by the presence of two chromodomains (chromatin organisation modifier), two SWI2/SNF2-like ATPase/helicase domains, a SANT DNA binding domain, and two BRK domains (see Figure 1, page 29). Based on the functions of these domains, CHD7 is thought to function as an ATP-dependent chromatin remodeler,[10] which mainly acts during embryonic development. CHD7 binds to histone H3 methylated at lysine 4 in the enhancer regions of numerous genes in different cell types.[12] CHD7 can form complexes with different proteins, thereby ensuring specific binding to different enhancer regions, and leading to time- and tissue-specific regulation of gene expression.[13] One example is the association of CHD7 with PBAF (polybromo- and BRG1-associated factor containing complex) that is essential for neural crest gene expression and cell migration.[14] CHD7 was also shown to interact both directly and indirectly with another CHD protein, CHD8.[15] In addition, *Chd7* was shown to cooperate with *Sox2* in regulating genes of the Notch pathway (*Jag1*, *Rbpj* and *Hes5*) and Sonic Hedgehog pathway (*Gli2*, *Gli3*, *Mycn* and *Tulp3*) in mouse neural stem cells.[16] Another study reported association of CHD7 with rDNA and it was suggested that CHD7 plays a role as a positive regulator of rRNA synthesis.[17]

Chd7 animal models

Several *Chd7* animal models have been generated, of which the mouse models have been studied most extensively. In the past, mouse lines were generated by subjecting male mice to a highly potent mutagen, N-ethyl-N-nitroso-urea (ENU). The progeny of these mice were then assessed for congenital anomalies or behavioural changes. Positional cloning and sequence analysis of candidate genes can ultimately lead to identification of the causative mutation. The first *Chd7* mutant mouse was generated by such an ENU mutagenesis programme in 1995[18,19] and showed circling behaviour (due to balance disturbance) and altered circadian activity. Ten years later, it was discovered that the causative mutation had occurred in the *Chd7* gene.[20] Mice with homozygous *Chd7* mutations die *in utero* (around E10.5 – E11.5) and half of the mice with a heterozygous *Chd7* mutation die before weaning.[20,21] The phenotype of the *Whirligig* mouse, which carries a heterozygous nonsense mutation in exon 11 of the *Chd7* gene, was studied in detail. *Whirligig* mice have several features in common with human CHARGE syndrome: fully penetrant semicircular canal anomalies and occasional congenital heart defects, cleft palate, choanal atresia, and reduced body weight.[20]

Recently, a Drosophila model of CHARGE syndrome was described.[22] Fruit flies with reduced expression of *kismet* (the Drosophila homologue of *Chd7*) have neuronal migration defects and reduced motor function and memory.[22] Another study showed that *kismet* mutants had ectopic expression of *hedgehog* in Drosophila wings leading to abnormal wings and it was concluded that *hedgehog* expression is regulated by Kismet.[23]

Chd7-deficient Xenopus (tadpole) embryos showed absent or malformed otolith (part of the vestibular system), ocular coloboma, microphthalmia, craniofacial malformations and heart defects.[14]

1.2 CHARGE SYNDROME

History

Reports of patients with CHARGE-like anomalies date back to 1961, when Angelman described a girl with unilateral coloboma, cleft palate, pulmonary stenosis, severe growth failure, and developmental delay.[24] Edwards and colleagues reported a boy with bilateral posterior staphylomata (protrusion of the sclera with deformation of the eye leading to myopia), unilateral facial palsy, a congenital heart defect (patent ductus arteriosus, right-sided aorta) and unilateral renal agenesis.[25] It was noted that the earlobes were poorly formed (later recognized as typical CHARGE ears). This boy had an abnormal EEG and developmental delay. He died at three months of age.

Almost twenty years later, in 1979, Hall and Hittner recognized clustering of certain congenital anomalies.[26,27] Bryan Hall investigated a cohort of 17 patients with choanal atresia and recognized that several features were regularly associated, including ocular coloboma, heart defects, mental and growth retardation, micropenis, small ears, micrognathia and microcephaly.[26] In some of his patients, cleft palate, facial palsy, tracheo-oesophageal fistula, feeding problems and seizures were present. Helen Hittner and colleagues described ten patients with colobomatous microphthalmia and associated heart disease, mental retardation, ear anomalies and hearing loss.[27] In addition, they saw facial palsy in six patients, whereas severe feeding difficulties, renal and skeletal anomalies only occurred in a minority.

In 1981, Roberta Pagon and colleagues examined 21 patients and reviewed the cases (n=41) described in the literature.[28] She proposed the acronym CHARGE; ocular coloboma, heart defects, choanal atresia, retardation of growth and development, genital hypoplasia and ear anomalies or deafness. In 2004, *CHD7* was identified as the causative gene and CHARGE association was changed to CHARGE syndrome.[2]

Prevalence

Different estimates of the incidence of CHARGE syndrome can be found in the literature and range from 1:8,500 in Canada[29] to 1:51,000 in Alberta.[30] Most frequently, an incidence of 1:10,000 is cited.[31] Unfortunately, the incidence estimates in the literature are based on a clinical diagnosis of CHARGE syndrome and DNA analysis was not used for establishing the diagnosis. In more than 90% of patients who fulfil the clinical criteria, a *CHD7* mutation can be identified. In addition, an increasing number of patients who do not fulfil the clinical criteria are found to have a *CHD7* mutation and can be diagnosed with CHARGE syndrome.[4] Because CHARGE syndrome is well known among clinical geneticists and has a very recognizable phenotype, we think that *CHD7* analysis will now be performed in all children suspected of having CHARGE syndrome. We therefore used the number of *CHD7* mutations found in the two DNA diagnostic laboratories in the Netherlands that perform *CHD7* analysis (Radboud University Nijmegen Medical Centre and Erasmus Medical Centre) to estimate the incidence of CHARGE syndrome in the Dutch population. Forty *CHD7* mutations were identified in 735,942 children born in the Netherlands in the four-year period 2006–2009 inclusive. We estimate

that the frequency of *CHD7* mutations is 1/18,399 in newborn children in the Netherlands. The birth incidence of CHARGE syndrome will be higher, because *CHD7* analysis will not have been performed in some of the very mildly affected children with CHARGE syndrome (e.g. without coloboma and choanal atresia) and we think we will have missed 10% of the typical CHARGE patients who do not have a detectable *CHD7* mutation. We therefore estimate the incidence of CHARGE syndrome in the Netherlands at 1:15,000 to 1:17,000. This is only a rough estimate, because random fluctuations could have distorted the numbers in this relatively short period of study.

Clinical features and clinical diagnosis

CHARGE syndrome has a very variable clinical presentation.[32] Some patients are severely affected with profound mental retardation or life-threatening congenital anomalies, whereas others are mildly affected and may be unaware that they have CHARGE syndrome. Multi-sensory impairment is a main feature of CHARGE syndrome.[1] Patients can suffer from hearing loss, blindness, balance disturbance and anosmia. The majority of patients with CHARGE syndrome have hypogonadotropic hypogonadism and anosmia, which means that these patients might also be diagnosed with Kallmann syndrome.[3-5] The most frequently occurring features in CHARGE syndrome are external ear anomalies (triangular conchae, absent ear lobes), cranial nerve dysfunction (e.g. facial palsy, sensorineural hearing loss, swallowing problems and anosmia) and semicircular canal anomalies (leading to balance disturbance). For an overview of the features occurring in CHARGE syndrome see Figure 1 on page 178 and Table 2 on page 181.

At present, there are two different sets of clinical criteria used for diagnosing CHARGE syndrome. The Blake criteria were compiled in 1998 and were updated by a consortium in 2006 and 2009.[31,33] The other set of criteria was drawn up by Verloes in 2005.[34] Both sets are composed of several major and minor criteria. Choanal atresia and coloboma are major criteria in both sets. The other major criteria are cranial nerve dysfunction and the characteristic ears in the Blake set of criteria and semicircular canal anomalies in the Verloes set. The two sets of diagnostic criteria are summarized in Table 1 on page 179.

Molecular diagnosis

Since 2004, CHARGE syndrome can also be diagnosed molecularly.[2] Sequence analysis of the *CHD7* gene detects mutations in more than 90% of patients who fulfil the clinical criteria for CHARGE syndrome.[4] Most *CHD7* mutations are nonsense or frameshift mutations and lead to haploinsufficiency, while missense mutations and deletions are found in only a minority of patients[4,35-41] (see Figure 2, page 30). Most mutations occur *de novo* and are predominantly of paternal origin.[42] However, familial recurrence of CHARGE syndrome has also been described[4,32,36,39,43-45] (see Table 3 on page 183).

Pathogenesis

Little is known about the pathogenesis of CHARGE syndrome. The current hypothesis is that decreased expression of *CHD7* during embryogenesis leads to altered expression of other, as yet mainly unidentified, target genes that are important in the development of the eye, inner ear, heart, choanae, brain and kidney (see also the section on "CHD7 protein and function" in this introduction). If CHD7 expression is below a critical threshold (which is developmental stage- and tissue-specific), congenital anomalies will occur.[13,46] This theory can explain the discordant phenotypes seen in monozygotic twins with CHARGE syndrome.[4,36,39]

Surveillance and genetic counselling

The complexity of CHARGE syndrome requires surveillance by an expert team. In 2005, an outpatient clinic for CHARGE syndrome was started in the Radboud University Nijmegen Medical Centre; it was moved to the University Medical Centre Groningen in 2007. More than 70 patients with CHARGE syndrome have visited this multidisciplinary outpatient clinic, in which 12 different specialists are involved. In the clinic, the patients are screened for congenital anomalies that can occur in CHARGE syndrome (e.g. heart defects and kidney anomalies), growth and development are recorded, and advice is given to the local doctors, parents and other caregivers (see Table 4 on pages 188-189).

In addition, genetic counselling for the patients and their families is important. When the *CHD7* mutation has occurred *de novo* in the patient, the recurrence risk for the parents is 2–3% due to somatic and germline mosaicism.[4,32,36,44] Patients with CHARGE syndrome have a 50% chance of transmitting the *CHD7* mutation to their offspring. Thus, reproductive options, like prenatal diagnosis and pre-implantation genetic diagnosis, should be discussed at an early stage.

1.3 KALLMANN SYNDROME

History

As long ago as 1856, a Spanish doctor named Aureliano Maestre de San Juan described a deceased male patient with features of Kallmann syndrome.[47] This patient had testicular atrophy, absence of olfactory neurons in the brain, and had no sense of smell. In 1944, in Germany, Dr. Frans Joseph Kallmann described three families with one female and 11 male patients, who had primary hypogonadism, colour blindness, anosmia, synkinesia (mirror movements of the upper limbs) and mental defects.[6] He established the hereditary nature of the disease, which was later named after him. The association between hypogonadism and anosmia due to olfactory bulb agenesis was termed olfacto-genital dysplasia by a Swiss anatomist, Dr. de Morsier, in 1954.[48]

Prevalence

Kallmann syndrome is a rare syndrome of which the true incidence is hard to ascertain. Different studies have estimated the incidence at 1:10,000 to 1:86,000 males.[49] The male to female ratio was estimated at 4:1. The reason for the male preponderance is currently unknown. Under-diagnosis in

female patients or sex-dependent penetrance of mutations in KS genes could play a role.

Clinical features and clinical diagnosis

Kallmann syndrome is characterized by the combination of hypogonadotropic hypogonadism (HH) and anosmia or hyposmia and is therefore a clinical diagnosis.[6] HH is defined as the absence of pubertal development at 18 years of age, prepubertal levels of sex steroid hormones, and low levels of gonadotropic hormones. Boys with Kallmann syndrome often have a micropenis and cryptorchidism, but many Kallmann patients are only recognized when puberty fails to occur spontaneously.[50] Adult male Kallmann patients have small testes, absence of facial and axillary hair growth, a high pitched voice, decreased muscle mass, diminished libido and erectile dysfunction. Adult female Kallmann patients have primary amenorrhea and have little or no breast development. Patients with Kallmann syndrome are infertile and may develop osteoporosis if untreated.[51,52] Patients are not always aware of their smell deficit and it is therefore recommended that formal smell testing be performed (e.g. the University of Pennsylvania Smell Identification Test[53,54]).

Other clinical features that can occur in Kallmann syndrome are bimanual synkinesia (mirror movements of the upper limbs), unilateral renal agenesis, cleft lip and/or palate, hypodontia, sensorineural hearing loss, loss of nasal cartilage, and occasionally hand or feet anomalies.[50,55] The phenotypes of Kallmann patients within the same family can be variable: some have only anosmia, while others have only HH or both features.

Molecular diagnosis

Kallmann syndrome is genetically heterogeneous and a genetic defect can at present be identified in less than 30% of patients.[7] At present, diagnostic testing is possible for five Kallmann genes in the Netherlands; *KAL1*, *FGFR1*, *PROK2*, *PROKR2* and *FGF8* (www.dnadiagnostiek.nl). Kallmann syndrome can be inherited in different ways: X-linked recessive (*KAL1*[56,57]), autosomal dominant with incomplete penetrance (*FGFR1*,[58] *FGF8*[59]), and autosomal recessive (*PROK2*, *PROKR2*[60]) (Table 1). Mutations in all these genes, except for *KAL1*, have also been found in patients with normosmic HH. Digenic inheritance has also been described, which means that mutations in two different Kallmann or HH genes are found in the same patient (a combination of mutations in *PROKR2* and *PROK2*,[60,61] *KAL1* and *PROKR2*,[60] *FGFR1* and *FGF8*,[59] *PROKR2* and *GNRH1*,[62] *FGFR1* and *GNRHR1*,[63] *PROKR2* and *KISS1R*,[62] and *FGFR1* and *NELF*.[63] It was suggested that a significant proportion (11.3%) of patients with Kallmann syndrome or normosmic HH can be attributed to oligogenic inheritance.[64,64] Recently, three more genes (*WDR11*, *NELF* and *CHD7*) were associated with Kallmann syndrome (Table 1). In 2010, heterozygous mutations in the *WDR11* gene were identified in patients with Kallmann syndrome or HH.[65] In 2011, three patients were reported with *NELF* mutations: one was compound heterozygous for two *NELF* mutations, whereas the other patients had a heterozygous *NELF* mutation in combination with an *FGFR1* mutation or a *TACR3* mutation, respectively.[66] In 2008, the *CHD7* gene was described as playing a role in HH and/or Kallmann syndrome. Two studies identified

CHD7 mutations in patients with Kallmann syndrome.[67,68] The first study found that heterozygous *CHD7* mutations could lead to isolated HH or Kallmann syndrome without other CHARGE features,[68] and the second study concluded that Kallmann patients with a *CHD7* mutation always have other features of CHARGE syndrome (chapter 4.1).[67] This thesis will further discuss the role of the *CHD7* gene in Kallmann syndrome in chapter 4.

Table 1 Molecular diagnosis in Kallmann syndrome

Gene	Inheritance pattern	Frequency in Kallmann patients	Additional clinical features
KAL1[56,57]	XLR/digenic	5-10%	Unilateral renal agenesis, bimanual synkinesia, hearing loss, high arched palate, nystagmus, hypodontia[55]
FGFR1[58]	AD/digenic	~10%	Cleft lip and/or palate (CLP), loss of nasal cartilage, hand/feet anomalies, synkinesia, hearing loss, hypodontia, agenesis of corpus callosum[55]
PROK2[60]	AR/digenic	<5%	Sleep disorder, pectus excavatum, obesity[60,69-71]
PROKR2[60]	AR/digenic	~5%	Sleep disorder, synkinesia, high arched palate, obesity, pes planus, pectus excavatum[61,69,71]
FGF8[59]	AD/digenic	<5%	CLP, hearing loss, camptodactyly, hypertelorism[59]
WDR11[65]	AD	<5%	No additional features reported[65]
NELF[66]	AR/digenic	<<5%	Unilateral renal agenesis (*NELF/KAL1*)[66], CLP and clinodactyly (*NELF/FGFR1*)[63]
CHD7[67,68]	AD	~5%	Additional features of CHARGE syndrome, e.g. CLP, hearing loss, external ear anomalies, coloboma, semicircular canal hypoplasia[67,68]

AD, autosomal dominant; AR, autosomal recessive; XLR, X-linked recessive

Pathogenesis

HH and anosmia in Kallmann syndrome are the result of hypothalamic gonadotropin releasing hormone (GnRH) deficiency and olfactory bulb agenesis, respectively.[72,73] In normal embryogenesis, the olfactory neurons extend their axons through the cribriform plate of the ethmoid bone and synapse in the olfactory bulb (Figure 1).[73-75] GnRH neurons originate in the nasal placode and migrate alongside the olfactory neurons into the brain until they eventually reach the hypothalamus. Hypothalamic GnRH neurons secrete GnRH into the portal blood circulation in a pulsatile manner, which leads to the production of gonadotropins in the pituitary; follicle stimulating hormone (FSH) and luteinising hormone (LH). FSH and LH are necessary for normal fertility and activate the testes in males and ovaries in females to produce testosterone and estrogens, respectively. The rising levels of sex steroids induce puberty development. In Kallmann syndrome, the neuronal migration of olfactory nerves and GnRH neurons is disturbed. There is some evidence from the description of a human foetus with X-linked Kallmann syndrome in 1989.[76] In this foetus, the olfactory nerves and GnRH neurons were found in a tangle beneath the forebrain. The olfactory bulb had not formed and the GnRH neurons had never reached the hypothalamus. These findings were confirmed in 2010,

when another human foetus with a *KAL1* nonsense mutation was described with an absence of GnRH neurons in the hypothalamus and similar pathological findings.[77]

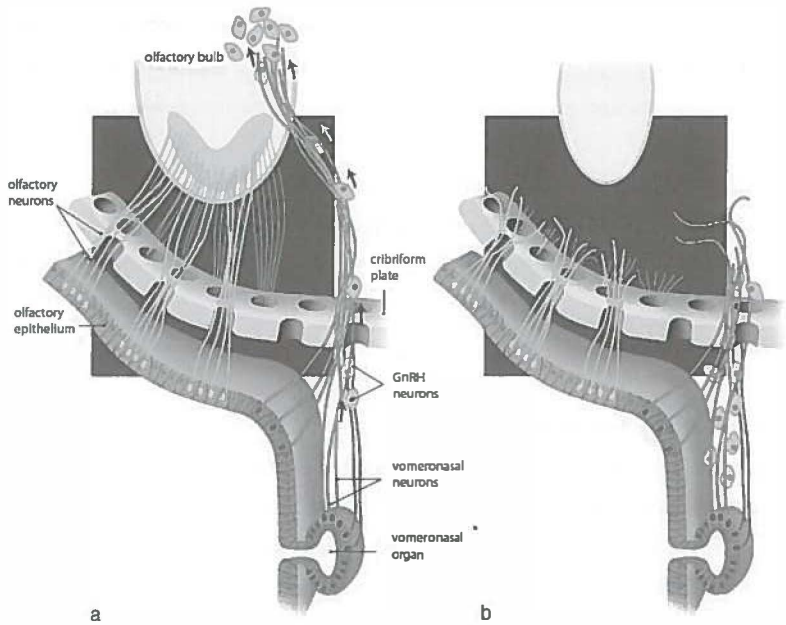

Figure 1. Schematic representation of normal (a) and defective (b) migration of olfactory neurons and GnRH neurons. (a) Normal migration. The olfactory and the vomeronasal neurons extend their axons through the cribriform plate of the ethmoid bone and the olfactory bulb is formed. GnRH neurons migrate alongside these axons and further into the brain towards their destination in the hypothalamus. (b) Defective migration in Kallmann syndrome. The olfactory and vomeronasal neurons extend their axons through the cribriform plate, but do not connect with the forebrain. The olfactory bulb is not formed. The GnRH neurons do not enter the brain but form a tangle with the olfactory and vomeronasal neurons beneath the forebrain. Reprinted with permission from the *Nederlands Tijdschrift voor de Geneeskunde*.[75] (see color image on page 234)

Surveillance and genetic counselling

HH can be treated with sex steroids, gonadotropins or pulsatile GnRH therapy. Sex steroid treatment (testosterone in males and estrogens/progestins in females) will induce secondary sexual characteristics, but testes volume in boys will not increase and patients will not be fertile.[52] Recombinant FSH or pulsatile GnRH therapy are used to restore fertility in patients with HH. Timely induction of puberty is important, because this will reduce the risk of osteoporosis. Bone mineral density studies can be considered, especially in patients with other risk factors for osteoporosis.

Patients should be screened for additional (non-reproductive) features of Kallmann syndrome, for example a smell deficit and unilateral renal agenesis (Table 1). Anosmia cannot be cured, but it is important that patients are aware of their smell deficit. Olfaction is important for detecting warning odours (e.g. gas, smoke and rotten food), is linked to memory (through the limbic system), plays a role in personal hygiene (detection of body odour), and determines the flavour of food and drinks.[3,54]

Genetic counselling in Kallmann syndrome is hampered by the high percentage (65-75%) of

patients in whom no genetic defect can be identified and by the possibility of digenic inheritance. DNA analysis can sometimes give an indication of the recurrence risk of Kallmann syndrome for offspring. One should discuss the reasons for DNA analysis, as Kallmann syndrome is generally not a reason for terminating a pregnancy (after prenatal diagnosis) or a reason to refrain from having children.

1.4 CLINICAL COMPARISON OF CHARGE AND KALLMANN SYNDROMES

In order to study the Kallmann features that can be part of CHARGE syndrome, standardised clinical phenotyping is important. Therefore, the quantitative clinical description of puberty development, the University of Pennsylvania Smell Identification Test and radiologic evaluation of the olfactory bulbs will be discussed below.

Clinical description of puberty development

Puberty development can best be evaluated by a paediatric endocrinologist. The Tanner stages are used to determine the stage of pubertal maturation (Table 2).[78] In boys, stretched penile length and testes volume can be measured. A micropenis is present when the stretched penile length is below -2.5 SD for a given age (reference values for the Dutch population can be found in Massa *et al).*[79] In both boys and girls, serum concentrations of gonadotropins and sex steroids should be determined.

HH is diagnosed when no sexual maturation has occurred at 18 years of age and when endocrine studies show prepubertal levels of sex steroids and gonadotropins. HH is invariably present in Kallmann syndrome and is also seen in approximately 85% of boys and 60% of girls with CHARGE syndrome.[4,6]

Table 2. Tanner stages of pubertal maturation

Tanner stage	Pubic hair (males and females)	Male genitalia	Female breast development
I	No pubic hair	Prepubertal	Prepubertal
II	Sparse, long and slightly pigmented pubic hair	Growth of testes, reddening of scrotum	Breast buds form, enlargement of areola
III	Darker, coarser and curlier hair	Further growth of testes, growth of penis	Further growth of breast buds and areola, areola confluent with breast contour
IV	Adult hair covering pubis	Further growth of testes and penis, darkening of scrotum	Further growth of breasts and areola, papilla and areola project above breast contour
V	Adult hair extending laterally	Adult scrotum and penis (TV ≥20 mL)	Adult breasts, areola confluent with breast contour

TV, testes volume

The University of Pennsylvania Smell Identification Test (UPSIT)

The UPSIT is a widely used smell test and was developed in the USA in 1984 (UPSIT, Sensonics Inc, Haddon Heights, New Jersey,[53] www.sensonics.com). The test consists of 40 scratch-and-sniff odorants and is forced-choice (with four options for each odour). The UPSIT can be used in children of five years or older. The UPSIT score varies between 0 and 40 (with 1 point for each correct answer) and age- and sex-specific reference scores are available. Anosmia is a key feature of Kallmann syndrome, while in CHARGE syndrome a smell deficit is present in the majority of patients.[3,5,80]

Radiological evaluation of the olfactory bulbs

A high resolution MRI scan with slices of 3 mm or less in the coronal plane is required for olfactory bulb analysis. Analysis should be done by an experienced neuroradiologist. Olfactory bulb hypoplasia or aplasia is frequently present in both Kallmann and CHARGE syndromes.[5,9,81-83]

1.5 SCOPE AND OUTLINE OF THIS THESIS

Chapter 1 provides background information on the *CHD7* gene and we discuss the history, clinical features, clinical and molecular diagnosis, pathogenesis, surveillance and genetic counselling aspects of CHARGE syndrome and Kallmann syndrome. However, several issues still remain to be elucidated. My general goal was to gain more insight into CHARGE syndrome and Kallmann syndrome, with a special focus on the *CHD7* gene. I have divided the results of my research into four chapters.

Chapter 2: CHD7

Many patients with *CHD7* mutations have been reported in the literature. Several patients have been reported more than once, but this is not always clearly indicated. Especially in review articles, this can lead to confounding data. Moreover, an increasing number of mutations are not being published. We therefore created an online *CHD7* mutation database that contains all the *CHD7* variants identified by the DNA diagnostic laboratories of the Radboud University Nijmegen Medical Centre, the Netherlands and the Department of Cellular and Molecular Medicine, the Panum Institute, University of Copenhagen, Denmark and all the *CHD7* variants that have been reported in the literature, in combination with the relevant clinical data. This database is presented in **chapter 2.1**. We also give an overview of the *CHD7* mutation spectrum and review the current knowledge on the function of the CHD7 protein.

CHD7 variants can be either pathogenic (disease-causing), benign (non-disease causing) or the effect of a variant can be unknown (unclassified variant). It can be difficult to distinguish between the different categories, especially in the case of variants that lead to an amino acid substitution (missense mutations). We have therefore created a classification system in **chapter 2.2** that reflects the chance that a given missense variant in the *CHD7* gene is pathogenic. To test the hypothesis that missense mutations are associated with a milder phenotype than truncating mutations (nonsense

and frameshift mutations and deletions), we also compared the clinical features of patients with missense mutations with those of patients with truncating mutations.

Only a few patients with partial or complete deletions of the *CHD7* gene have been reported in the literature. The frequency of *CHD7* deletions is therefore considered to be low. We have performed multiplex ligation-dependent probe amplification (MLPA) in 54 patients suspected of CHARGE syndrome but without an identified *CHD7* mutation upon sequence analysis and present the results in **chapter 2.3**.

Chapter 3: CHARGE syndrome

Mouse models have been helpful in gaining insight into the pathogenesis of human disease. The *Whirligig* mouse seemed to be an excellent model for studying CHARGE syndrome, because many CHARGE features are present in these mice. We hypothesised that the *Whirligig* mouse could be a good model to study the pathogenesis of anosmia and HH in CHARGE syndrome. We therefore analysed sense of smell, olfactory bulb anatomy, gonads, GnRH neuron migration and reproductive performance in these mice. The results are presented in **chapter 3.1**.

Anosmia and HH often occur in association, for example in Kallmann syndrome. We hypothesised that anosmia and HH would also be associated in CHARGE syndrome. If this proved to be the case, an easily administered smell test could be used to predict the occurrence of HH in patients with CHARGE syndrome. This would offer the opportunity to start hormone replacement therapy at a more appropriate age in these patients. In **chapter 3.2**, we present the results of our study of smell and pubertal development in a cohort of 35 adolescent patients with CHARGE syndrome who were seen at our outpatient clinic.

The meticulous follow-up of patients with CHARGE syndrome at our outpatient clinic has resulted in a valuable source of clinical information. This helps to improve the surveillance of these patients, who often have a complex combination of impairments and medical complications. Via the outpatient clinic, we heard that three patients had died unexpectedly after the neonatal period. We were able to collect clinical data on four additional patients (from the Netherlands and Canada) who also died after the neonatal period and we investigated the causes of death and risk factors that could identify those children at risk for sudden death. The results are summarised in **chapter 3.3**.

Chapter 4: Kallmann syndrome

Because of the considerable clinical overlap between CHARGE and Kallmann syndromes, we hypothesised that *CHD7* mutations might be present in patients with Kallmann syndrome. We therefore analysed the *CHD7* gene in 36 patients with Kallmann syndrome and 20 patients with normosmic HH from North America and Japan in whom mutations in *KAL1*, *FGFR1*, *PROK2* and *PROKR2* had been excluded. The results are presented in **chapter 4.1**.

A concurrent study also performed *CHD7* analysis in patients with Kallmann syndrome or

normosmic HH, but their results partly conflicted with our results. We therefore repeated our study in a Dutch cohort with clinically well-characterised patients with Kallmann syndrome and present the results in **chapter 4.2**.

Chapter 5: Implications and general discussion

During the follow-up of our patients in the presented studies, we were impressed by the clinical variability of CHARGE syndrome. Especially in those patients who initially presented as Kallmann syndrome, the clinical presentation was sometimes extremely mild. We therefore decided to explore the complete phenotypic spectrum with a special focus on the mild end of the spectrum and to discuss the implications of the expanding phenotypic spectrum for *CHD7* analysis and clinical surveillance. In **chapter 5.1**, we evaluate the clinical characteristics of our own cohort of 280 *CHD7*-positive patients and of previously reported patients with *CHD7* mutations, and compare these with previously reported patients with CHARGE syndrome but an unknown *CHD7* status. In addition, we propose guidelines for *CHD7* analysis and present updated recommendations for the clinical surveillance of patients with a *CHD7* mutation.

In **chapter 5.2**, I further explore the implications of the results of my studies and give recommendations for ongoing and future studies.

REFERENCES

1 Sanlaville D, Verloes A. CHARGE syndrome: an update. *Eur J Hum Genet* 2007;15(4):389-399.

2 Vissers LE, van Ravenswaaij CM, Admiraal R, Hurst JA, De Vries BB, Janssen IM, et al. Mutations in a new member of the chromodomain gene family cause CHARGE syndrome. *Nat Genet* 2004;36(9):955-957.

3 Chalouhi C, Faulcon P, Le Bihan C, Hertz-Pannier L, Bonfils P, Abadie V. Olfactory evaluation in children: application to the CHARGE syndrome. *Pediatrics* 2005;116(1):e81-e88.

4 Jongmans MC, Admiraal RJ, van der Donk KP, Vissers LE, Baas AF, Kapusta L, et al. CHARGE syndrome: the phenotypic spectrum of mutations in the *CHD7* gene. *J Med Genet* 2006;43(4):306-314.

5 Pinto G, Abadie V, Mesnage R, Blustajn J, Cabrol S, Amiel J, et al. CHARGE syndrome includes hypogonadotropic hypogonadism and abnormal olfactory bulb development. *J Clin Endocrinol Metab* 2005;90(10):5621-5626.

6 Kallmann FJ, Schoenfeld WA, Barrera SE. The genetic aspects of primary eunuchoidism. *Am J Ment Def* 1944;48:203-236.

7 Dode C, Hardelin JP. Kallmann syndrome. *Eur J Hum Genet* 2009;17(2):139-146.

8 Hurst JA, Meinecke P, Baraitser M. Balanced t(6;8)(6p8p;6q8q) and the CHARGE association. *J Med Genet* 1991;28(1):54-55.

9 Sanlaville D, Etchevers HC, Gonzales M, Martinovic J, Clement-Ziza M, Delezoide AL, et al. Phenotypic spectrum of CHARGE syndrome in fetuses with *CHD7* truncating mutations correlates with expression during human development. *J Med Genet* 2006;43(3):211-217.

10 Hall JA, Georgel PT. CHD proteins: a diverse family with strong ties. *Biochem Cell Biol* 2007;85(4):463-476.

11 Marfella CG, Imbalzano AN. The Chd family of chromatin remodelers. *Mutat Res* 2007;618(1-2):30-40.

12 Schnetz MP, Bartels CF, Shastri K, Balasubramanian D, Zentner GE, Balaji R, et al. Genomic distribution of CHD7 on chromatin tracks H3K4 methylation patterns. *Genome Res* 2009;19(4):590-601.

13 Layman WS, Hurd EA, Martin DM. Chromodomain proteins in development: lessons from CHARGE syndrome. *Clin Genet* 2010;78(1):11-20.

14 Bajpai R, Chen DA, Rada-Iglesias A, Zhang J, Xiong Y, Helms J, et al. CHD7 cooperates with PBAF to control multipotent neural crest formation. *Nature* 2010;463(7283):958-962.

15 Batsukh T, Pieper L, Koszucka AM, von Velsen N, Hoyer-Fender S, Elbracht M, et al. CHD8 interacts with CHD7, a protein which is mutated in CHARGE syndrome. *Hum Mol Genet* 2010;19(14):2858-2866.

16 Engelen E, Akinci U, Bryne JC, Hou J, Gontan C, Moen M, et al. Sox2 cooperates with Chd7 to regulate genes that are mutated in human syndromes. *Nat Genet* 2011;43(6):607-611.

17 Zentner GE, Hurd EA, Schnetz MP, Handoko L, Wang C, Wang Z, et al. CHD7 functions in the nucleolus as a positive regulator of ribosomal RNA biogenesis. *Hum Mol Genet* 2010;19(18):3491-3501.

18 Nolan PM, Sollars PJ, Bohne BA, Ewens WJ, Pickard GE, Bucan M. Heterozygosity mapping of partially congenic lines: mapping of a semidominant neurological mutation, Wheels (Whl), on mouse chromosome 4. *Genetics* 1995;140(1):245-254.

19 Pickard GE, Sollars PJ, Rinchik EM, Nolan PM, Bucan M. Mutagenesis and behavioral screening for altered circadian activity identifies the mouse mutant, Wheels. *Brain Res* 1995;705(1-2):255-266.

20 Bosman EA, Penn AC, Ambrose JC, Kettleborough R, Stemple DL, Steel KP. Multiple mutations in mouse *Chd7* provide models for CHARGE syndrome. *Hum Mol Genet* 2005;14(22):3463-3476.

21 Hurd EA, Capers PL, Blauwkamp MN, Adams ME, Raphael Y, Poucher HK, et al. Loss of Chd7 function in gene-trapped reporter mice is embryonic lethal and associated with severe defects in multiple developing tissues. *Mamm Genome* 2007;18(2):94-104.

22 Melicharek DJ, Ramirez LC, Singh S, Thompson R, Marenda DR. Kismet/CHD7 regulates axon morphology, memory, and locomotion in a Drosophila model of CHARGE Syndrome. *Hum Mol Genet* 2010;19(21):4253-4264.

23 Terriente-Felix A, Molnar C, Gomez-Skarmeta JL, de Celis JF. A conserved function of the chromatin ATPase Kismet in the regulation of hedgehog expression. *Dev Biol* 2011;350(2):382-392.

24 Angelman H. Syndrome of coloboma with multiple congenital abnormalities in infancy. *Br Med J* 1961;1(5234):1212-1214.

25 Edwards JH, Finlay HVL, Young RB. Coloboma with Multiple Congenital Anomalies. *Br Med J* 1961;2(525):586-587.

26 Hall BD. Choanal atresia and associated multiple anomalies. *J Pediatr* 1979;95(3):395-398.

27 Hittner HM, Hirsch NJ, Kreh GM, Rudolph AJ. Colobomatous microphthalmia, heart disease, hearing loss, and mental retardation--a syndrome. *J Pediatr Ophthalmol Strabismus* 1979;16(2):122-128.

28 Pagon RA, Graham JM, Jr., Zonana J, Yong SL. Coloboma, congenital heart disease, and choanal atresia with multiple anomalies: CHARGE association. *J Pediatr* 1981;99(2):223-227.

29 Issekutz KA, Graham JM, Jr., Prasad C, Smith IM, Blake KD. An epidemiological analysis of CHARGE syndrome: preliminary results from a Canadian study. *Am J Med Genet A* 2005;133(3):309-317.

30 Lowry RB, Sibbald B, Bamforth JS. RE: An epidemiological analysis of CHARGE syndrome: preliminary results from a Canadian study [Issekutz et al., 2005]. *Am J Med Genet A* 2005;139A(2):169.

31 Blake KD, Davenport SL, Hall BD, Hefner MA, Pagon RA, Williams MS, et al. CHARGE association: an update and review for the primary pediatrician. *Clin Pediatr (Phila)* 1998;37(3):159-173.

32 Jongmans MC, Hoefsloot LH, van der Donk KP, Admiraal RJ, Magee A, van de Laar I, et al. Familial CHARGE syndrome and the *CHD7* gene: a recurrent missense mutation, intrafamilial recurrence and variability. *Am J Med Genet A* 2008;146(1):43-50.

33 Lalani SR, Hefner MA, Belmont JW, Davenport SLH. CHARGE Syndrome. GeneReviews 2009. http://www.ncbi.nlm.nih.gov/books/NBK1117/ (accessed 2011).

34 Verloes A. Updated diagnostic criteria for CHARGE syndrome: a proposal. *Am J Med Genet A* 2005;133(3):

306-308.

35 Aramaki M, Udaka T, Kosaki R, Makita Y, Okamoto N, Yoshihashi H, et al. Phenotypic spectrum of CHARGE syndrome with *CHD7* mutations. *J Pediatr* 2006;148(3):410-414.

36 Lalani SR, Safiullah AM, Fernbach SD, Harutyunyan KG, Thaller C, Peterson LE, et al. Spectrum of *CHD7* mutations in 110 individuals with CHARGE syndrome and genotype-phenotype correlation. *Am J Hum Genet* 2006;78(2):303-314.

37 Lee YW, Kim SC, Shin YL, Kim JW, Hong HS, Lee YK, et al. Clinical and genetic analysis of the *CHD7* gene in Korean patients with CHARGE syndrome. *Clin Genet* 2009;75(3):290-293.

38 Vuorela P, la-Mello S, Saloranta C, Penttinen M, Poyhonen M, Huoponen K, et al. Molecular analysis of the *CHD7* gene in CHARGE syndrome: identification of 22 novel mutations and evidence for a low contribution of large *CHD7* deletions. *Genet Med* 2007;9(10):690-694.

39 Wincent J, Holmberg E, Stromland K, Soller M, Mirzaei L, Djureinovic T, et al. *CHD7* mutation spectrum in 28 Swedish patients diagnosed with CHARGE syndrome. *Clin Genet* 2008;74(1):31-38.

40 Wincent J, Schulze A, Schoumans J. Detection of *CHD7* deletions by MLPA in CHARGE syndrome patients with a less typical phenotype. *Eur J Med Genet* 2009;52(4):271-272.

41 Zentner GE, Layman WS, Martin DM, Scacheri PC. Molecular and phenotypic aspects of *CHD7* mutation in CHARGE syndrome. *Am J Med Genet A* 2010;152A(3):674-686.

42 Pauli S, von Velsen N, Burfeind P, Steckel M, Manz J, Buchholz A, et al. *CHD7* mutations causing CHARGE syndrome are predominantly of paternal origin. *Clin Genet* 2011; Epub ahead of print, doi: 10.1111/j.1399-0004.2011.01701.x.

43 Delahaye A, Sznajer Y, Lyonnet S, Elmaleh-Berges M, Delpierre I, Audollent S, et al. Familial CHARGE syndrome because of *CHD7* mutation: clinical intra- and interfamilial variability. *Clin Genet* 2007;72(2):112-121.

44 Pauli S, Pieper L, Haeberle J, Grzmil P, Burfeind P, Steckel M, et al. Proven germline mosaicism in a father of two children with CHARGE syndrome. *Clin Genet* 2009;75(5):473-479.

45 Vuorela PE, Penttinen MT, Hietala MH, Laine JO, Huoponen KA, Kaariainen HA. A familial CHARGE syndrome with a *CHD7* nonsense mutation and new clinical features. *Clin Dysmorphol* 2008;17(4):249-253.

46 Martin DM. Chromatin Remodeling in Development and Disease: Focus on CHD7. *PLoS Genet* 2010;6(7):e1001010.

47 Maestre de San Juan A. [Total lack of the olfactory nerve with anosmia in an individual in whom there was congenital atrophy of the testicles and penis]. *Siglo Medico* 1856;131:211-211.

48 De Morsier G. [Studies in cranio-encephalic dysraphia. I. Agenesia of the olfactory lobe (lateral telencephaloschisis) and of the callous and anterior commissures (median telencephaloschisis); olfacto-genital dysplasia]. *Schweiz Arch Neurol Psychiatr* 1954;74(1-2):309-361.

49 Seminara SB, Hayes FJ, Crowley WF,Jr. Gonadotropin-releasing hormone deficiency in the human (idiopathic hypogonadotropic hypogonadism and Kallmann's syndrome): pathophysiological and genetic considerations. *Endocr Rev* 1998;19(5):521-539.

50 Kaplan JD, Bernstein JA, Kwan A, Hudgins L. Clues to an early diagnosis of Kallmann syndrome. *Am J Med Genet A* 2010;152A(11):2796-2801.

51 Fechner A, Fong S, McGovern P. A review of Kallmann syndrome: genetics, pathophysiology, and clinical management. *Obstet Gynecol Surv* 2008;63(3):189-194.

52 Pallais JC, Au M, Pitteloud N, Seminara S, Crowley WF. Kallmann Syndrome. GeneReviews 2011. http://www.ncbi.nlm.nih.gov/books/NBK1334/ (accessed 2011).

53 Doty RL, Shaman P, Dann M. Development of the University of Pennsylvania Smell Identification Test: a standardized microencapsulated test of olfactory function. *Physiol Behav* 1984;32(3):489-502.

54 Doty RL. Olfactory dysfunction and its measurement in the clinic and workplace. *Int Arch Occup Environ*

Health 2006;79(4):268-282.

55 Tsai PS, Gill JC. Mechanisms of disease: insights into X-linked and autosomal-dominant Kallmann syndrome. *Nat Clin Pract Endocrinol Metab* 2006;2(3):160-171.

56 Franco B, Guioli S, Pragliola A, Incerti B, Bardoni B, Tonlorenzi R, et al. A gene deleted in Kallmann's syndrome shares homology with neural cell adhesion and axonal path-finding molecules. *Nature* 1991;353(6344):529-536.

57 Legouis R, Hardelin JP, Levilliers J, Claverie JM, Compain S, Wunderle V, et al. The candidate gene for the X-linked Kallmann syndrome encodes a protein related to adhesion molecules. *Cell* 1991;67(2):423-435.

58 Dode C, Levilliers J, Dupont JM, De Paepe A, Le Du N, Soussi-Yanicostas N, et al. Loss-of-function mutations in *FGFR1* cause autosomal dominant Kallmann syndrome. *Nat Genet* 2003;33(4):463-465.

59 Falardeau J, Chung WC, Beenken A, Raivio T, Plummer L, Sidis Y, et al. Decreased FGF8 signaling causes deficiency of gonadotropin-releasing hormone in humans and mice. *J Clin Invest* 2008;118(8):2822-2831.

60 Dode C, Teixeira L, Levilliers J, Fouveaut C, Bouchard P, Kottler ML, et al. Kallmann syndrome: mutations in the genes encoding prokineticin-2 and prokineticin receptor-2. *PLoS Genet* 2006;2(10):1648-1652.

61 Cole LW, Sidis Y, Zhang C, Quinton R, Plummer L, Pignatelli D, et al. Mutations in prokineticin 2 and prokineticin receptor 2 genes in human gonadotrophin-releasing hormone deficiency: molecular genetics and clinical spectrum. *J Clin Endocrinol Metab* 2008;93(9):3551-3559.

62 Sarfati J, Guiochon-Mantel A, Rondard P, Arnulf I, Garcia-Pinero A, Wolczynski S, et al. A comparative phenotypic study of Kallmann syndrome patients carrying monoallelic and biallelic mutations in the prokineticin 2 or prokineticin receptor 2 genes. *J Clin Endocrinol Metab* 2010;95(2):659-669.

63 Pitteloud N, Quinton R, Pearce S, Raivio T, Acierno J, Dwyer A, et al. Digenic mutations account for variable phenotypes in idiopathic hypogonadotropic hypogonadism. *J Clin Invest* 2007;117(2):457-463.

64 Sykiotis GP, Plummer L, Hughes VA, Au M, Durrani S, Nayak-Young S, et al. Oligogenic basis of isolated gonadotropin-releasing hormone deficiency. *Proc Natl Acad Sci U S A* 2010;107(34):15140-15144.

65 Kim HG, Ahn JW, Kurth I, Ullmann R, Kim HT, Kulharya A, et al. WDR11, a WD protein that interacts with transcription factor EMX1, is mutated in idiopathic hypogonadotropic hypogonadism and Kallmann syndrome. *Am J Hum Genet* 2010;87(4):465-479.

66 Xu N, Kim HG, Bhagavath B, Cho SG, Lee JH, Ha K, et al. Nasal embryonic LHRH factor (*NELF*) mutations in patients with normosmic hypogonadotropic hypogonadism and Kallmann syndrome. *Fertil Steril* 2011;95(5):1613-1620.

67 Jongmans MC, Ravenswaaij-Arts CM, Pitteloud N, Ogata T, Sato N, Claahsen-van der Grinten HL, et al. *CHD7* mutations in patients initially diagnosed with Kallmann syndrome--the clinical overlap with CHARGE syndrome. *Clin Genet* 2009;75(1):65-71.

68 Kim HG, Kurth I, Lan F, Meliciani I, Wenzel W, Eom SH, et al. Mutations in *CHD7*, encoding a chromatin-remodeling protein, cause idiopathic hypogonadotropic hypogonadism and Kallmann syndrome. *Am J Hum Genet* 2008;83(4):511-519.

69 Abreu AP, Trarbach EB, de Castro M, Frade Costa EM, Versiani B, Matias Baptista MT, et al. Loss-of-function mutations in the genes encoding prokineticin-2 or prokineticin receptor-2 cause autosomal recessive Kallmann syndrome. *J Clin Endocrinol Metab* 2008;93(10):4113-4118.

70 Pitteloud N, Zhang C, Pignatelli D, Li JD, Raivio T, Cole LW, et al. Loss-of-function mutation in the prokineticin 2 gene causes Kallmann syndrome and normosmic idiopathic hypogonadotropic hypogonadism. *Proc Natl Acad Sci U S A* 2007;104(44):17447-17452.

71 Sarfati J, Dode C, Young J. Kallmann syndrome caused by mutations in the *PROK2* and *PROKR2* genes: pathophysiology and genotype-phenotype correlations. *Front Horm Res* 2010;39:121-132.

72 Cadman SM, Kim SH, Hu Y, Gonzalez-Martinez D, Bouloux PM. Molecular pathogenesis of Kallmann's syndrome. *Horm Res* 2006;67(5):231-242.

73 Cariboni A, Maggi R. Kallmann's syndrome, a neuronal migration defect. *Cell Mol Life Sci* 2006;63(21):2512-

2526.

74 Balasubramanian R, Dwyer A, Seminara SB, Pitteloud N, Kaiser UB, Crowley WF,Jr. Human GnRH deficiency: a unique disease model to unravel the ontogeny of GnRH neurons. *Neuroendocrinology* 2010;92(2):81-99.

75 Voorhoeve PG, Delemarre-van de Waal. [From gene to disease; hypogonadotrophic hypogonadism and anosmia: Kallmann's syndrome]. *Ned Tijdschr Geneeskd* 2004;148(23):1142-1144.

76 Schwanzel-Fukuda M, Bick D, Pfaff DW. Luteinizing hormone-releasing hormone (LHRH)-expressing cells do not migrate normally in an inherited hypogonadal (Kallmann) syndrome. *Brain Res Mol Brain Res* 1989;6(4):311-326.

77 Teixeira L, Guimiot F, Dode C, Fallet-Bianco C, Millar RP, Delezoide AL, et al. Defective migration of neuroendocrine GnRH cells in human arrhinencephalic conditions. *J Clin Invest* 2010;120(10):3668-3672.

78 Tanner JM. Normal growth and techniques of growth assessment. *Clin Endocrinol Metab* 1986;15(3):411-451.

79 Massa GG, Langenhorst V, Oostdijk W, Wit JM. [Micropenis in children: etiology, diagnosis and therapy]. *Ned Tijdschr Geneeskd* 1997;141(11):511-515.

80 Layman WS, McEwen DP, Beyer LA, Lalani SR, Fernbach SD, Oh E, et al. Defects in neural stem cell proliferation and olfaction in *Chd7* deficient mice indicate a mechanism for hyposmia in human CHARGE syndrome. *Hum Mol Genet* 2009;18(11):1909-1923.

81 Asakura Y, Toyota Y, Muroya K, Kurosawa K, Fujita K, Aida N, et al. Endocrine and radiological studies in patients with molecularly confirmed CHARGE syndrome. *J Clin Endocrinol Metab* 2008;93(3):920-924.

82 Truwit CL, Barkovich AJ, Grumbach MM, Martini JJ. MR imaging of Kallmann syndrome, a genetic disorder of neuronal migration affecting the olfactory and genital systems. *Am J Neuroradiol* 1993;14(4):827-838.

83 Yousem DM, Turner WJ, Li C, Snyder PJ, Doty RL. Kallmann syndrome: MR evaluation of olfactory system. *Am J Neuroradiol* 1993;14(4):839-843.

CHAPTER 2.1

Mutation update on the *CHD7* gene involved in CHARGE syndrome

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Submitted

ABSTRACT

CHD7 is a member of the chromodomain helicase DNA-binding (CHD) protein family that plays a role in transcription regulation by chromatin remodelling. Loss-of-function mutations in *CHD7* are known to cause CHARGE syndrome, an autosomal dominant malformation syndrome in which several organ systems, for example the central nervous system, eye, ear, nose and mediastinal organs, are variably involved. In this paper, we review all the currently described *CHD7* variants, including 184 new pathogenic mutations found by our laboratories. In total, we compiled 531 different pathogenic *CHD7* alterations from 515 previously published patients with CHARGE syndrome and 296 unpublished patients analysed by our laboratories. The mutations are equally distributed along the coding region of *CHD7* and most are nonsense or frameshift mutations. Most mutations are unique, but we identified 96 recurrent mutations, predominantly arginine to stop codon mutations. We built a locus-specific database listing all the variants that is easily accessible at www.CHD7.org. In addition, we summarize the latest data on CHD7 expression studies, animal models and functional studies, and we discuss the latest clinical insights into CHARGE syndrome.

INTRODUCTION

Chromodomain helicase DNA-binding (CHD) proteins play a role in transcription activation and repression by chromatin remodelling. For this function, all members of the CHD protein family possess two chromodomains (chromatin organisation modifier domains) located on the N-terminal and a centrally located SNF-like helicase motif. The human CHD family consists of nine members that can be subdivided into three subfamilies based on differences in their structure and sequence.[1,2] Members of subfamily I contain a DNA-binding domain located in the C-terminal region. Subfamily II members harbour paired N-terminal PHD (plant homeo domain) Zinc-finger-like domains. Members of subfamily III are characterised by C-terminal paired BRK (Brahma and Kismet) domains and a SANT-like domain (switching-defective protein 3, adaptor 2, nuclear receptor co-repressor, transcription factor IIIB). CHD7 is one of the CHD proteins of subfamily III.[1,2]

CHD7 is located at chromosome 8 (8q12) starting 61.59 Mb from the p-arm telomere. *CHD7* has a genomic size of 188 kb and consists of 38 exons, of which the first is non-coding. The encoded protein (2997 amino acids, Figure 1) is localized in both the nucleoplasm and nucleolus.[3] CHD7 is highly conserved across species and orthologs have been identified in *Xenopus*, zebrafish, mouse and chicken, amongst others.[4-6] This, in combination with the observation that homozygous *Chd7* mutant mice do not survive beyond an early embryonic stage, suggests strong selective pressure and a high functional importance of CHD7.[7,8] Indeed, recent reports about CHD7 function suggest a role in controlling gene expression programs by ATP-dependent chromatin remodelling in embryonic stem cells and other cell types.[3,5]

Heterozygous mutations and deletions of *CHD7* (OMIM *608892) result in CHARGE syndrome (OMIM #214800), a complex of multiple congenital malformations involving the central nervous

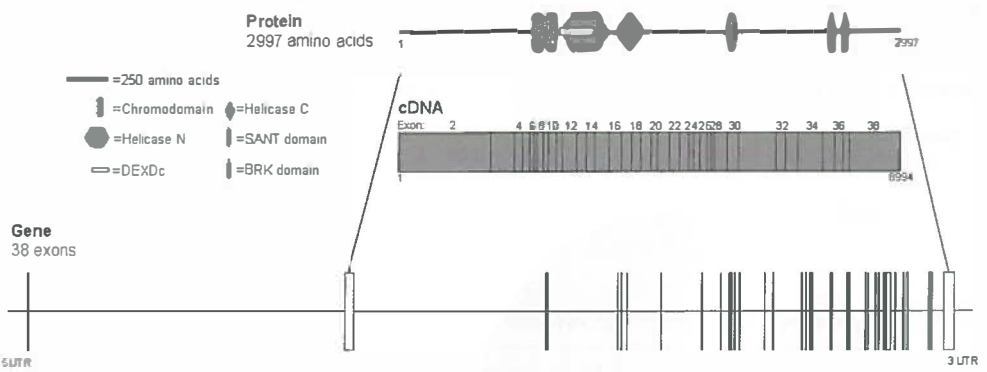

Figure 1. Overview of the *CHD7* gene and protein

Overview of *CHD7* with its 38 exons and introns. The sizes of the exons and introns are drawn to scale. The cDNA of *CHD7* consists of 37 exons: the first exon and also part of genomic exon 2 and 38 are non-coding. The CHD7 protein consists of 2997 amino acids and has several conserved domains which are drawn to scale.

Chromodomain, chromatin organisation modifier domain; Helicase N, helicase N-lobe; DEXDc, DEAD-like helicase superfamily including an ATP-binding domain; Helicase C, helicase C-lobe; SANT domain, switching-defective protein 3, adaptor 2, nuclear receptor co-repressor, transcription factor IIIB domain; BRK domain, Brahma and Kismet domain.

system, eye, ear, nose and mediastinal organs.[9] CHARGE syndrome has been estimated to occur in 1/10,000 births worldwide and has a broad clinical variability.[10,11] Clinical features include ocular coloboma, heart defects, choanal atresia, retarded growth and development, genital hypoplasia, ear anomalies, deafness and semicircular canal hypoplasia.[12-14] Based on these characteristics, clinical criteria for CHARGE syndrome have been defined by Blake *et al*[15] and Verloes.[16] *CHD7* analysis is a major contributor to the diagnosis today, although not all clinically diagnosed patients with CHARGE syndrome carry a mutation in this gene.[12,13,17] *CHD7* mutations have also been found in patients initially diagnosed with Kallmann syndrome, which supports the well-known observation that Kallmann syndrome is part of the phenotypic spectrum of CHARGE syndrome.[18-20]

In this study, we provide an overview of all *CHD7* sequence variants, submicroscopic genomic rearrangements and translocations that were published before June 15th 2011. In addition, we present all the unpublished *CHD7* variants that have been identified in the DNA diagnostic laboratories of the Radboud University Nijmegen Medical Centre (RUNMC) and the Department of Cellular and Molecular Medicine (ICMM), University of Copenhagen (Supplementary Methods). All *CHD7* variants, including relevant clinical data, were entered into the new locus-specific database at www.CHD7.org. Furthermore, we summarize the latest data on the function of CHD7 and discus the clinical implications of identifying a *CHD7* mutation. The interpretation of missense variants is discussed in another paper (Bergman *et al*, submitted).

MUTATION SPECTRUM

Intragenic *CHD7* mutations in CHARGE syndrome

Per June 15th 2011, a total of 531 pathogenic and unique *CHD7* alterations had been identified in 811 index patients with CHARGE syndrome, including 184 new mutations identified by the DNA diagnostic laboratories of the RUNMC and ICMM.[8,9,12,13,17-68] The majority of the pathogenic *CHD7* variants are intragenic mutations (Figure 2). A schematic presentation of *CHD7* and the locations of

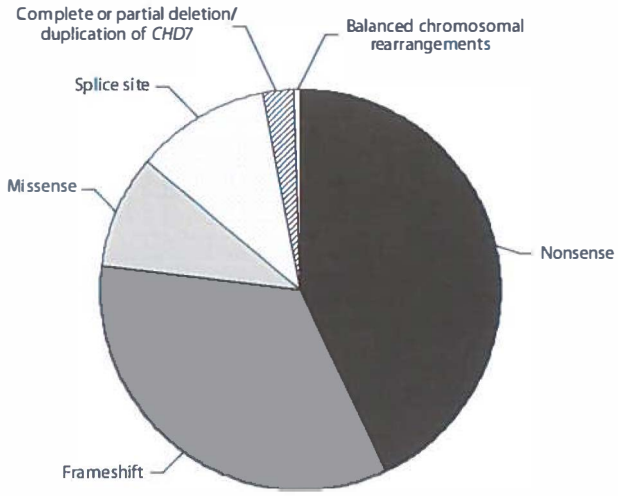

Figure 2. Distribution of mutation types in the *CHD7* gene
An overview of the distribution of the different pathogenic mutation types found in *CHD7*. Nonsense and frameshift mutations occur in over 75% of the patients. Missense and splice site mutations comprise an additional 20%, while complete and partial deletions/duplications and chromosomal re-arrangements are rare.

the unique pathogenic mutations within the gene are presented in Figure 3, grouped by mutation type. In addition to the pathogenic mutations, 89 unique unclassified variants have been described in 108 patients in the literature and from our laboratories; these are mostly missense variants and intronic variants near the splice sites. In Supplementary Table S1, we provide a complete overview of all the *CHD7* variants (pathogenic mutations and unclassified variants) found by our laboratories in patients that have not been reported before, including their phenotypic information. Detailed information on all the *CHD7* mutations, including the unclassified variants, can also be accessed at the online locus-specific database (www.CHD7.org).

The mutations are distributed along the entire coding region and splice sites of *CHD7* and all types of mutations are found (Figures 2 and 3). The most prevalent types are nonsense mutations (43%), and frameshift mutations (34%). Splice site and missense mutations are found in 11% and 9%, respectively, while small in-frame deletions rarely occur (<1%). The remainder comprises the larger deletions and duplication (2%) and translocations (<1%), which will be discussed in the next sections. No mutations were found in exon 7 and only one mutation each was found in exons 9 and 28. This is probably due to the small genomic sizes of these exons, which are 56, 84 and 58 nucleotides, respectively (2.2% of the coding genomic size).

Approximately 28-32% of the mutations are found in the regions of *CHD7* that encode for the functional domains. The encoded region of these domains is approximately 20-25% of *CHD7*, so the frequency of mutations within these domains is only slightly higher than would be expected if the mutations were distributed equally (Supplementary Table S2). This observation could be due to a predilection of missense mutations for the functional domains. Pathogenic missense mutations were predominantly found in the highly conserved middle exons of the gene, while they were not found in the first seven or last five exons of the gene. In contrast, benign missense variants occurred more often in the first and last exons, which are non-conserved regions (Bergman *et al*, submitted). Nonsense and frameshift mutations were found scattered throughout the whole gene.

Most mutations are unique for a patient or family, but *de novo* recurrent mutations do occur. In total, 96 different recurrent mutations were found in 363 index patients. The two most frequently reported mutations to date (both n=12) are the c.1480C>T in exon 2 and the c.7879C>T in exon 36. They both result in the substitution of an arginine by a stop codon, at codon 494 and codon 2627, respectively. Of all the recurrent mutations, a remarkable number involves an arginine transition to a stop codon (27 different mutations in 187 patients). This was also observed by Bartels *et al*[26] and is in agreement with previous observations that the CG-nucleotide pair is hyper-mutable to TG.[69] This makes the arginine CGA codon, which occurs 27 times in *CHD7*, uniquely vulnerable to mutating into a stop codon.

Whole gene deletions and exon deletions or duplications of *CHD7*

Chromosomal microdeletions including *CHD7* have been described in only eight index cases in the literature.[8,9,24,26,44,55,63] In addition, we recently identified two whole gene deletions of *CHD7*. A loss of

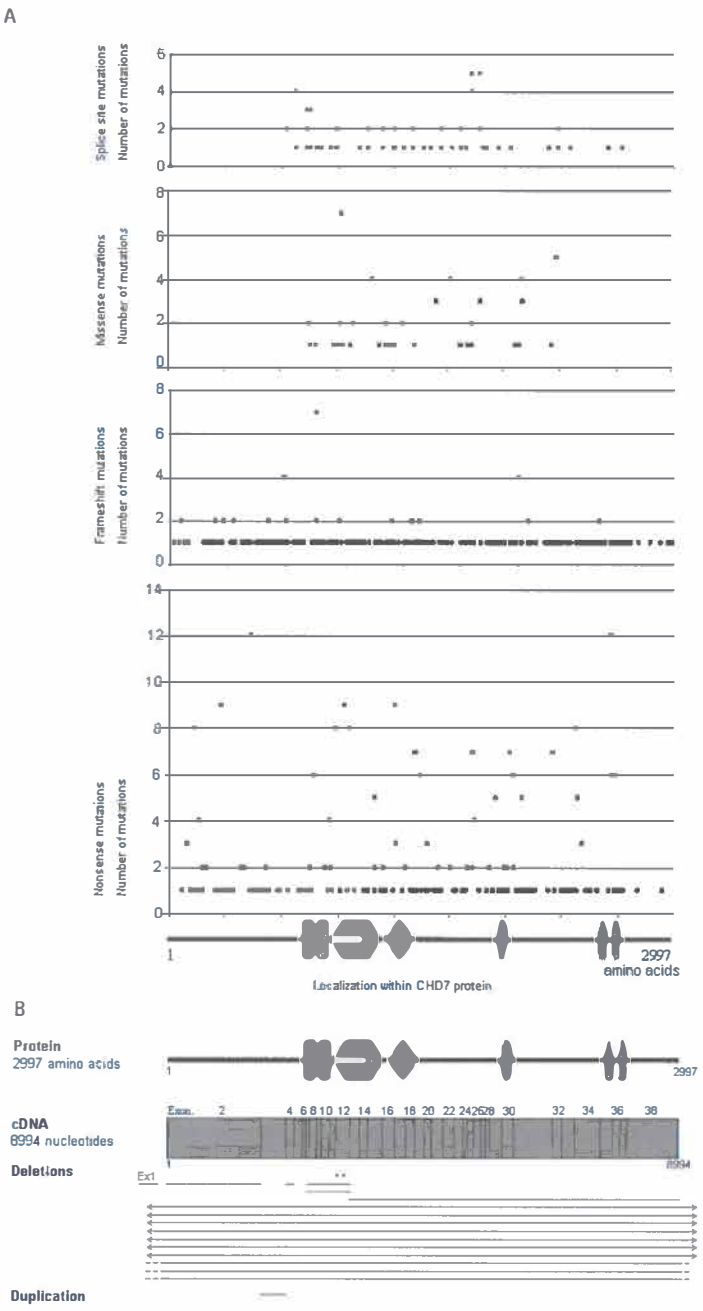

Figure 3. Overview of pathogenic *CHD7* mutations and copy number variants in index patients
The location of *CHD7* mutations in 811 index patients. **A.** Overview of the location of nonsense, frameshift, missense and splice site mutations. The mutations are spread over the *CHD7* gene, but missense mutations occur more often in the middle of the gene. The first splice site mutation is located in intron 3 and the last splice site mutation in intron 37. Several recurrent mutations occur. **B.** The deletions and duplication found in *CHD7*. Arrow = deletion extends further, dashed line = the exact size of deletion is unknown.

exons 2 to 38 was identified by MLPA (not further defined by whole-genome array) in a patient with bilateral choanal atresia, semicircular canal hypoplasia and a heart defect, and a 7.7 Mb deletion including *CHD7* was found in a patient with bilateral coloboma, external ear anomalies and a heart defect.

Whole exon deletions and duplications were found in seven index cases in the literature.[22,26,29,58,63,64] Therefore, aberrations of *CHD7* detected by MLPA or whole genome array comprise only 2% of the defects in patients with molecularly confirmed CHARGE syndrome (17 of 811 patients). In contrast, in cohorts of CHARGE or CHARGE-like patients without a *CHD7* mutation these aberrations are detected in 0-22%. However, the analytical method, number of exons screened, and clinical inclusion criteria differed between the studies. Compiling all the studies, six whole exon deletions, one whole exon duplication, and two whole gene deletions were identified in 152 patients who showed no *CHD7* mutation upon sequencing (6%).[22,26,29,58,60,63,64]

Although typical CHARGE patients without a *CHD7* mutation are more likely to have a deletion of *CHD7* than mildly affected patients, deletions have also been demonstrated in four atypical patients.[8,64] Therefore, MLPA analysis of *CHD7* is advisable in all patients suspected of CHARGE syndrome in whom no *CHD7* mutation is found by sequencing.

Translocations

Translocations involving chromosome 8q12 have been described in two cases in the literature. The first patient with an apparently balanced translocation t(6;8)(6p8p;6q8q) was later found to have a cryptic deletion including *CHD7*.[9,44] The second *de novo* translocation t(8;13)(q11.2;q22) was reported in monozygotic twins and disrupted *CHD7*.[46] We report here an additional translocation t(2;8)(q11.2;q11.2) in a typical CHARGE patient. The breakpoint was defined at 8q12.2 between FISH probes RP11-414L17 at 61.40 Mb and RP3-491L6 at 61.83 Mb (*CHD7* is located at 61.59 - 61.77 Mb). Thus, it is highly likely that *CHD7* is disrupted by the translocation. Unfortunately, MLPA and array CGH could not be performed due to insufficient DNA, so a deletion of *CHD7* could not be excluded.

***CHD7* mutation detection rate**

The mean mutation detection rate reported so far for patients suspected of CHARGE syndrome in a research setting is 58%, with a range of 33-100%, depending on the selection criteria and molecular techniques used.[9,13,17,22,25,37,49,57,60,62,63] Most studies also included atypical CHARGE patients and whole exon or whole gene deletions were not always excluded in the patients.

In a diagnostic setting, the mutation detection rate is lower because *CHD7* analysis is also commonly used to exclude CHARGE syndrome in patients with an atypical presentation. GeneDx (Gaithersburg, Maryland, USA) reported a mutation detection rate of 32% in the patients referred to them (n=203/642),[26] while in the RUNMC laboratory the mutation detection rate is 41% (n=382/922). As pointed out by Jongmans *et al*,[13] the mutation detection rate rises above 90% if only those CHARGE patients who meet the clinical diagnostic criteria of Blake *et al*[15] and/or Verloes[16]

are taken into account. On the other hand, *CHD7* mutations have been identified in atypical CHARGE patients.[12,13,22,25,49,62,63]

Benign *CHD7* variants

Many benign variants have been described in *CHD7*, mostly in intronic regions. In the NCBI Single Nucleotide Polymorphism database (http://www.ncbi.nlm.nih.gpv/SNP, dbSNP build 132), over 1500 variants are reported in the *CHD7* region. In the literature, 72 unique benign variants have been described in patients with CHARGE syndrome, their unaffected parents and controls.[17,20,23,26,37,43,54,60,63,70] Occasionally, benign variants were initially misclassified as pathogenic mutations in the literature. For example, c.6103+8C>T had occurred *de novo* and was classified as pathogenic,[17,26] but later proved to be a benign variant.[20] In Supplementary Table S3 we give an overview of the benign variants in the coding region and in the first or last 50 nucleotides of an intron that have either been published, or found by the RUNMC or ICMM, or published with frequency data in the NCBI SNP database.

Familial CHARGE syndrome and somatic and germline mosaicism

CHARGE syndrome is typically a sporadic condition. Familial recurrence is rare and almost all *CHD7* mutations occur *de novo*. Seventeen families with multiple affected members due to a segregating *CHD7* mutation have been reported to date.[12,13,17,34,47,53,61,63] In addition, we identified a presumed pathogenic missense mutation (c.6221T>C; p.Leu2074Pro) in two sisters with Kallmann syndrome, whose clinical features were previously reported by Levy and Knudtzon.[71] In all CHARGE families, a remarkable clinical variability is seen. Especially the parents are relatively mildly affected, and do not fulfil the clinical diagnostic criteria.[12] The type of mutations seen in familial CHARGE syndrome varies: mainly nonsense mutations are found in monozygotic twins and affected sibs with unaffected parents (germline mosaicism), whereas a preponderance of missense and splice site mutations is seen in the two-generation families.[12] A likely explanation is that missense and splice site mutations give rise to a milder phenotype (Bergman *et al*, submitted).

Germline and somatic mosaicism have been suggested in some families in the literature.[13,17,26,47,63] More recently, germline mosaicism was proven in a father who had a *CHD7* truncating mutation (c.7302dupA) in his spermatozoa, but not in his peripheral blood cells and who had two children with CHARGE syndrome.[53] Somatic mosaicism could be demonstrated in three families: in an unaffected mother who had two sons with CHARGE syndrome (c.5982G>A; p.Trp1994X);[13] in an unaffected father who had a son and daughter with CHARGE syndrome (c.2520G>A; p.Trp840X);[47] and in a father whose child had CHARGE syndrome (c.7636G>T; p.Glu2546X).[26] We identified an even more complicated case of somatic mosaicism in a child affected with CHARGE syndrome. *CHD7* sequence analysis in a blood sample showed 3 alleles at the c.5534+1 position (intron 26); the wild-type allele was present in half of the sequence, while two mutations were also found at that same position (c.5534+1G>A and c.5534+1G>T). MLPA analysis showed no exon copy number variations.

CHD7 analysis in blood samples from both parents was normal. The most likely explanation is that two somatic mutations occurred on one allele, creating two mutant cell lines.

Disease-causing *CHD7* variants in non-CHARGE syndrome patients

CHD7 mutations have been identified in patients with Kallmann syndrome (KS), which is a syndrome that partially overlaps with CHARGE syndrome. KS is characterised by the combination of hypogonadotropic hypogonadism (HH) and a smell deficit. Occasionally, other features, like renal anomalies, dental agenesis, cleft lip/palate and hearing loss can occur in KS.[72,73] Two groups have analysed *CHD7* in patients with normosmic idiopathic hypogonadotropic hypogonadism (nlHH) or KS. The first study analysed 197 patients and identified seven *CHD7* mutations.[20] Four patients with a *CHD7* mutation were diagnosed with nlHH and the other three patients had KS. No additional anomalies were reported in three patients, while two patients had a facial cleft in combination with cryptorchidism or hearing loss, one patient had myopia, and another had cryptorchidism. It should be noted, however, that the authors did not report whether the patients had undergone a formal smell test or if they were clinically re-evaluated after the *CHD7* mutation was identified.[20] The second study identified three *CHD7* mutations in 56 patients with nlHH or KS.[19] All three *CHD7*-positive patients were proven to be anosmic by formal smell tests and therefore had received the diagnosis KS. All patients had additional CHARGE features, and two could be re-diagnosed as CHARGE syndrome after clinical re-evaluation.[19] All nlHH/KS cases with a *CHD7* mutation were sporadic in both studies. The chance of finding a *CHD7* mutation in patients with HH seems highest if at least anosmia and one other feature of CHARGE syndrome is present, especially since HH and anosmia have been proven to be highly correlated in CHARGE syndrome patients with a proven *CHD7* mutation.[18] At least two mutations found in the studies described above were also found in CHARGE patients. The combined results of the two studies suggest that Kallmann syndrome can be seen as a mild clinical presentation of CHARGE syndrome. Recently, *CHD7* analysis was performed in a third cohort of 30 Finnish patients with Kallmann syndrome, but no pathogenic mutations were identified.[74]

Studies of *CHD7* have also been done in several cohorts of patients with one feature of CHARGE syndrome, e.g. scoliosis, cleft lip/palate or congenital heart defects. Scoliosis develops in late childhood in more than 60% of patients with CHARGE syndrome.[75] In 53 families with isolated scoliosis, a genome-wide scan showed linkage and association with 8q12 loci.[70] Further analysis revealed a potentially functional polymorphism in *CHD7* (IVS2-3238A>G), which is hypothesized to disrupt normal spinal growth patterns and predispose to spinal deformity. So far, this association has not been confirmed by a second independent study and the polymorphism has not been described in other patients or controls.

Cleft lip/palate occurs in 30-48% of patients with CHARGE syndrome with a *CHD7* mutation.[12,13,17,76] In 184 cases with non-syndromic cleft lip/palate, a role for *CHD7* could not be proven, although some variants were found.[37]

Congenital heart disease occurs in approximately 75% of CHARGE patients.[12,13,17,76] Analysis of *CHD7* in 67 patients with a congenital heart defect and in 100 controls revealed seven intronic variants.[77] Remarkably, one variant was detected in patients only (IVS14-35C>G), while another variant (IVS12-5T>C) had a lower frequency in the patient group, suggesting it has a protective effect. No *CHD7* mutations were found in the coding region and it was concluded that *CHD7* mutations do not contribute substantially to non-syndromic congenital heart defects.

Other causes of CHARGE syndrome

The cause of CHARGE syndrome remains unclear in 5-10% of typical CHARGE patients and in 40-60% of patients suspected of CHARGE syndrome. Non-detectable rearrangements in *CHD7* (e.g. deep intronic mutations that affect splicing, intragenic rearrangements or mutations in regulatory regions), and whole gene or exon deletions/duplications (which are not always screened for) might explain CHARGE syndrome in some of these patients. It is also possible that there are other genes involved in CHARGE syndrome.

The only other gene that was shown to be implicated in CHARGE syndrome, the *SEMA3E* gene, was found to be mutated in one CHARGE patient and disrupted in another patient with a *de novo* chromosomal translocation between chromosomes 2 and 7.[78] No mutation in *CHD7* was found in these patients.[17] Thus far, no additional *SEMA3E* mutations have been reported in CHARGE patients. Other candidate genes have also been tested without revealing any pathogenic mutations, e.g. *PITX2* and *PAX* in 29 and 34 patients with CHARGE syndrome, respectively.[79,80] *CHD7* results are not known for these patients. It is further worth noting that analysis of *CHD8*, whose protein product interacts with CHD7, revealed no mutations in 25 *CHD7*-negative CHARGE patients.[27]

Phenocopies of CHARGE syndrome due to chromosomal imbalances have been reported. Unfortunately, most cases were published before 2004 so that *CHD7* analysis was not performed. Chromosomal imbalances reported in patients with a CHARGE-like phenotype are shown in Table 1. Some chromosomal aberrations, for example duplication 1(q25q32) and deletion 4(q31qter), have been reported as causes of CHARGE syndrome.[81,82] However, our review of the clinical features revealed that these patients had neither choanal atresia nor coloboma, and thus did not fulfil the clinical diagnostic criteria for CHARGE syndrome.[5,16]

In contrast to the unique chromosomal cases mentioned in Table 1, a recurrent clinical overlap has been reported for 22q11.2 deletion syndrome and CHARGE syndrome.[8,12,40,45,56,66,83,84] The overlapping clinical features include cleft palate, cardiac malformations, ear abnormalities, hearing loss, growth deficiency, developmental delay, renal abnormalities, hearing loss, hypocalcaemia and immune deficiency.[8,45,56,66,83,85,86] *CHD7* mutations are more often, but not exclusively, associated with coloboma, choanal atresia, facial nerve palsy, tracheo-oesophageal fistula and micropenis compared to 22q11.2 deletions.[86] Hypoplastic semicircular canals are suggestive for CHARGE syndrome, as they are present in almost all patients with CHARGE syndrome.[12,16,87,88] However, semicircular canal abnormalities cannot exclude 22q11.2 deletion syndrome, since this feature has been described in

patients with a 22q11 deletion, albeit very rarely.[12,89] Defects of the lateral semicircular canals were also noted in a mouse model for 22q11.2 deletion syndrome, the *Tbx1*$^{+/-}$ mouse.[8]

In conclusion, *CHD7* is the major causative gene in CHARGE syndrome. If sequence analysis does not reveal a *CHD7* mutation, MLPA and genome-wide array studies should be performed in patients suspected of CHARGE syndrome. In the future this will probably be extended with whole genome sequencing.

Table 1. Unique chromosomal imbalances mimicking CHARGE syndrome

Chromosomal imbalance	Reference
der(2)t(2;21)(q37;qter)	114
der(3)t(3;22)(p25.1;q11.1)	115
del(3)(p12p21.2)	116
der(4)t(4;8)(q34,3;q22,1)	117
der(6)t(4;6)(q34;q25)	118
der(9)t(9;13)(p23;q33)	118
inv dup(14)(q22q24.3)	119
der(18)t(2;18)(q37.3;q22.3)	115
trisomy 18	120
der(21)t(19;21)(q13.1q22.3)	121
der(X)t(X;2)(p22.1;q33)	122

THE *CHD7* MUTATION DATABASE

We have established a web-based, locus-specific database which gives a complete overview of the variants identified in *CHD7*. This *CHD7* mutation database has been constructed to aid both clinicians and scientists. The database contains all the *CHD7* mutations, unclassified variants and benign variants, which have been published in the medical literature, including those presented in this article. The database is patient-based and contains information about the clinical phenotype of the patient, if provided. For missense variants a prediction of pathogenicity is given (Bergman *et al*, submitted).

The database software was constructed by the Genomics Coordination Centre, a joint venture of the Department of Genetics, UMCG, and the Groningen Bioinformatics Centre, University of Groningen, the Netherlands. The software is based on the online patient registry for dystrophic epidermolysis bullosa.[90] All the software has been built using the open-source MOLGENIS framework[91-93] and is freely available to others working on locus-specific databases at http://www.molgenis.org.

Mutations are numbered according to the current reference sequence (RefSeq NM_017780.2), and the mutation nomenclature is according to the Human Genome Variation Society (HGVS) recommendations (http://www.hgvs.org/rec.html). The database will be freely accessible online at www.CHD7.org. It can be updated with any reported variant from any team, worldwide. It is highly

recommended that new as well as previously reported variants are submitted to the database, because additional data will improve its value, e.g. for the interpretation of unclassified variants and phenotype-genotype correlations.

NOVEL INSIGHTS INTO CHD7 FUNCTION

Expression patterns of CHD7

The expression of CHD7 has been studied in human, mouse and chicken embryos, amongst others.[6,7,17,57,94-97] In all species, Chd7 expression patterns correlate with the developmental abnormalities observed in CHARGE syndrome.[4,6,17,57] The expression of Chd7 is tissue- and embryonic stage-dependent. Neural crest derived cells express CHD7 in different tissues in all the studied species, while no major differences in expression pattern are observed across species.[4-7,17,57] Expression has been observed in several areas of the brain, including the pituitary, olfactory bulb, and ganglia of the cranial nerves, and has also been demonstrated in the otic and optic pits, developing inner ear, nasal and oral epithelium.[4,6,7,17,57,94,95] CHD7 expression was also noted in the vascular plexus of the yolk sac, cardiac outflow tract, pharyngeal and brachial arches, and the heart, although not in all studies.[4,6,7,17,97] It was also seen in the enteric neurons, kidneys and epithelium of the stomach, gut and lungs.[6,7]

Animal models for CHARGE syndrome

Different models exist, of which the mouse models have been studied most extensively.[6-8,94,95,98-100] The mouse *Chd7* gene sequence is 97% similar to the human sequence. The first nine *Chd7* mutant mice, including the most-studied *Whirligig* mouse (*Chd7*$^{Whi/+}$) with a heterozygous nonsense mutation in exon 11, were identified in a large-scale ENU mutagenesis program by their dominantly inherited head bobbing and circling behaviour due to inner ear defects.[6] Later, *Chd7*-deficient mice were generated using gene-trap technology, where a beta-galactosidase expression vector was introduced between exons 1 and 2 of the gene (*Chd7*$^{Gt/+}$).[7]

Mice with homozygous *Chd7* mutations die *in utero* and in heterozygous mice a reduced survival at weaning is seen.[6,7] Most abnormalities frequently observed in human CHARGE syndrome have been found in mice as well. All mutant mice show a balance disturbance due to semicircular canal defects consistent with the phenotype in humans.[6-8,98] In addition, in most heterozygous mice, low postnatal body weight or reduced growth was found.[6,7] Genital defects in *Chd7*$^{Whi/+}$ mice include vulval hypoplasia, clitoral abnormalities, and abnormal uterine horns in females, and hypoplastic testes in males.[6,94] In *Chd7*$^{Gt/+}$ mice, delayed puberty, erratic oestrus cycles, decreased levels of circulating LH and FSH, and a reduced GnRH neuron count in the hypothalamus were observed.[100] Furthermore, hyposmia and olfactory bulb anomalies were observed in *Chd7*-deficient mice.[94,95] Heart defects in mice include interventricular septum defects and pharyngeal arch anomalies, like interrupted aortic arch,[6,8] while choanal atresia and cleft palate have also been observed in some mice.[6] Remarkably, optic coloboma has not been reported in mice, but some do have a keratoconjunctivitis sicca.[6]

External ear anomalies and tracheo-oesophageal defects have also not been described previously in *Chd7*-deficient mice. Why mice with *Chd7* mutations display some, but not all CHARGE features is unclear, but may indicate species-specific differences in the developmental requirement for Chd7 or differences in genetic background.[76]

The effect of *Chd7* deficiency has also been studied in *Xenopus* and *Drosophila*. In *Chd7*-deficient *Xenopus* embryos, otolith malformations, ocular coloboma, microphthalmia, craniofacial malformations and heart defects were observed.[5] Null mutations in *Kismet*, the homologue of *Chd7* and *Chd8* in *Drosophila*, were found to be embryonically lethal. Decreased Kismet expression was associated with abnormal wings, neuro-anatomical defects, and defects in memory and motor function.[101,102]

The combination of a heterozygous *Chd7* mutation with a heterozygous mutation in another gene might cause more severe defects. These double heterozygous effects have been studied in mouse models for Kallmann syndrome (*Chd7*$^{Whi/+}$;*Fgfr1*$^{Hspy/+}$) and 22q11.2 deletion syndrome (*Chd7*$^{+/-}$;*Tbx1*$^{+/-}$).[8,94] Double heterozygous *Chd7*$^{Whi/+}$;*Fgfr1*$^{Hspy/+}$ mice showed reduced survival, but their anatomical abnormalities were the same as in the *Chd7*$^{Whi/+}$ mice.[94] In double heterozygous *Chd7*$^{+/-}$;*Tbx1*$^{+/-}$ mice, the heart, inner ear and thymus were found to be more frequently and/or more severely affected. In addition, the postnatal viability of double heterozygotes was significantly reduced.[8] Thus, the double heterozygous models studied so far were indeed less viable and thus more severely affected.

Function of the CHD7 protein

Before the discovery of *CHD7* mutations as the cause of CHARGE syndrome, already several theories had been proposed to explain the pathogenesis of the various malformations seen in CHARGE syndrome. The postulated pathogenic mechanisms included maldevelopment of the neural crest cells,[103] disruption of the interaction between mesoderm and neural crest cells,[104] and disruption of mesenchymal-epithelial interaction.[105] Upon the discovery of *CHD7* as the major actor in CHARGE syndrome, a critical role in chromatin remodelling during development was suggested, based on the domains of CHD7 and the known function of CHD family members.[9] Recently, more insight into the function of CHD7 has been obtained.

Several studies have focused on CHD7 binding sites and function. Schnetz *et al* showed that CHD7 binds in a cell type- and stage-specific manner to methylated histone H3 lysine 4 in enhancer regions (i.e. regions associated with transcriptional activation) of numerous genes.[3] They concluded that CHD7 may have a function in enhancer-mediated transcription based on four observations: the CHD7 binding sites are predominantly located distal to transcription start sites, most often contain DNase hypersensitive sites, are frequently conserved, and are located near genes expressed in relatively high levels similar to gene enhancer elements. In mouse embryonic stem (ES) cells, Chd7 was shown to co-localize at suspected gene enhancer elements together with a known gene-enhancer binding protein element, p300, and other proteins which are core components of the

transcriptual circuitry of ES cells, for example Oct4, Sox2, Nanog, Smad1 and Stat3.[106] The expression profiles of *Chd7* wild-type, heterozygous and null ES cells from mice indicate that Chd7 modulates or fine tunes the levels of genes that are specifically expressed in mouse ES cells in both a positive and negative direction, but it has no effect on ES cell pluripotency, self-renewal or reprogramming.[106] A study of the CHD7 *Drosophila* ortholog, Kismet, showed that Kismet also regulates the transcription of genes by promoting early elongation by RNA polymerase II and by recruiting the ASH1 and TRX histone methyltransferases to chromatin to counteract the epigenetic silencing of genes by the Polycomb group proteins.[107]

In addition to a role in the transcription regulation of nuclear genes, CHD7 was also reported to be involved in the regulation of ribosomal RNA (rRNA) biogenesis in the nucleolus.[108] The involvement of CHD7 in rRNA regulation was suspected because chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq) showed a high enrichment of CHD7 at the rDNA. Results from further tests, including analysis of pre-rRNA levels in wild-type, heterozygous and null ES cells from mouse embryos, as well as the effect on cell proliferation and protein synthesis in *CHD7* knockdown cells, supported the idea that CHD7 functions as a positive regulator of rRNA synthesis. It was also shown that the role of Chd7 is tissue-specific by comparing pre-rRNA expression between wild-type and heterozygous cells of different tissues. In addition, it was noticed that Chd7 co-associates with treacle, the protein involved in Treacher Collins syndrome, and that the binding of treacle with rDNA partly depends on the presence of Chd7.[108]

A tissue-specific effect of CHD7 was demonstrated in a study in *Xenopus* and human neural crest cells, which showed that CHD7 is essential for the formation of multipotent migratory neural crest cells.[5] The transcription factors Sox9, Twist and Slug, which are critical for the formation of multipotent migratory neural crest cells, were diminished after knockdown of Chd7 in *Xenopus* embryos. Among others, PBAF (polybromo- and BRG1-associated factor containing complex) is a molecular partner for CHD7 in human neural crest cells: CHD7 and PBAF bind together to the neural crest cell-specific regulatory elements of *TWIST1* and *SOX9*. The cooperation of CHD7 with PBAF promotes neural crest gene expression and cell migration.

Other tissue-specific studies show that Chd7 is required for the proliferation and differentiation of neural stem cells in the basal olfactory epithelium of *Chd7*$^{Gt/+}$ mice.[95] *Chd7*-haploinsufficiency was associated with decreased expression of *Fgfr1*, *Bmp4* and *Otx2* in the embryonic olfactory placode of *Chd7*$^{Gt/+}$ mice, whereas in the adult hypothalamus *Otx2* and *GnRH1* expression were diminished.[100] These results suggest that Chd7 affects GnRH neurogenesis and signalling by influencing the transcriptional regulation of target genes involved in the BMP and FGF pathways. In the inner ear of mice, Chd7 is necessary for proliferation of neuroblasts and the expression of genes known to be involved in inner ear morphogenesis, like *Ngn1, Itx2* and *Fgf10*.[96] In mesenchymal stem cells of bone marrow, Chd7 forms a complex with Nlk, Setdb1, and Ppar-gamma that promotes osteoblast formation in preference to adipogenesis.[109] In the mouse pharyngeal arch, Chd7 and Tbx1 are both required in ectoderm during embryogenesis for normal great vessel development.[8] In the

wing development of *Drosophila*, Kismet is a component of the *hedgehog* transcriptual repression mechanism in anterior compartment cells.[102] In mouse neural stem cells, it was shown that Chd7 cooperates with Sox2 (an HMG-box transcription factor) in activating the expression of common target genes, which include effectors of the Sonic Hedgehog (*Gli2*, *Gli3*, *Mycn* and *Tulp3*) and Notch pathway (*Jag1*, *Rbpj* and *Hes5*).[110,111] CHARGE syndrome and SOX2 anophthalmia syndrome show considerable clinical overlap and also the phenotypes of the diseases caused by mutations in the common target genes overlap with CHARGE syndrome and SOX2 anophthalmia syndrome: pituitary and genital anomalies occur in Pallister Hall syndrome (*GLI3*), tracheo-oesophageal anomalies are seen in Feingold syndrome (*MYCN*), and semicircular canal anomalies are present in Alagille syndrome (*JAG1*).

We conclude from all recent studies on the function of CHD7 that its haploinsufficiency alters the transcription of tissue-specific target genes that are normally regulated by CHD7 or complexes in which CHD7 is involved.[3,106,108,110] Since the effect of CHD7 is tissue- and developmental stage-dependent due to different binding sites, protein complexes and target genes,[3,108] the current hypothesis is that the broad clinical variability of congenital anomalies seen in CHARGE syndrome may be explained by subtle alterations of the CHD7 level in time and place. In this respect, frequently affected organs, like the inner ear, may be more sensitive to CHD7-dosage than, for example, the palate which is more variably affected.[108,112] However, the precise gene targets and complexes for each tissue are still unknown, and the common factors, targets, and molecular genetic pathways are only slowly starting to emerge.[112,113] Further studies are needed to identify the genetic, epigenetic and environmental factors that modify the phenotype in CHARGE syndrome.

Reviewing the theories on the pathogenesis of CHARGE syndrome postulated before the identification of *CHD7*, we conclude that the role of the neural crest has been confirmed by recent studies.[5,103] CHD7 may also indirectly influence the interaction between the neural crest and other tissues, or have an additional direct effect on other cell types, so the theories on mesenchymal-epithelial and mesoderm-neural crest cell interaction might be correct as well.[104,105] These theories deserve further study since not all the defects seen in CHARGE syndrome can be explained by neural crest cell involvement.[105] Furthermore, a recent study in heterozygous *Chd7* mice showed that rescue of Chd7 in neural crest cells did not improve the phenotype of pharyngeal arch defects, while rescue of Chd7 in pharyngeal ectoderm did.[8]

LATEST CLINICAL INSIGHTS

We estimated a new incidence of CHARGE syndrome, which is lower than the previous estimate of 1 in 10,000 live births worldwide.[10,11] Our estimate was based on the number of *CHD7* mutations that were identified in children born in the Netherlands between 2006 and 2009 and the overall birth prevalence in those years. This gives a good estimate of the incidence of CHARGE syndrome, because most children who are suspected of CHARGE syndrome will undergo *CHD7* analysis (DNA testing is insurance covered in the Netherlands). Forty *CHD7* mutations were found in 735,942 live-

born children, which gives a birth incidence of 1 in 18,400. However, because we based our estimate on the number of *CHD7* mutations, the patients with CHARGE syndrome who had not yet undergone *CHD7* analysis because of a very mild phenotype (14-17% of all patients with a *CHD7* mutation) and the patients with typical CHARGE syndrome but without a mutation in *CHD7* (a maximum of 10%) were not included.[12,13] If we take this into account, we estimate the incidence of CHARGE syndrome in the Netherlands at 1 in 15,000 to 1 in 17,000 live births.

Now that CHARGE syndrome can be diagnosed molecularly, it is possible to delineate the phenotypic consequences of *CHD7* mutations. The penetrance of *CHD7* mutations is generally complete, but their expression is highly variable.[12] This is underscored by the observation of discordant features in monozygotic twins, and by the occurrence of identical mutations in patients with Kallmann and CHARGE syndromes.[13,17,63] The most consistent clinical features in patients with a *CHD7* mutation are semicircular canal hypoplasia, external ear abnormalities and cranial nerve dysfunction, which are present in over 90% of the patients.[12,76] In addition, most patients have some degree of developmental delay, but their cognitive function can be normal.[12,76] Coloboma and choanal atresia are found in 75-81% and 38-55% of patients with a *CHD7* mutation, respectively.[12,76] Minor features of CHARGE syndrome vary in their incidence: congenital heart defects occur in 76-77%, genital hypoplasia in 62-81%, cleft lip and/or palate in 33-48%, and tracheo-oesophageal anomalies in 19-29%.[12,76] No clear genotype-phenotype correlations have been found for *CHD7* mutations, although missense mutations can be associated with a milder phenotype (Bergman *et al*, submitted).[12,13,18] With the rapidly growing information on the clinical effects of *CHD7* mutations, it is likely that the clinical spectrum of CHARGE syndrome will expand further and the definition of CHARGE syndrome may need to be redefined. CHARGE syndrome remains a clinical diagnosis, as long as no mutations can be found in 5-10% of the clinical typical CHARGE patients. Nonetheless, a pathogenic mutation in *CHD7* confirms the diagnosis and gives tools for counselling about reproductive options. In patients who do not completely fulfil the clinical CHARGE diagnostic criteria, identifying a *CHD7* mutation is important in order to guarantee accurate clinical surveillance, which can possibly lead to the identification of additional CHARGE features (e.g. balance problems, endocrine dysfunction, and anosmia).[12] Recently, a guideline for *CHD7* analysis was published, which will help clinicians to decide if *CHD7* analysis or imaging of the semicircular canals should be performed in the diagnostic work-up of the patients that are suspected of CHARGE syndrome.[12]

For counselling of recurrence risks, it is important to know the genetic status of the parents. Most *CHD7* mutations occur *de novo* and are predominantly located on the paternal allele of the patient.[54] In these cases, the recurrence risk for the parents is 2-3%, as both germline and somatic mosaicism have been described.[13,47,53] In a minority of cases, however, one of the parents carries the *CHD7* mutation and the recurrence risk is 50%.[12] Reproductive options should be discussed with the parents of patients with CHARGE syndrome and with the patients themselves at an appropriate age. The severity and diversity of CHARGE syndrome features cannot be predicted in the offspring by molecular diagnosis. Foetal ultrasound has an additional value, but the full clinical presentation,

may only become evident after birth.

CONCLUSIONS

Loss-of-function mutations in *CHD7* cause CHARGE syndrome, a highly variable multiple congenital anomaly syndrome. We have established a web-based database (www.CHD7.org) which gives an up-to-date overview of all the described *CHD7* mutations and clinical phenotype of patients, and includes 184 new mutations and 296 patients presented in this paper. Our database will also allow inclusion of new cases. In this paper we have summarized the latest data on expression studies, animal models and molecular studies of CHD7. The function of CHD7 and its interaction with other genes is emerging, indicating that it regulates the expression of genes in a cell type- and embryonic stage-dependent manner.

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REFERENCES

1 Hall JA, Georgel PT. CHD proteins: a diverse family with strong ties. *Biochem Cell Biol* 2007;85(4):463-476.

2 Marfella CG, Imbalzano AN. The Chd family of chromatin remodelers. *Mutat Res* 2007;618(1-2):30-40.

3 Schnetz MP, Bartels CF, Shastri K, Balasubramanian D, Zentner GE, Balaji R, et al. Genomic distribution of CHD7 on chromatin tracks H3K4 methylation patterns. *Genome Res* 2009;19(4):590-601.

4 Aramaki M, Kimura T, Udaka T, Kosaki R, Mitsuhashi T, Okada Y, et al. Embryonic expression profile of chicken CHD7, the ortholog of the causative gene for CHARGE syndrome. *Birth Defects Res A Clin Mol Teratol* 2007;79(1):50-57.

5 Bajpai R, Chen DA, Rada-Iglesias A, Zhang J, Xiong Y, Helms J, et al. CHD7 cooperates with PBAF to control multipotent neural crest formation. *Nature* 2010;463(7283):958-962.

6 Bosman EA, Penn AC, Ambrose JC, Kettleborough R, Stemple DL, Steel KP. Multiple mutations in mouse *Chd7* provide models for CHARGE syndrome. *Hum Mol Genet* 2005;14(22):3463-3476.

7 Hurd EA, Capers PL, Blauwkamp MN, Adams ME, Raphael Y, Poucher HK, et al. Loss of Chd7 function in gene-trapped reporter mice is embryonic lethal and associated with severe defects in multiple developing tissues. *Mamm Genome* 2007;18(2):94-104.

8 Randall V, McCue K, Roberts C, Kyriakopoulou V, Beddow S, Barrett AN, et al. Great vessel development requires biallelic expression of Chd7 and Tbx1 in pharyngeal ectoderm in mice. *J Clin Invest* 2009;119(11):3301-3310.

9 Vissers LE, van Ravenswaaij CM, Admiraal R, Hurst JA, De Vries BB, Janssen IM, et al. Mutations in a new member of the chromodomain gene family cause CHARGE syndrome. *Nat Genet* 2004;36(9):955-957.

10 Issekutz KA, Graham JM,Jr., Prasad C, Smith IM, Blake KD. An epidemiological analysis of CHARGE syndrome: preliminary results from a Canadian study. *Am J Med Genet A* 2005;133(3):309-317.

11 Sanlaville D, Verloes A. CHARGE syndrome: an update. *Eur J Hum Genet* 2007;15(4):389-399.

12 Bergman JE, Janssen N, Hoefsloot LH, Jongmans MC, Hofstra RM, van Ravenswaaij-Arts CM. *CHD7* mutations and CHARGE syndrome: the clinical implications of an expanding phenotype. *J Med Genet* 2011;48(5):334-342.

13 Jongmans MC, Admiraal RJ, van der Donk KP, Vissers LE, Baas AF, Kapusta L, et al. CHARGE syndrome: the phenotypic spectrum of mutations in the *CHD7* gene. *J Med Genet* 2006;43(4):306-314.

14 Pagon RA, Graham JM, Jr., Zonana J, Yong SL. Coloboma, congenital heart disease, and choanal atresia with multiple anomalies: CHARGE association. *J Pediatr* 1981;99(2):223-227.

15 Blake KD, Davenport SL, Hall BD, Hefner MA, Pagon RA, Williams MS, et al. CHARGE association: an update and review for the primary pediatrician. *Clin Pediatr (Phila)* 1998;37(3):159-173.

16 Verloes A. Updated diagnostic criteria for CHARGE syndrome: a proposal. *Am J Med Genet A* 2005;133(3):306-308.

17 Lalani SR, Safiullah AM, Fernbach SD, Harutyunyan KG, Thaller C, Peterson LE, et al. Spectrum of *CHD7* mutations in 110 individuals with CHARGE syndrome and genotype-phenotype correlation. *Am J Hum Genet* 2006;78(2):303-314.

18 Bergman JE, Bocca G, Hoefsloot LH, Meiners LC, Ravenswaaij-Arts CM. Anosmia predicts hypogonadotropic hypogonadism in CHARGE syndrome. *J Pediatr* 2011;158(3):474-479.

19 Jongmans MC, Ravenswaaij-Arts CM, Pitteloud N, Ogata T, Sato N, Claahsen-van der Grinten HL, et al. *CHD7* mutations in patients initially diagnosed with Kallmann syndrome--the clinical overlap with CHARGE syndrome. *Clin Genet* 2009;75(1):65-71.

20 Kim HG, Kurth I, Lan F, Meliciani I, Wenzel W, Eom SH, et al. Mutations in *CHD7*, encoding a chromatin-remodeling protein, cause idiopathic hypogonadotropic hypogonadism and Kallmann syndrome. *Am J Hum Genet* 2008;83(4):511-519.

21 Alazami AM, Alzahrani F, Alkuraya FS. Expanding the "E" in CHARGE. *Am J Med Genet A* 2008;146A(14):1890-1892.

22 Aramaki M, Udaka T, Kosaki R, Makita Y, Okamoto N, Yoshihashi H, et al. Phenotypic spectrum of CHARGE syndrome with *CHD7* mutations. *J Pediatr* 2006;148(3):410-414.

23 De Arriba Munoz A, Monge Galindo L, Lopez Pison J, Lafuente Hidalgo M, Perez Delgado R, Dominguez Cajal M, et al. CHARGE syndrome and *CHD7* gene mutation. *Neurologia* 2011;26(4):255.

24 Arrington CB, Cowley BC, Nightingale DR, Zhou H, Brothman AR, Viskochil DH. Interstitial deletion 8q11.2-q13 with congenital anomalies of CHARGE association. *Am J Med Genet A* 2005;133(3):326-330.

25 Asakura Y, Toyota Y, Muroya K, Kurosawa K, Fujita K, Aida N, et al. Endocrine and radiological studies in patients with molecularly confirmed CHARGE syndrome. *J Clin Endocrinol Metab* 2008;93(3):920-924.

26 Bartels CF, Scacheri C, White L, Scacheri PC, Bale S. Mutations in the *CHD7* Gene: The Experience of a Commercial Laboratory. *Genet Test Mol Biomarkers* 2010;14(6):881-891.

27 Batsukh T, Pieper L, Koszucka AM, von Velsen N, Hoyer-Fender S, Elbracht M, et al. CHD8 interacts with CHD7, a protein which is mutated in CHARGE syndrome. *Hum Mol Genet* 2010;19(14):2858-2866.

28 Bech AP, op den Akker JO, Matthijsse PR. Isolation of the left subclavian artery from the pulmonary artery in a patient with CHARGE association. *Congenit Anom (Kyoto)* 2010;50(3):200-202.

29 Bergman JE, de Wijs I, Jongmans MC, Admiraal RJ, Hoefsloot LH, Ravenswaaij-Arts CM. Exon copy number alterations of the *CHD7* gene are not a major cause of CHARGE and CHARGE-like syndrome. *Eur J Med Genet* 2008;51(5):417-425.

30 Bergman JE, Blake KD, Bakker MK, du Marchie Sarvaas GJ, Free RH, Ravenswaaij-Arts CM. Death in CHARGE syndrome after the neonatal period. *Clin Genet* 2010;77(3):232-240.

31 Chang TM, Ke YY, Chen WL, Chen M, Yang AD. Unilateral agenesis of the internal carotid artery in CHARGE syndrome. *Pediatr Neonatol* 2010;51(6):363-366.

32 Chiu CH, Thakuria J, Agrawal PB. Novel *CHD7* and *FBN1* mutations in an infant with multiple congenital anamolies. *Indian J Pediatr* 2010;77(2):208-209.

33 Dauber A, Hirschhorn JN, Picker J, Maher TA, Milunsky A. Delayed puberty due to a novel mutation in *CHD7* causing CHARGE syndrome. *Pediatrics* 2010;126(6):e1594-e1598.

34 Delahaye A, Sznajer Y, Lyonnet S, Elmaleh-Berges M, Delpierre I, Audollent S, et al. Familial CHARGE syndrome because of *CHD7* mutation: clinical intra- and interfamilial variability. *Clin Genet* 2007;72(2):112-121.

35 Douglas AG, Lam W. Extending the phenotypic spectrum of CHARGE syndrome: a case with preaxial polydactyly. *Clin Dysmorphol* 2009;19(1):33-34.

36 Ellison J. Gene symbol: *CHD7*. Disease: CHARGE syndrome. *Hum Genet* 2008;124(3):323.

37 Felix TM, Hanshaw BC, Mueller R, Bitoun P, Murray JC. *CHD7* gene and non-syndromic cleft lip and palate. *Am J Med Genet A* 2006;140(19):2110-2114.

38 Foppiani L, Maffe A, Forzano F. CHARGE syndrome as unusual cause of hypogonadism: endocrine and molecular evaluation. *Andrologia* 2010;42(5):326-330.

39 Fujita K, Aida N, Asakura Y, Kurosawa K, Niwa T, Muroya K, et al. Abnormal basiocciput development in CHARGE syndrome. *Am J Neuroradiol* 2009;30(3):629-634.

40 Gennery AR, Slatter MA, Rice J, Hoefsloot LH, Barge D, Lean-Tooke A, et al. Mutations in *CHD7* in patients with CHARGE syndrome cause T-B + natural killer cell + severe combined immune deficiency and may cause Omenn-like syndrome. *Clin Exp Immunol* 2008;153(1):75-80.

41 Glueckert R, Rask-Andersen H, Sergi C, Schmutzhard J, Mueller B, Beckmann F, et al. Histology and synchrotron radiation-based microtomography of the inner ear in a molecularly confirmed case of CHARGE syndrome. *Am J Med Genet A* 2010;152A(3):665-673.

42 Holak HM, Kohlhase J, Holak SA, Holak NH. New recognized ophthalmic morphologic anomalies in CHARGE syndrome caused by the R2319C mutation in the *CHD7* gene. *Ophthalmic Genet* 2008;29(2):79-84.

43 Hoover-Fong J, Savage WJ, Lisi E, Winkelstein J, Thomas GH, Hoefsloot LH, et al. Congenital T cell deficiency in a patient with CHARGE syndrome. *J Pediatr* 2009;154(1):140-142.

44 Hurst JA, Meinecke P, Baraitser M. Balanced t(6;8)(6p8p;6q8q) and the CHARGE association. *J Med Genet* 1991;28(1):54-55.

45 Inoue H, Takada H, Kusuda T, Goto T, Ochiai M, Kinjo T, et al. Successful cord blood transplantation for a CHARGE syndrome with *CHD7* mutation showing DiGeorge sequence including hypoparathyroidism. *Eur J Pediatr* 2010;169(7):839-844.

46 Johnson D, Morrison N, Grant L, Turner T, Fantes J, Connor JM, et al. Confirmation of *CHD7* as a cause of CHARGE association identified by mapping a balanced chromosome translocation in affected monozygotic twins. *J Med Genet* 2006;43(3):280-284.

47 Jongmans MC, Hoefsloot LH, van der Donk KP, Admiraal RJ, Magee A, van de Laar I, et al. Familial CHARGE syndrome and the *CHD7* gene: a recurrent missense mutation, intrafamilial recurrence and variability. *Am J Med Genet A* 2008;146(1):43-50.

48 Kaliakatsos M, Giannakopoulos A, Fryssira H, Kanariou M, Skiathitou AV, Siahanidou T, et al. Combined microdeletions and *CHD7* mutation causing severe CHARGE/DiGeorge syndrome: clinical presentation and molecular investigation by array-CGH. *J Hum Genet* 2010;55(11):761-763.

49 Lee YW, Kim SC, Shin YL, Kim JW, Hong HS, Lee YK, et al. Clinical and genetic analysis of the *CHD7* gene in Korean patients with CHARGE syndrome. *Clin Genet* 2009;75(3):290-293.

50 Lobete Prieto CJ, Llano Rivas I, Fernandez Toral J, Madero Barrajon P. [CHARGE syndrome]. *Arch Argent Pediatr* 2010;108(1):e9-e12.

51 Michelucci A, Ghirri P, Iacopetti P, Conidi ME, Fogli A, Baldinotti F, et al. Identification of three novel mutations in the *CHD7* gene in patients with clinical signs of typical or atypical CHARGE syndrome. *Int J Pediatr Otorhinolaryngol* 2010;74(12):1441-1444.

52 Ogata T, Fujiwara I, Ogawa E, Sato N, Udaka T, Kosaki K. Kallmann syndrome phenotype in a female patient with CHARGE syndrome and *CHD7* mutation. *Endocr J* 2006;53(6):741-743.

53 Pauli S, Pieper L, Haeberle J, Grzmil P, Burfeind P, Steckel M, et al. Proven germline mosaicism in a father of two children with CHARGE syndrome. *Clin Genet* 2009;75(5):473-479.

54 Pauli S, von Velsen N, Burfeind P, Steckel M, Manz J, Buchholz A, et al. *CHD7* mutations causing CHARGE syndrome are predominantly of paternal origin. *Clin Genet* 2011;Epub ahead of print, doi: 10.1111/j.1399-0004.2011.01701.x.

55 Pedersen AM, Skovby F. [Molecular diagnosis of CHARGE syndrome]. *Ugeskr Laeger* 2007;169(5):402-406.

56 Sanka M, Tangsinmankong N, Loscalzo M, Sleasman JW, Dorsey MJ. Complete DiGeorge syndrome associated with *CHD7* mutation. *J Allergy Clin Immunol* 2007;120(4):952-954.

57 Sanlaville D, Etchevers HC, Gonzales M, Martinovic J, Clement-Ziza M, Delezoide AL, et al. Phenotypic spectrum of CHARGE syndrome in fetuses with *CHD7* truncating mutations correlates with expression during human development. *J Med Genet* 2006;43(3):211-217.

58 Udaka T, Okamoto N, Aramaki M, Torii C, Kosaki R, Hosokai N, et al. An Alu retrotransposition-mediated deletion of *CHD7* in a patient with CHARGE syndrome. *Am J Med Genet A* 2007;143(7):721-726.

59 Van de Laar I, Dooijes D, Hoefsloot L, Simon M, Hoogeboom J, Devriendt K. Limb anomalies in patients with CHARGE syndrome: an expansion of the phenotype. *Am J Med Genet A* 2007;143(22):2712-2715.

60 Vuorela P, la-Mello S, Saloranta C, Penttinen M, Poyhonen M, Huoponen K, et al. Molecular analysis of the *CHD7* gene in CHARGE syndrome: identification of 22 novel mutations and evidence for a low contribution of large *CHD7* deletions. *Genet Med* 2007;9(10):690-694.

61 Vuorela PE, Penttinen MT, Hietala MH, Laine JO, Huoponen KA, Kaariainen HA. A familial CHARGE syndrome with a *CHD7* nonsense mutation and new clinical features. *Clin Dysmorphol* 2008;17(4):249-253.

62 Wessels K, Bohnhorst B, Luhmer I, Morlot S, Bohring A, Jonasson J, et al. Novel *CHD7* mutations contributing to the mutation spectrum in patients with CHARGE syndrome. *Eur J Med Genet* 2010;53(5):280-285.

63 Wincent J, Holmberg E, Stromland K, Soller M, Mirzaei L, Djureinovic T, et al. *CHD7* mutation spectrum in 28 Swedish patients diagnosed with CHARGE syndrome. *Clin Genet* 2008;74(1):31-38.

64 Wincent J, Schulze A, Schoumans J. Detection of *CHD7* deletions by MLPA in CHARGE syndrome patients with a less typical phenotype. *Eur J Med Genet* 2009;52(4):271-272.

65 Wright EMMB, O'Connor R, Kerr BA. Radial aplasia in CHARGE syndrome: A new association. *Eur J Med Genet* 2009;52(4):239-241.

66 Writzl K, Cale CM, Pierce CM, Wilson LC, Hennekam RC. Immunological abnormalities in CHARGE syndrome. *Eur J Med Genet* 2007;50(5):338-345.

67 Yagihashi T, Hatori K, Ishii K, Torii C, Momoshima S, Takahashi T, et al. Juvenile muscular atrophy of a unilateral upper extremity (Hirayama disease) in a patient with CHARGE syndrome. *Mol Syndromol* 2010;1(2):91-94.

68 Yamada K, Fukushi D, Ono T, Kondo Y, Kimura R, Nomura N, et al. Characterization of a *de novo* balanced t(4;20)(q33;q12) translocation in a patient with mental retardation. *Am J Med Genet A* 2010;152A(12):3057-3067.

69 Antonarakis SE, Krawczak M, Cooper DN. Disease-causing mutations in the human genome. *Eur J Pediatr* 2000;159 Suppl 3:S173-S178.

70 Gao X, Gordon D, Zhang D, Browne R, Helms C, Gillum J, et al. *CHD7* gene polymorphisms are associated with susceptibility to idiopathic scoliosis. *Am J Hum Genet* 2007;80(5):957-965.

71 Levy CM, Knudtzon J. Kallmann syndrome in two sisters with other developmental anomalies also affecting their father. *Clin Genet* 1993;43(1):51-53.

72 Pallais JC, Au M, Pitteloud N, Seminara S, Crowley WF. Kallmann syndrome. GeneReviews 2011. http://www.ncbi.nlm.nih.gov/books/NBK1334/ (accessed 2011).

73 Tsai PS, Gill JC. Mechanisms of disease: Insights into X-linked and autosomal-dominant Kallmann

syndrome. *Nat Clin Pract Endocrinol Metab* 2006;2(3):160-171.

74 Laitinen EM, Vaaralahti K, Tommiska J, Eklund E, Tervaniemi M, Valanne L, et al. Incidence, phenotypic features and molecular genetics of Kallmann syndrome in Finland. *Orphanet J Rare Dis* 2011;6:41.

75 Doyle C, Blake K. Scoliosis in CHARGE: a prospective survey and two case reports. *Am J Med Genet A* 2005;133A(3):340-343.

76 Zentner GE, Layman WS, Martin DM, Scacheri PC. Molecular and phenotypic aspects of *CHD7* mutation in CHARGE syndrome. *Am J Med Genet A* 2010;152A(3):674-686.

77 Qi Q, Yi L, Yang C, Chen H, Shen L, Mo X, et al. [Mutation analysis of the *CHD7* gene in patients with congenital heart disease]. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi* 2008;25(6):637-641.

78 Lalani SR, Safiullah AM, Molinari LM, Fernbach SD, Martin DM, Belmont JW. *SEMA3E* mutation in a patient with CHARGE syndrome. *J Med Genet* 2004;41(7):e94.

79 Martin DM, Probst FJ, Fox SE, Schimmenti LA, Semina EV, Hefner MA, et al. Exclusion of *PITX2* mutations as a major cause of CHARGE association. *Am J Med Genet* 2002;111(1):27-30.

80 Tellier AL, Amiel J, Delezoide AL, Audollent S, Auge J, Esnault D, et al. Expression of the *PAX2* gene in human embryos and exclusion in the CHARGE syndrome. *Am J Med Genet* 2000;93(2):85-88.

81 Dev VG, Butler MG, Phillips JA. 1q duplication due to inequal crossover in a patient with CHARGE association and Di George sequence. *Am J Hum Genet* 1985;37:A90.

82 Shroff M, Israel J, Rosenthal F. Congenital anomalies associated with partial deletion of the long arm of chromosome 4 46,XY,del(4)(q31). *Am J Hum Genet* 1981;33(6):122A.

83 Devriendt K, Swillen A, Fryns JP. Deletion in chromosome region 22q11 in a child with CHARGE association. *Clin Genet* 1998;53(5):408-410.

84 Emanuel BS, Budarf ML, Sellinger B, Goldmuntz E, Driscoll DA. Detection of microdeletions of 22q11.2 with fluorescence *in situ* hybrydization (FISH): diagnosis of DiGeorge (DGS), velo-cardial-facial syndrome, CHARGE association and conotruncal cardiac malformations. *Am J Hum Genet* 1992;51:AB0.

85 de Lonlay-Debeney P, Cormier-Daire V, Amiel J, Abadie V, Odent S, Paupe A, et al. Features of DiGeorge syndrome and CHARGE association in five patients. *J Med Genet* 1997;34(12):986-989.

86 Jyonouchi S, McDonald-Mcginn DM, Bale S, Zackai EH, Sullivan KE. CHARGE (coloboma, heart defect, atresia choanae, retarded growth and development, genital hypoplasia, ear anomalies/deafness) syndrome and chromosome 22q11.2 deletion syndrome: a comparison of immunologic and nonimmunologic phenotypic features. *Pediatrics* 2009;123(5):e871-e877.

87 Admiraal RJ, Joosten FB, Huygen PL. Temporal bone CT findings in the CHARGE association. *Int J Pediatr Otorhinolaryngol* 1998;45(2):151-162.

88 Amiel J, Attiee-Bitach T, Marianowski R, Cormier-Daire V, Abadie V, Bonnet D, et al. Temporal bone anomaly proposed as a major criteria for diagnosis of CHARGE syndrome. *Am J Med Genet* 2001;99(2):124-127.

89 Hopsu E, Markkola A, Pitkaranta A. Labyrinthine malformation in the 22q11.2 deletion syndrome. *Clin Dysmorphol* 2007;16(1):67-68.

90 Van den Akker PC, Jonkman MF, Rengaw T, Bruckner-Tuderman L, Has C, Bauer JW, et al. The international dystrophic epidermolysis bullosa patient registry: an online database of dystrophic epidermolysis bullosa patients and their *COL7A1* mutations. *Hum Mutat* 2011;Epub ahead of print, doi: 10.1002/humu.21551.

91 Swertz MA, De Brock EO, Van Hijum SA, De Jong A, Buist G, Baerends RJ, et al. Molecular genetics information system (MOLGENIS): alternatives in developing local experimental genomics databases. *Bioinformatics* 2004;20(13):2075-2083.

92 Swertz MA, Jansen RC. Beyond standardization: dynamic software infrastructures for systems biology. *Nat Rev Genet* 2007;8(3):235-243.

93 Swertz MA, Dijkstra M, Adamusiak T, van der Velde JK, Kanterakis A, Roos ET, et al. The MOLGENIS toolkit: rapid prototyping of biosoftware at the push of a button. *BMC Bioinformatics* 2010;11 Suppl 12:S12.

94 Bergman JE, Bosman EA, Ravenswaaij-Arts CM, Steel KP. Study of smell and reproductive organs in a mouse model for CHARGE syndrome. *Eur J Hum Genet* 2010;18(2):171-177.

95 Layman WS, McEwen DP, Beyer LA, Lalani SR, Fernbach SD, Oh E, et al. Defects in neural stem cell proliferation and olfaction in *Chd7* deficient mice indicate a mechanism for hyposmia in human CHARGE syndrome. *Hum Mol Genet* 2009;18(11):1909-1923.

96 Hurd EA, Poucher HK, Cheng K, Raphael Y, Martin DM. The ATP-dependent chromatin remodeling enzyme CHD7 regulates pro-neural gene expression and neurogenesis in the inner ear. *Development* 2010;137(18):3139-3150.

97 Kosaki K. Role of rare cases in deciphering the mechanisms of congenital anomalies: CHARGE syndrome research. *Congenit Anom (Kyoto)* 2011;51(1):12-15.

98 Adams ME, Hurd EA, Beyer LA, Swiderski DL, Raphael Y, Martin DM. Defects in vestibular sensory epithelia and innervation in mice with loss of Chd7 function: Implications for human CHARGE syndrome. *J Comp Neurol* 2007;504(5):519-532.

99 Cleary JO, Price AN, Thomas DL, Scambler PJ, Kyriakopoulou V, McCue K, et al. Cardiac phenotyping in ex vivo murine embryos using microMRI. *NMR Biomed* 2009;22(8):857-866.

100 Layman WS, Hurd EA, Martin DM. Reproductive dysfunction and decreased GnRH neurogenesis in a mouse model of CHARGE syndrome. *Hum Mol Genet* 2011;20(16):3138-3150.

101 Melicharek DJ, Ramirez LC, Singh S, Thompson R, Marenda DR. Kismet/CHD7 regulates axon morphology, memory, and locomotion in a Drosophila model of CHARGE Syndrome. *Hum Mol Genet* 2010;19(21):4253-4264.

102 Terriente-Felix A, Molnar C, Gomez-Skarmeta JL, de Celis JF. A conserved function of the chromatin ATPase Kismet in the regulation of hedgehog expression. *Dev Biol* 2011;350(2):382-392.

103 Siebert JR, Graham JM,Jr., MacDonald C. Pathologic features of the CHARGE association: support for involvement of the neural crest. *Teratology* 1985;31(3):331-336.

104 Van Meter TD, Weaver DD. Oculo-auriculo-vertebral spectrum and the CHARGE association: clinical evidence for a common pathogenetic mechanism. *Clin Dysmorphol* 1996;5(3):187-196.

105 Williams MS. Speculations on the pathogenesis of CHARGE syndrome. *Am J Med Genet A* 2005;133(3):318-325.

106 Schnetz MP, Handoko L, Akhtar-Zaidi B, Bartels CF, Pereira CF, Fisher AG, et al. CHD7 targets active gene enhancer elements to modulate ES cell-specific gene expression. *PLoS Genet* 2010;6(7):e1001023.

107 Srinivasan S, Dorighi KM, Tamkun JW. Drosophila Kismet regulates histone H3 lysine 27 methylation and early elongation by RNA polymerase II. *PLoS Genet* 2008;4(10):e1000217.

108 Zentner GE, Hurd EA, Schnetz MP, Handoko L, Wang C, Wang Z, et al. CHD7 functions in the nucleolus as a positive regulator of ribosomal RNA biogenesis. *Hum Mol Genet* 2010;19(18):3491-3501.

109 Takada I, Mihara M, Suzawa M, Ohtake F, Kobayashi S, Igarashi M, et al. A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR-gamma transactivation. *Nat Cell Biol* 2007;9(11):1273-1285.

110 Engelen E, Akinci U, Bryne JC, Hou J, Gontan C, Moen M, et al. Sox2 cooperates with Chd7 to regulate genes that are mutated in human syndromes. *Nat Genet* 2011;43(6):607-611.

111 Puc J, Rosenfeld MG. SOX2 and CHD7 cooperatively regulate human disease genes. *Nat Genet* 2011;43(6):505-506.

112 Layman WS, Hurd EA, Martin DM. Chromodomain proteins in development: lessons from CHARGE syndrome. *Clin Genet* 2010;78(1):11-20.

113 Martin DM. Chromatin remodeling in development and disease: Focus on CHD7. *PLoS Genet* 2010;6(7):e1001010.

114 Fernandez-Rebollo E, Perez O, Martinez-Bouzas C, Cotarelo-Perez MC, Garin I, Ruibal JL, et al. Two cases of

deletion 2q37 associated with segregation of an unbalanced translocation 2;21: choanal atresia leading to misdiagnosis of CHARGE syndrome. *Eur J Endocrinol* 2009;160(4):711-717.

115 Clementi M, Tenconi R, Turolla L, Silvan C, Bortotto L, Artifoni L. Apparent CHARGE association and chromosome anomaly: chance or contiguous gene syndrome. *Am J Med Genet* 1991;41(2):246-250.

116 Wieczorek D, Bolt J, Schwechheimer K, Gillessen-Kaesbach G. A patient with interstitial deletion of the short arm of chromosome 3 (pter-->p21.2::p12-->qter) and a CHARGE-like phenotype. *Am J Med Genet* 1997;69(4):413-417.

117 Khalifa OA, Walter CU, Rahbeeni ZA, Verloes A. Terminal 4q deletion and 8q duplication in a patient with CHARGE-like features. *Eur J Med Genet* 2011;54(2):173-176.

118 Sanlaville D, Romana SP, Lapierre JM, Amiel J, Genevieve D, Ozilou C, et al. A CGH study of 27 patients with CHARGE association. *Clin Genet* 2002;61(2):135-138.

119 North KN, Wu BL, Cao BN, Whiteman DA, Korf BR. CHARGE association in a child with de novo inverted duplication (14)(q22-->q24.3). *Am J Med Genet* 1995;57(4):610-614.

120 Lee WT, Hou JW, Yau KI, Wang TR. Trisomy 18 in a patient with CHARGE association. *J Formos Med Assoc* 1995;94(1-2):60-62.

121 De Krijger RR, Mooy CM, Van Hemel JO, Sulkers EJ, Kros JM, Bartelings MM, et al. CHARGE association-related ocular pathology in a newborn with partial trisomy 19q and partial monosomy 21q, from a maternal translocation (19;21)(q13.1;q22.3). *Pediatr Dev Pathol* 1999;2(6):577-581.

122 Lev D, Nakar O, Bar-Am I, Zudik A, Watemberg N, Finkelstien S, et al. CHARGE association in a child with *de novo* chromosomal aberration 46, X,der(X)t(X;2)(p22.1;q33) detected by spectral karyotyping. *J Med Genet* 2000;37(12):E47.

Supplementary Table S1. *CHD7* mutations and unclassified variants found in unpublished patients

A. All pathogenic *CHD7* mutations found by our laboratories in previously unpublished patients, including their phenotypic information

CHD7 c	CHD7 p	Mutation type	Segregation	Phenotypic information												
				C	H	A	RG	RD	G	E	SCC	C(L)P	HL	FD	VII	TE
del		del	U	no	yes	yes	U	U	U	U	yes	U	U	U	U	U
del		del	de novo	yes	yes	U	U	U	U	yes	U	U	U	U	U	U
transloc		transloc	de novo	no	yes	yes	yes	U	yes	yes	U	yes	yes	yes	yes	no
160del	Leu54fs	fs	U	yes	yes	U	yes	U	yes	yes	yes	U	yes	U	no	U
191_194del	Thr64fs	fs	U	yes	yes	U	U	U	U	U	U	U	yes	U	U	U
232C>T	Gln78X	non	U	U	no	U	U	poss	yes	yes	U	yes	yes	poss	yes	U
257del	Pro86fs	fs	de novo	no	no	no	U	yes	No	yes	U	yes	yes	yes	yes	no
282del	Asn96fs	fs	de novo	U	no	yes	U	poss	yes	U	U	U	yes	U	U	U
317delA	His106fs	fs	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
334C>T	Gln112X	non	U	yes	yes	yes	yes	U	yes	U	no	U	yes	U	yes	U
388C>T	Gln130X	non	not maternal	yes	U	no	yes	U	yes	U	yes	U	yes	yes	U	U
406C>T	Gln136X	non	U	U	U	U	U	yes	U	U	U	U	U	U	U	U
469C>T	Arg157X	non	U	yes	yes	yes	U	U	yes	yes	yes	U	U	U	U	yes
469C>T	Arg157X	non	U	yes	U	U	U	U	U	yes	U	yes	U	U	U	U
550C>T	Gln184X	non	U	no	yes	yes	U	U	U	U	U	U	U	U	U	yes
601C>T	Gln201X	non	U	yes	yes	no	yes	yes	U	yes	U	no	yes	U	U	U
604C>T	Gln202X	non	de novo	yes	yes	yes	U	U	U	yes	U	U	U	U	U	no
608dup	His203fs	fs	U	yes	yes	yes	U	U	U	yes	yes	U	U	U	U	yes
619C>T	Gln207X	non	de novo	yes	yes	U	U	yes	U	yes	U	U	yes	yes	yes	U
619C>T	Gln207X	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
627del	Met210X	fs	U	yes	yes	yes	U	yes	yes	yes	U	U	yes	U	yes	U
635delA	Gln212fs	fs	U	yes	yes	U	no	yes	yes	yes	U	U	yes	poss	yes	no
674del	Pro225fs	fs	U	yes	yes	yes	U	U	U	yes	U	U	U	U	U	U
689C>G	Ser230X	non	de novo	yes	no	U	U	U	yes	yes	U	yes	U	U	U	U
718del	Gln240fs	fs	U	no	yes	yes	no	U	yes	yes	yes	no	yes	U	U	yes
781del	Ser261fs	fs	de novo	U	yes	U	U	U	yes	yes	U	yes	U	U	U	U

791_792del	Leu264fs	fs	U	U	yes	yes	U	U	U	yes	U	U	U	U	U	U
844C>T	Gln282X	non	U	U	yes	U	U	U	U	yes	U	yes	yes	U	U	yes
921_922del	Gly308fs	fs	U	U	yes	yes	U	U	U	yes	U	U	yes	U	yes	U
934C>T	Arg312X	non	U	yes	yes	yes	yes	U	U	yes	U	U	U	U	U	U
934C>T	Arg312X	non	U	U	U	U	U	U	U	yes	U	U	yes	yes	U	U
995T>G	Leu332X	non	de novo	yes	yes	U	U	poss	yes	yes	U	U	yes	poss	yes	U
1066_1069 dup	Ser357fs	fs	U	yes	no	no	yes	yes	yes	yes	yes	U	yes	U	yes	no
1093C>T	Gln365X	non	U	yes	no	U	U	poss	yes	yes	U	yes	yes	poss	U	yes
1123C>T	Gln375X	non	de novo	yes	no	U	yes	yes	yes	yes	yes	yes	yes	yes	U	U
1135C>T	Gln379X	non	U	yes	yes	U	U	U	yes	U	yes	U	yes	U	yes	U
1159C>T	Gln387X	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
1170T>G	Tyr390X	non	U	U	yes	yes	U	U	U	yes	U	U	U	U	U	U
1247insG	Ser417fs	fs	U	yes	yes	yes	U	U	U	yes	U	U	U	U	U	no
1312C>T	Gln438X	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
1480C>T	Arg494X	non	U	yes	yes	no	no	U	yes	yes	yes	no	yes	yes	U	no
1480C>T	Arg494X	non	U	yes	yes	yes	no	yes	yes	yes	yes	U	yes	U	U	yes
1480C>T	Arg494X	non	U	U	yes	yes	U	U	U	yes	U	U	U	yes	U	U
1480C>T	Arg494X	non	de novo	yes	yes	yes	yes	U	U	yes	yes	U	yes	U	U	no
1480C>T	Arg494X	non	not maternal	U	yes	no	U	U	No	yes	U	no	U	U	U	U
1528del	Gln510fs	fs	U	yes	yes	U	U	U	yes	U	U	yes	yes	U	U	U
1645C>T	Gln549X	non	U	yes	yes	U	yes	yes	U	U	U	U	U	U	U	U
1678delG	Glu560fs	fs	U	yes	yes	U	U	yes	yes	U	U	yes	yes	U	U	U
1683_1684 delCT	Phe562fs	fs	U	yes	U	yes	yes	yes	yes	U	yes	U	yes	U	U	U
1735C>T	Gln579X	non	U	yes	yes	yes	U	U	U	yes	U	U	U	U	yes	U
1735C>T	Gln579X	non	U	yes	U	no	U	U	yes	yes	U	U	U	poss	U	yes
1740del	Val581fs	fs	U	yes	U	U	U	yes	U	yes	U	yes	yes	poss	yes	U
1926_1929 del	Lys643fs	fs	U	poss	U	U	no	yes	yes	yes	U	no	yes	yes	yes	U
1932_1935 delGAAA	Lys645fs	fs	not maternal	yes	no	yes	U	yes	yes	yes	U	yes	no	yes	U	yes
1953dup	Asp652fs	fs	de novo	yes	yes	no	yes	U	U	yes	U	no	yes	yes	no	yes
1953dup	Asp652fs	fs	U	U	yes	U	no	yes	yes	yes	U	U	yes	yes	U	U
1953dup	Asp652fs	fs	U	yes	no	U	yes	yes	yes	yes	U	U	yes	poss	U	U
1953dup	Asp652fs	fs	de novo	yes	yes	no	no	U	no	yes	yes	yes	yes	no	yes	no

1972G>T	Glu658X	non	U	yes	yes	U	yes	yes	yes	yes	yes	U	yes	yes	yes	U
1983dup	Lys662fs	fs	U	yes	yes	U	U	yes	U	yes	U	U	yes	U	U	U
1988-1989dup	Glu664fs	fs	de novo	U	yes	U	U	U	U	U	U	U	U	U	U	U
1989dup	Glu664fs	fs	U	yes	no	U	U	yes	yes	yes	yes	U	yes	U	U	U
1990G>T	Glu664X	non	U	yes	no	U	U	yes	U	U	yes	yes	yes	yes	U	U
2019_2023 delinsGGG	Pro674fs	fs	U	yes	yes	no	U	yes	U	yes	U	U	yes	yes	U	yes
2096+2T>A	?	splice	de novo	yes	yes	no	yes	U	yes	yes	yes	U	U	U	yes	no
2097-1G>A	?	splice	U	yes	yes	U	U	yes	U	yes	yes	U	U	yes	yes	U
2118del	Ala707fs	fs	de novo	no	yes	yes	U	poss	U	yes	U	U	U	U	U	U
2157del	Glu1720fs	fs	de novo	yes	yes	yes	yes	yes	U	U	U	U	yes	yes	U	U
2181dup	Asp728fs	fs	de novo	U	yes	U	U	poss	U	U	yes	U	U	U	yes	yes
2196dup	Pro733fs	fs	de novo	U	yes	U	U	U	U	U	U	yes	U	U	U	yes
2236delC	Gln746fs	fs	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
2238+1G>T	?	splice	U	yes	no	no	no	yes	no	yes	yes	no	yes	no	yes	no
2238+2T>G	?	splice	U	U	U	U	U	U	U	U	U	U	U	U	U	U
2238+1G>A	?	splice	U	U	yes	yes	U	U	U	yes	U	U	U	U	U	U
2247_2260 del	Ser750fs	fs	U	yes	yes	yes	U	U	U	U	yes	U	U	U	U	U
2374C>T	Gln792X	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
2443-2A>T	?	splice	de novo	no	yes	no	yes	yes	yes	yes	U	no	yes	U	yes	no
2443-1G>A	?	splice	U	U	U	U	U	U	U	U	U	U	U	U	U	U
2498+2T>C	?	splice	U	yes	yes	U	yes	poss	U	yes	U	yes	yes	U	U	U
2498+1G>T	?	splice	de novo	U	yes	yes	U	U	U	U	U	U	yes	U	U	U
2498+2dup	?	splice	U	U	yes	U	U	U	U	yes	U	yes	U	U	U	poss
2504_2508 del	Tyr835fs	fs	de novo	U	yes	yes	U	U	no	yes	yes	U	yes	poss	U	U
2504_2508 del	Leu836fs	fs	U	yes	yes	U	U	U	U	yes	U	U	U	U	U	U
2504_2508 del	Tyr835fs	fs	U	U	yes	U	U	U	U	U	U	yes	U	U	U	no
2520G>C	Trp840Cys	mis	de novo	yes	no	no	no	yes	yes	yes	U	no	no	U	no	U
2571insA	Arg858fs	fs	de novo	yes	yes	no	yes	U	yes	U	yes	U	yes	U	U	yes
2572C>T	Arg858X	non	de novo	U	yes	yes	U	U	yes	yes	U	U	U	U	U	yes
2577dup	Lys860X	fs	U	yes	yes	no	U	yes	U	yes	U	U	yes	U	yes	U
2601-2605del	Lys867fs	fs	U	U	yes	U	U	U	U	yes	U	U	U	U	U	U
2620delG	Asp874fs	fs	U	yes	yes	U	yes	yes	yes	yes	U	no	yes	yes	yes	no

2707_2710 delCACT	His903fs	fs	de novo	yes	yes	U	U	U	U	U	U	U	U	U	U	U
2815G>T	Glu939X	non	U	no	yes	no	U	yes	yes	yes	yes	yes	yes	yes	yes	no
2829del	glu943fs	fs	U	yes	yes	yes	U	U	U	yes	U	U	U	U	U	U
2839C>T	Arg947X	non	U	U	no	yes	U	U	U	U	yes	U	U	U	U	U
2859del	Trp953X	fs	U	yes	yes	no	yes	yes	U	yes	yes	U	yes	yes	U	no
2887A>T	Lys963X	non	not maternal	U	U	U	U	U	U	U	U	U	U	U	U	U
2905_2906 del	Arg969fs	fs	U	U	U	U	U	U	U	U	U	U	U	U	U	U
2957+2T>C	?	splice	U	yes	yes	no	U	yes	U	yes	U	U	yes	poss	U	U
2957+5G>A	?	splice	de novo	yes	yes	no	yes	no	yes	yes	U	yes	yes	U	yes	no
2959C>T	Arg987X	non	not maternal	yes	yes	U	U	U	yes	yes	U	U	U	poss	U	U
3023_3024 del	Tyr1008X	fs	de novo	yes	yes	no	yes	U	yes	yes	U	U	yes	poss	U	no
3024T>A	Tyr1008X	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
3059T>C	Leu1020Ser	mis	U	U	yes	yes	U	U	U	U	U	U	U	U	U	yes
3082A>G	Ile1028Val	mis	de novo	yes	no	U	U	yes	yes	yes	U	yes	yes	U	U	U
3082A>G	Ile1028Val	mis	de novo	yes	yes	no	U	yes	U	U	U	U	U	yes	U	U
3082A>G	Ile1028Val	mis	U	no	yes	U	U	no	yes	U	yes	yes	yes	yes	U	no
3091T>C	Trp1031Arg	mis	de novo	yes	yes	U	U	U	yes	yes	U	U	U	U	yes	U
3106C>T	Arg1036X	non	de novo	yes	yes	yes	U	yes	U	yes	yes	yes	U	U	U	yes
3106C>T	Arg1036X	non	de novo	yes	yes	yes	U	U	U	U	U	U	U	U	U	U
3117dup	Glu1040fs	fs	U	yes	U	U	U	U	U	U	U	U	U	U	poss	yes
3165del	Ile1056fs	fs	U	U	U	U	U	U	U	U	U	U	U	U	U	U
3173T>A	Leu1058X	non	de novo	yes	yes	no	no	U	yes	yes	yes	no	yes	yes	yes	no
3205C>T	Arg1069X	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
3205C>T	Arg1069X	non	U	no	yes	U	U	poss	yes	yes	U	yes	yes	U	U	U
3222_3223 ins5	Tyr1075fs	fs	de novo	no	yes	yes	no	U	U	yes	U	U	U	U	U	U
3245C>A	Thr1082Asn	mis	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
3301T>C	Cys1101Arg	mis	de novo	U	U	U	U	U	U	yes	U	U	yes	U	U	U
3318del	Ala1107fs	fs	de novo	U	yes	U	U	U	U	U	U	yes	U	U	U	U
3322delC	His1108fs	fs	de novo	U	U	yes	U	U	yes	U	U	U	yes	U	U	U
3336del	Asn1112fs	fs	U	yes	yes	yes	yes	U	U	U	U	U	yes	U	U	U
3378+5G>C	?	splice	de novo	yes	yes	yes	yes	yes	U	yes	U	no	yes	U	U	U
3379-1G>A	?	splice	U*	yes	yes	yes	U	poss	U	U	U	U	U	U	U	yes

3379-3C>G	?	splice	U	yes	U	yes	U	yes	yes	U	U	yes	yes	poss	U	U
3514_3515 del	Glu1172fs	fs	de novo	yes	no	U	yes	poss	yes	yes	U	yes	yes	yes	U	U
3522+2T>G	?	splice	U	yes	U	U	U	U	U	yes	yes	U	U	U	yes	U
3522+2T>C	?	splice	de novo	U	no	U	U	U	U	U	U	U	yes	U	U	U
3523-1G>C	?	splice	U	yes	yes	no	yes	yes	yes	yes	U	no	yes	U	yes	no
3573dup	Glu1192fs	fs	U	yes	yes	yes	U	yes	U	U	U	U	yes	U	U	U
3616dup	Ile1206fs	fs	de novo	yes	yes	U	U	U	yes	yes	yes	yes	yes	U	U	U
3641A>G	Gln1214Arg	mis	de novo	yes	no	no	yes	yes	yes	yes	U	U	yes	U	U	no
3641A>G	Gln1214Arg	mis	de novo	U	U	U	U	yes	U	yes	U	U	yes	U	U	U
3655C>T	Arg1219X	non	de novo	yes	yes	U	U	U	U	U	U	U	U	U	U	U
3655C>T	Arg1219X	non	U	yes	yes	U	yes	U	U	U	U	U	yes	U	U	U
3734del	Met1245fs	fs	de novo	yes	yes	yes	no	yes	yes	yes	yes	yes	yes	yes	U	no
3807del	Phe1269fs	fs	U	yes	no	no	yes	yes	yes	yes	U	no	yes	U	poss	no
3847C>T	Gln1283X	non	U	U	U	yes	U	U	yes	yes	U	U	yes	U	U	U
3875T>C	Leu1292Pro	mis	de novo	U	yes	U	U	U	U	U	U	U	U	U	U	U
3937del	Ser1313fs	fs	de novo	yes	no	no	no	yes	U	yes	U	yes	yes	poss	U	no
3952T>C	Cys1318Arg	mis	de novo	yes	yes	no	U	poss	yes	yes	U	no	U	yes	yes	no
3990-2G>A	?	splice	de novo	yes	yes	no	no	U	yes	yes	yes	U	yes	yes	yes	no
3993C>G	Tyr1331X	non	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
4015C>T	Arg1339X	non	de novo	U	yes	U	U	U	U	U	U	U	U	U	U	U
4034G>A	Arg1345His	mis	de novo	no	no	U	U	yes	no	yes	U	yes	yes	poss	U	yes
4113-4114 insCA	Gly1372fs	fs	de novo	yes	yes	no	yes	yes	U	yes	U	no	yes	U	yes	U
4213C>T	Gln1405X	non	U	yes	no	yes	U	U	U	yes	no	U	yes	poss	U	U
4213C>T	Gln1405X	non	de novo	yes	no	U	yes	yes	U	yes	U	U	yes	poss	U	U
4253del	Asn1418fs	fs	not maternal	yes	no	U	U	yes	U	yes	U	yes	yes	yes	U	U
4257del	Tyr1420fs	fs	U	yes	yes	U	U	U	U	yes	U	yes	U	poss	U	U
4270del	Met1424fs	fs	U	yes	yes	yes	U	U	yes	yes	yes	U	U	U	U	yes
4295_4296 del	Leu1432fs	fs	U	U	U	U	U	U	U	U	U	U	U	U	U	U
4353+2T>C	?	splice	U	U	yes	yes	U	U	U	U	U	U	yes	U	U	U
4357_4358 dupCA	Gln1453fs	fs	not maternal	U	no	U	U	yes	yes	yes	U	U	yes	yes	yes	U
4375G>T	Glu1459X	non	de novo	yes	yes	yes	U	U	yes	U	U	no	U	U	no	no
4393del	Arg1465fs	fs	not maternal	yes	yes	yes	yes	yes	U	yes	U	U	U	U	U	no
4393C>T	Arg1465X	non	U	yes	yes	no	no	yes	no	yes	yes	yes	yes	yes	U	no

4393C>T	Arg1465X	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
4393C>T	Arg1465X	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
4424del	Glu1475fs	fs	U	U	U	U	U	U	U	U	U	U	U	U	U	U
4480C>T	Arg1494X	non	de novo	yes	yes	U	yes	yes	U	yes	U	U	yes	yes	yes	U
4480C>T	Arg1494X	non	U	U	U	U	U	U	U	U	yes	U	U	U	U	U
4644+5G>A	?	splice	de novo	no	no	yes	no	yes	U	no	yes	U	yes	no	no	no
4665del	Pro1556fs	fs	U	U	yes	U	U	U	yes	yes	U	yes	U	U	yes	U
4731del	Asp1578fs	fs	de novo	no	no	yes	U	U	yes	yes	yes	no	yes	yes	U	U
4783C>T	Gln1595X	non	U	no	no	yes	U	U	yes	yes	yes	no	yes	yes	yes	no
4850+1dup	Trp1618fs	fs	U	no	yes	yes	U	U	U	yes	no	no	U	U	yes	no
4850+2T>A	?	splice	de novo	U	U	U	U	U	U	U	U	U	U	U	yes	U
4850+1G>A	?	splice	not maternal	U	U	U	U	U	U	U	U	U	U	U	U	U
4851-2A>G	?	splice	U	yes	no	no	yes	yes	yes	yes	yes	U	yes	yes	yes	poss
4851-2A>T	?	splice	U	yes	yes	no	yes	U	yes	yes	yes	U	yes	yes	yes	U
4854G>A	Trp1618X	non	de novo	yes	yes	U	U	no	U	yes	U	yes	yes	U	yes	U
5012_5018 del	Thr1671fs	fs	U	U	yes	U	U	U	U	U	U	U	U	U	U	U
5050G>A	Gly1684Ser	mis	de novo	no	yes	no	yes	yes	U	U	yes	yes	yes	U	U	no
5069dup	Arg1691fs	fs	de novo	yes	yes	yes	U	U	U	yes	yes	no	yes	no	yes	U
5136G>A	Trp1712X	non	not maternal	U	yes	U	U	U	U	U	U	U	U	U	U	U
5205_5206 insT	Asn1736X	non	U	yes	poss	yes	yes	yes	U	U	U	U	yes	yes	U	U
5211-1G>C	?	splice	U	yes	yes	yes	U	U	yes	yes	U	U	yes	yes	U	no
5241-5244del	Tyr1747X	fs	de novo	no	yes	U	no	yes	yes	yes	yes	no	yes	yes	yes	no
5297C>G	Ser1766X	non	de novo	yes	yes	U	yes	yes	U	yes	yes	U	yes	yes	yes	no
5316G>A	Trp1772X	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
5390G>T	Gly1797Val	mis	de novo	yes	no	U	no	yes	yes	yes	yes	U	yes	yes	yes	U
5405-17G>A	?	splice	de novo	no	no	no	yes	yes	yes	yes	yes	yes	yes	yes	U	U
5405-7G>A	?	splice	U	yes	no	prob	yes	yes	No	yes	yes	U	yes	U	yes	U
5405-17G>A	?	splice	U	no	no	no	no	yes	U	yes	yes	no	yes	yes	U	U
5405-7G>A	?	splice	de novo	no	no	no	U	U	yes	no	yes	yes	yes	U	U	no
5405-17G>A	?	splice	U	yes	no	no	U	U	U	yes	U	yes	yes	U	U	no
5405-7G>A	?	splice	U	U	yes	U	U	yes	yes	U	yes	yes	yes	U	U	U
5418C>G	Tyr1806X	non	U	yes	yes	yes	U	U	yes	yes	U	no	U	U	yes	U

5428C>T	Arg1810X	non	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
5434G>C	Asp1812His	mis	de novo	no	no	yes	no	poss	U	yes	yes	no	yes	yes	U	no
5435A>G	Asp1812Gly	mis	de novo	yes	yes	U	no	no	yes	U	U	U	yes	poss	U	yes
5444T>C	Leu1815Pro	mis	U	yes	yes	U	U	U	U	yes	U	yes	U	U	yes	U
5453dup	Glu1819fs	fs	de novo	U	U	yes	U	U	U	U	U	U	U	U	U	U
5458C>T	Arg1820X	non	de novo	yes	yes	yes	U	yes	U	yes	U	U	yes	U	U	yes
5458C>T	Arg1820X	non	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
5534+1G>T	?	splice	de novo	yes	yes	no	U	U	U	yes	U	yes	U	U	U	U
5534+1G>A/T	?	splice	de novo	U	yes	prob	U	U	U	yes	U	U	U	U	U	U
5539G>T	Glu1847X	non	de novo	U	yes	U	U	yes	yes	U	U	U	yes	U	U	U
5592del	Phe1864fs	fs	U	yes	yes	U	yes	yes	yes	yes	U	yes	yes	U	U	U
5597A>G	Asp1866Gly	mis	de novo	yes	yes	U	U	U	U	U	U	U	U	U	U	U
5607+1G>A	?	splice	de novo	yes	yes	no	U	U	U	yes	U	no	U	yes	yes	U
5706C>A	Tyr1902X	non	U	yes	yes	U	U	yes	U	U	U	U	yes	U	U	U
5709G>A	Trp1903X	non	U	U	yes	yes	U	yes	U	U	U	U	yes	U	yes	yes
5866dup	Glu1956fs	fs	U	U	U	U	U	U	U	U	U	U	U	U	U	U
5898G>A	Trp1966X	non	U	yes	yes	yes	yes	yes	yes	yes	yes	U	yes	U	yes	no
5908G>T	Glu1970X	non	de novo	yes	yes	yes	U	U	U	U	U	U	U	U	U	U
5910dup	Glu1971fs	fs	not maternal	yes	yes	no	yes	yes	U	yes	yes	U	yes	U	U	yes
5944_5989 dup	Phe1997fs	fs	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
5968C>T	Gln1990X	non	de novo	yes	yes	no	U	yes	yes	yes	U	yes	yes	yes	U	U
6018-6019insG	Ser2007fs	fs	U	no	yes	yes	yes	yes	U	yes	U	no	yes	poss	U	no
6018dup	Ser2007fs	fs	U	yes	no	no	yes	yes	yes	yes	U	no	U	U	yes	no
6070del	Arg2024fs	fs	U	yes	yes	U	U	yes	U	U	yes	yes	yes	poss	U	U
6070C>T	Arg2024X	non	U	yes	no	yes	yes	yes	U	U	U	no	U	poss	yes	no
6070C>T	Arg2024X	non	U	yes	yes	yes	U	yes	U	yes	U	U	U	U	U	yes
6070C>T	Arg2024X	non	U	yes	yes	U	U	yes	U	yes	U	U	yes	U	poss	U
6070C>T	Arg2024X	non	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
6079C>T	Arg2027X	non	de novo	yes	no	no	U	yes	U	yes	yes	no	yes	yes	yes	U
6079C>T	Arg2027X	non	U	yes	yes	yes	no	yes	U	yes	U	U	yes	poss	yes	no
6079C>T	Arg2027X	non	U	yes	yes	U	U	U	U	yes	U	yes	U	U	yes	U
6079C>T	Arg2027X	non	U	yes	yes	yes	U	yes	yes	yes	no	no	yes	U	yes	no

6079C>T	Arg2027X	non	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
6103+1G>A	?	splice	U	yes	U	yes	U	U	U	yes	yes	U	U	U	U	U
6104-2A>T	?	splice	U	yes	yes	U	U	U	yes	U	U	yes	U	poss	yes	U
6148C>T	Arg2050X	non	U	yes	yes	U	U	yes	yes	yes	U	no	yes	yes	U	poss
6148C>T	Arg2050X	non	de novo	yes	yes	yes	U	U	U	yes	U	yes	yes	U	yes	U
6148C>T	Arg2050X	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
6157C>T	Arg2053X	non	U	no	yes	no	yes	yes	U	yes	U	no	yes	yes	U	no
6157C>T	Arg2053X	non	U	yes	U	U	U	U	U	yes	U	U	U	yes	yes	yes
6157C>T	Arg2053X	non	U	yes	yes	yes	yes	yes	U	yes	yes	U	yes	U	yes	no
6165_6166 delGT	Tyr2056fs	fs	de novo	yes	yes	U	U	U	U	yes	U	U	yes	U	yes	U
6165_6166 delGT	Tyr2056fs	fs	de novo	U	yes	U	U	U	U	U	U	U	yes	yes	U	U
6179del	Leu2060fs	fs	U	yes	yes	U	U	U	U	yes	U	U	U	U	yes	U
6196G>T	Glu2066X	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
6221T>C[a]	Leu2074Pro	mis	not maternal[a]	no	U	U	U	U	yes	U	U	U	yes	U	U	U
6221T>C[a]	Leu2074Pro	mis	not maternal[a]	yes	U	U	U	U	yes	U	U	U	yes	U	U	U
6224del	Gly2075fs	fs	U	yes	yes	yes	U	U	U	yes	yes	U	yes	U	U	U
6271T>C	Trp2091Arg	mis	de novo	yes	no	no	yes	poss	U	yes	U	no	yes	yes	U	no
6292C>T	Arg2098X	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
6316A>T	Lys2106X	non	U	yes	yes	no	U	yes	U	yes	yes	U	yes	U	U	no
6322G>A	Gly2108Arg	mis	de novo	yes	no	U	U	no	yes	yes	U	yes	yes	poss	U	U
6473C>G	Ser2158X	non	U	yes	yes	U	U	yes	U	U	U	U	yes	U	U	U
6520A>T	Lys2174X	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
6526G>T	Glu2176X	non	U	yes	yes	U	U	yes	U	yes	yes	yes	yes	U	yes	no
6620dup	Cys2207fs	fs	U	yes	yes	U	U	U	U	yes	U	no	yes	poss	U	no
6651del	Glu2217fs	fs	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
6652_6656 del	Leu2218fs	fs	U	U	U	U	U	U	yes	yes	U	U	U	U	U	U
6667del	Val2223fs	fs	U	U	yes	U	U	U	U	yes	yes	yes	U	U	U	U
6681_6685 dup	Ser2229fs	fs	de novo	yes	yes	U	U	U	yes	yes	yes	yes	U	U	U	U
6688delA	Lys2230fs	fs	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
6705del	Gly2236fs	fs	U	yes	yes	yes	U	U	U	U	U	U	yes	U	U	U
6857dup	Phe2287fs	fs	de novo	no	no	yes	U	yes	yes	yes	yes	yes	yes	yes	yes	no
6857G>C	Gly2286Ala	mis	de novo	yes	yes	no	no	yes	U	yes	no	U	yes	U	yes	no

6888_6889 insT	Ala2297fs	fs	U	U	yes	yes	U	yes	U	U	U	U	yes	U	U	U
6935A>C	Lys2312Thr	mis	Not maternal	no	U	yes	U	yes	U	yes	U	U	yes	U	yes	U
6937-1G>C	?	splice	de novo	yes	yes	no	yes	yes	U	yes	U	no	U	poss	yes	no
6937-2A>G	?	splice	U	yes	yes	no	yes	U	yes	yes	U	U	no	U	yes	U
6937-2A>G	?	splice	U	yes	no	no	yes	yes	yes	U	yes	yes	U	U	U	no
7075C>T	Gln2359X	non	U	no	yes	yes	no	U	yes	yes	yes	no	yes	yes	U	no
7106del	Val2369fs	fs	U	yes	yes	no	yes	U	No	yes	yes	no	yes	yes	yes	no
7160C>A	Ser2387X	non	de novo	yes	yes	yes	no	U	No	yes	yes	no	yes	yes	yes	yes
7164+1G>A	?	splice	de novo	U	yes	yes	no	no	No	yes	yes	U	yes	U	U	no
7252C>T	Arg2418X	non	de novo	yes	yes	no	U	yes	U	yes	U	yes	yes	U	yes	U
7252C>T	Arg2418X	non	not maternal*	yes	U	U	U	U	U	U	yes	U	U	U	U	U
7252C>T	Arg2418X	non	de novo	U	yes	U	U	U	yes	yes	U	U	yes	U	U	U
7252C>T	Arg2418X	non	de novo	yes	yes	no	yes	yes	yes	yes	U	yes	yes	U	U	no
7276C>T	Gln2426X	non	de novo	yes	U	U	U	U	U	U	U	U	yes	U	U	U
7282C>T	Arg2428X	non	U	yes	yes	U	U	poss	U	yes	U	yes	yes	poss	U	U
7282C>T	Arg2428X	non	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
7282C>T	Arg2428X	non	U	yes	U	U	U	U	U	yes	yes	yes	U	U	U	U
7320del	Val2441fs	fs	U	yes	no	no	U	U	U	no	yes	U	yes	U	U	U
7344_7345 del	Glu2450fs	fs	U	yes	yes	yes	U	U	U	yes	yes	U	U	U	yes	U
7400del	Leu2467fs	fs	U	U	U	U	U	U	U	U	U	U	U	U	U	U
7441C>T	Gln2481X	non	de novo	yes	yes	yes	U	yes	yes	yes	U	U	yes	U	U	U
7451dupT	Leu2485fs	fs	U	no	yes	no	yes	yes	yes	yes	yes	no	yes	yes	U	no
7454dup	Asn2486fs	fs	U	U	U	U	U	U	U	U	U	U	U	U	U	U
7577del	Phe2526fs	fs	U	yes	yes	U	U	U	U	U	U	U	U	U	yes	U
7593dup	Thr2532fs	fs	U	no	yes	no	yes	yes	yes	yes	yes	yes	yes	yes	U	U
7717-7720 del	Asn2573fs	fs	U	yes	no	U	yes	yes	yes	U	U	U	U	yes	U	U
7879C>T	Arg2627X	non	U	U	no	U	U	yes	yes	yes	U	U	U	yes	yes	U
7879C>T	Arg2627X	non	de novo	yes	yes	U	yes	yes	U	yes	U	no	U	U	U	yes
7879C>T	Arg2627X	non	U	yes	yes	yes	U	U	yes	U	U	yes	U	U	U	yes
7879C>T	Arg2627X	non	de novo	U	yes	U	U	U	U	yes	yes	U	yes	U	U	U
7879C>T	Arg2627X	non	de novo	yes	no	yes	U	U	yes	yes	yes	no	yes	no	U	no
7879C>T	Arg2627X	non	U	yes	U	yes	no	no	U	yes	U	U	no	U	yes	U

7879C>T	Arg2627X	non	U	U	yes	U	U	poss	U	yes	yes	yes	yes	poss	yes	U
7879C>T	Arg2627X	non	U	yes	yes	yes	U	U	U	yes	U	U	U	U	U	U
7891C>T	Arg2631X	non	U	yes	yes	U	U	yes	No	U	no	yes	yes	U	yes	no
7895del	Asn2632fs	fs	U	U	U	U	U	U	U	U	U	U	U	U	U	U
7933G>T	Glu2645X	non	U	yes	yes	U	yes	U	U	yes	U	yes	yes	U	U	U
7957C>T	Arg2653X	non	U	yes	yes	yes	yes	yes	No	yes	yes	no	yes	yes	U	no
7957C>T	Arg2653X	non	U	U	yes	U	U	U	yes	yes	U	yes	U	U	U	U
7957C>T	Arg2653X	non	U	yes	yes	U	U	yes	U	U	U	U	yes	U	U	U
8077-1G>A	?	splice	de novo	no	yes	no	no	yes	U	yes	U	U	yes	yes	yes	U
8356G>T	Gly2786X	non	U	yes	yes	no	no	yes	yes	no	U	no	yes	U	yes	yes
8613_8622 delins14	Asn2871fs	fs	de novo	yes	yes	no	U	U	U	yes	yes	yes	yes	U	U	U
8630_8634 del	Ala2877fs	fs	U	yes	yes	yes	U	U	yes	yes	U	no	U	yes	U	yes
8737dup	Leu2913fs	fs	U	yes	U	yes	U	poss	yes	yes	no	U	yes	poss	U	U
8962dup	Asp2988fs	fs	de novo	yes	yes	yes	U	U	yes	U	U	yes	yes	yes	U	U

B. All unclassified variants (UVs) found by our laboratories in previously unpublished patients, including their phenotypic information

				Phenotypic information											
CHD7 c	**CHD7 p**	**Domain**	**Segregation**	**C**	**H**	**A**	**RD**	**RG**	**G**	**E**	**SCC**	**C(L)P**	**HL**	**FD**	**VII**	**TE**
-380C>T[*]	?		U	U	yes	yes	U	U	U	yes	U	U	U	U	U	U
-360C>T	?		U	U	yes	U	U	U	U	U	U	U	yes	U	U	U
215A>G	Tyr72Cys		U	yes	yes	no	yes	yes	U	yes	U	no	no	U	U	U
295G>C	Ala99Pro		paternal	U	yes	no	yes	U	yes	yes	U	no	yes	U	poss	U
760C>G	Gln254Glu		U	U	yes	yes	yes	U	U	yes	yes	U	yes	U	U	yes
1315C>T	Pro439Ser		U	U	yes	U	U	U	U	U	U	U	U	U	U	U
1672C>G	Pro558Ala		U	U	U	U	U	U	U	U	U	U	U	U	U	U
2095A>G	Ser699Gly		U	yes	yes	yes	yes	U	U	U	U	U	U	U	U	U
2436A>T	Lys812Asn		U	U	yes	yes	yes	U	U	yes	yes	U	yes	U	U	yes
2436A>T	Lys812Asn	CD1	maternal	U	yes	U	yes	U	U	U	U	U	U	U	U	U
2824A>G	Thr942Ala	CD2	paternal	yes	no	U	U	no	yes	yes	U	yes	yes	U	U	U
2923G>A	Gly975Arg	HD	U	U	U	yes	yes	U	U	U	yes	U	yes	U	U	U
3242T>G	Ile1081Ser		U	U	U	U	U	U	U	U	U	U	U	U	U	U
3751T>C	Cys1251Arg	HD	U	yes	yes	U	U	U	U	yes	yes	U	yes	U	yes	U
3949C>T	Arg1317Cys			yes	U	U	U	U	U	U	U	U	U	U	U	U
4850G>A	Gly1617Asp		U	U	yes	yes	U	U	U	U	U	U	U	U	yes	U
4850G>A	Gly1617Asp		not maternal	yes	yes	U	yes	yes	yes	yes	U	U	yes	poss	yes	U
4856G>T	Gly1619Val		U	no	yes	yes	yes	yes	U	yes	yes	U	U	U	U	U
5894+5G>A	?		U	U	U	U	U	U	U	U	U	U	U	U	U	U
5915C>G	Ala1972Gly	SANT	paternal	no	yes	no	U	yes	U	U	no	no	U	yes	U	U
6290A>G	Asp2097Gly		U	U	U	U	U	U	U	U	U	U	U	U	U	U
6775G>A	Ala2259Thr		U	U	U	U	U	U	U	U	U	U	U	U	U	U
6777A>G	Ala2259Ala		not maternal	yes	U	U	no	U	yes	yes	U	yes	yes	U	U	U
7097T>G	Leu5066Arg		U	U	U	no	yes	U	U	U	yes	U	yes	U	U	U
7390A>G	Lys2464Glu		maternal	U	U	U	no	U	yes	yes	U	yes	yes	no	U	no
7463G>A	Gly2488Asp		paternal	U	U	U	U	U	U	U	U	U	U	U	U	U
8416C>G	Leu2806Val		maternal	no	no	yes	U	U	U	yes	U	U	U	poss	U	U
8416C>G	Leu2806Val		U	U	yes	U	U	U	U	U	U	U	U	U	U	U
6777A>G	Ala2259Ala		not maternal	yes	yes	yes	U	U	yes	no	U	U	yes	yes	U	U

VII, facial palsy; A, atresia of choanae; C, coloboma; CD, chromodomain; C(L)P, cleft lip and/or palate; del, deletion; E, ear anomaly; FD, feeding difficulties necessitating tube feeding; fs, frameshift mutation; G, genital hypoplasia; H, heart defect; HD, helicase domain; HL, hearing loss; mis, missense mutation; non, nonsense mutation; poss, possibly; prob, probably; RG, retarded growth; RD, retarded development; SANT, SANT domain; splice, splice site mutation; SCC, semicircular canal hypoplasia; TE, tracheo-oesophageal anomaly; U, unknown; [*], sib with features of CHARGE syndrome but unknown *CHD7* status; [#], sibs with *CHD7* mutation; [§], UV found together with a frameshift mutation (c.791_792del; p.Leu264fs).

Supplementary Table S2. The domains of CHD7: localisation, sizes and mutations

A. Localisation and sizes of the domains of CHD7 according to different protein databases[a]

Domain	Uniprot	Alamut	NCBI	EMBL-EBI 1	EMBL-EBI 2	EMBL- EBI 3	Smallest region	Largest region
	Amino acids	*Amino acids*	*Amino acids*	*Amino acids*	*Amino acids*	*Amino acids*	*Amino acids*	*Amino acids*
CD1	800-867	799-867	801-862	801-861	800-867	799-864	801-861	799-867
CD2	882-947	880-947	883-935	883-934	882-947	880-937	883-934	880-947
Helicase N		971-1258	971-1256	971-1256			971-1256	971-1258
DEXDc		964-1165	987-1136	964-1165			987-1136	964-1165
Helicase C	1294-1464	1294-1464	1290-1412	1325-1404	1294-1464	1320-1404	1325-1404	1290-1464
SANT		1962-2021		1962-2021			1962-2021	1962-2021
BRK1		2564-…	2562-2604	2562-2606	2564-2613		2564-2604	2562-2613
BRK2		…-2686	2642-2686	2641-2686	2642-2686		2642-2686	2641-2686

[a]This table shows the predicted size of CHD7 domains from Uniprot (www.uniprot.org/uniprot/Q9P2D1), Alamut (mutation interpretation software, version 1.5), NCBI Protein (reference sequence NP_060250.2) and EMBL-EBI (European Bioinformatics Institute, www.ebi.ac.uk/interpro/, protein accession: Q9p2d1). The EMBL-EBI database gives more than one prediction for several domains, these additional predictions are depicted in the columns EMBL-EBI 2 and 3. The smallest region of overlap and largest region of overlap are shown in the last two columns.

B. The number and percentage of mutations per domain

Domain	Percentage of CHD7[b]	Number of mutations	Percentage of mutations[c]
	Smallest region - Largest region (%)	*Smallest region-Largest region (N)*	*Smallest region- Largest region (%)*
CD1	2.0 -2.3	38 -39	4.8 -4.9
CD2	1.7 -2.3	9 -13	1.1 -1.6
Helicase N and DEXDc	9.5 -9.6	100 -101	12.6 -12.8
Helicase C	2.7 -5.8	33 -55	4.2 -6.9
SANT	2.0 -2.0	18 -18	2.3 -2.3
BRK1	1.4 -1.7	4 -6	0.5 -0.8
BRK2	1.5 -1.5	20 -21	2.5 -2.7
Total	20.9 -25.5	222 -253	28.0 -31.9

[b] Percentage of CHD7 = the number of amino acids in each domain / the total number of amino acids of CHD7 (N=2997)

[c] Percentage of mutations = the number of pathogenic mutation in each domain / the total number of pathogenic mutations in *CHD7* (excluding whole or partial gene deletion and translocation, N=792).

CD = chromodomain (chromatin organisation modifier domain); Helicase N = helicase N-lobe; DEXDc = DEAD-like helicase superfamily including an ATP-binding domain; Helicase C = helicase C-lobe; SANT = switching-defective protein 3, adaptor 2, nuclear receptor co-repressor, transcription factor IIIB domain; BRK = Brahma and Kismet domain.

Supplementary Table S3. Benign variants detected in *CHD7*[a]

CHD7 variant		Exon	NCBI SNP database	MAF[b]	Inherited[c]	Other mutation[d]	Phenotype of index[e]	Number of controls[f]	Reference
c.109A>T	p.Met37Leu	2			+		CH		
c.216T>C	p.Tyr72Tyr	2	rs16926453	0.0048	U	fs	CH		38
c.277A>G	p.Thr93Ala	2			+		CH		
c.307T>A	p.Ser103Thr	2	rs41272435	0.0155	+	fs, mis, hom	CH	12	20,26,63
c.309G>A	p.Ser103Ser	2	rs115293759	0.0055	U		CH		26
c.350G>A	p.Gly117Asp	2			+		CH		63
c.363T>G	p.Gly121Gly	2	rs79158412		U		CH		
c.500C>T	p.Pro167Leu	2	rs61742851		U		CH		
c.657C>T	p.Gly219Gly	2	rs113483301	0.0201	U		CH		26
c.712G>A	p.Val238Met	2			U		CH		36
c.712G>C	p.Val238Leu	2			U	fs	CH		
c.715C>G	p.Leu239Ala	2			U	fs	CH		
c.856A>G	p.Arg286Gly	2	rs61995713		U		CH		
c.1018A>G	p.Met340Val	2	rs41305525	0.0053	+	non	CH, SC	3, 2/69 SC	20,26,60,70
c.1105C>G	p.Pro369Ala	2			+		CH		26
c.1179A>G	p.Pro393Pro	2	rs111238892		U		CH		
c.1323T>G	p.Gly441Gly	2			U		CLP	2	37
c.1397C>T	p.Ser466Leu	2	rs71640285		U		CH	6	20,26,37
c.1467A>G	p.Gln489Gln	2	rs71640286		U		C	1	20
c.1536A>G	p.Pro512Pro	2			U		CH		26
c.1565G>T	p.Gly522Val	2			+	hom	CLP, CH	12	26,37
c.1571A>C	p.His524Pro	2	rs78962949		U		CH		
c.1579T>G	p.Ser527Ala	2			U		CLP	2, 1/92 CLP	37

c.1632C>G	p.Pro544Pro	2	rs45536935		U		SC	4/69 SC	70
c.1665+34G>A	p.?	intron 2	rs7836586	0.2114	+	fs, non, del	SC, CH	13/69 SC	17,54,70,77
c.1786C>A	p.Gln596Lys	3	rs75653665		U		CH		
c.1803G>A	p.Lys601Lys	3	rs74999330		U		CH		
c.1907G>T	p.Gly636Val	3			+	non	CH		26
c.2049G>A	p.Lys683Lys	3	rs78107494		U		CH		
c.2050_2055dupAAAGCA	p.Lys684_ Ala685dup	3			+	del	CH	3	43
c.2053_2058dupGCAAAA	p.Ala685_ Lys686dup	3			+	del, non	CH	3	26
c.2067G>A	p.Thr689Thr	3	rs34979623	0.0024	U		CH		
c.2096+15insA	p.?	intron 3	rs111577577		U		U		
c.2124T>C	p.Ser708Ser	4	rs79302359	0.0127	U		CH		126
c.2230G>A	p.Gly744Ser	4			U	mis	CH		26,60
c.2238+39G>A	p.?	intron 4	rs4540437	0.1351	U		SC, CH	13/69 SC	17,70,77
c.2361C>A	p.Ser787Ser	5			U		CH		26
c.2376+49insTGGACT	p.?	intron 5	rs33909822		U		CH		
c.2376+41G>A	p.?	intron 5	rs74407310		U		U		
c.2376+42insTGGACT	p.?	intron 5	rs112021443		U		U		
c.2376+43insTGGACT	p.?	intron 5	rs35504039		+	fs	CH		54
c.2376+48insTGGACT	p.?	intron 5	rs5891777		+	non, fs	CH		
c.2376+49A>T	p.?	intron 5	rs77952475		U		U		
c.2377-3dupT	p.?	intron 5			U		U		60
c.2436A>T	p.Lys812Asn	6	rs61978638	0.0060	U		CH		
c.2442+38A>T	p.?	intron 6	rs41272438	0.0163	U		CH	17	17,20
c.2443-43C>T	p.?	intron 6	rs74693288	0.0052	U		U		
c.2614-48C>G	p.?	intron 8	rs79276682	0.0690	U		U		
c.2614-45G>A	p.?	intron 8	rs6471902	0.1852	U		CH		17

c.2831G>A	p.Arg944Hls	10	rs117506164	0.0077	+		CH		26
c.2957+20C>T	p.?	intron 11			U		CH		
c.3201+21T>G	p.?	intron 12		0,0075	U		CH		77
c.3202-5T>C	p.?	intron 12		0,1505	U		CH		77
c.3379-33A>G	p.?	intron 13	rs45461501		U		CH		26
c.3522+13T>A	p.?	intron 14			U	fs	CH		
c.3523-39C>G	p.?	intron 14		0,011	U		CH		17
c.3523-35C>G	p.?	intron 14	rs41272442	0.0588	+	non	CHD		
c.3778+17C>T	p.?	intron 15	rs111863846	0.0044	U		U		
c.3879G>A	p.Val1293Val	16			U		CH		
c.4533+34T>C	p.?	intron 19			U	fs	CH		
c.4533+46A>G	p.?	intron 19	rs7844902	0.1987	U	non, fs, del	CH		17
c.4534-13G>T	p.?	intron 19	rs114996731		U		U		
c.4614T>C	p.Ala1535Ala	20			U	mis	CH		23
c.4644+26C>T	p.?	intron 20	rs115999896		U		U		
c.4644+36C>T	p.?	intron 20	rs71640287		U		CH	1	20
c.4780C>T	p.Pro15945er	21			U	fs	CH		
c.5015C>T	p.Ala1672Val	22	rs61737194		U		CH		
c.5051-4C>A	p.?	intron 22			U		CH		60
c.5051-4C>T	p.?	intron 22	rs71640288		+	mis	CH	2	20,26,60
c.5147insGCCAGCTG	p.	23			U	fs	CH		26
c.5307C>T	p.Ala1769Ala	25	rs16926499	0.0246	U		CH		26
c.5404+41C>T	p.?	Intron 25	rs115544727		U		U		
c.5607+27A>T	p.?	intron 27	rs77988197		U		U		
c.5754T>C	p.Thr1918Thr	29	rs61746542		U		CH		
c.5757C>G	p.Ala1919Ala	29	rs79203206	0.0086	U		CH		

c.5894+32C>G/T	p.?	intron 29	rs41265252	0.0100	U		U		
c.5895-23A>G	p.?	intron 29			U		CH		60
c.6103+8C>T	p.?	intron 30	rs3763592	0.1391	+	fs	CH		17,20,26
c.6111C>T	p.Pro2037Pro	31	rs41312170	0.0053	U		CH	2	20,26
c.6135G>A	p.Pro2045Pro	31	rs6999971	0.0304	+	non, fs	CH	8	20,26,63
c.6167C>A	p.Arg2062Arg	31			U	non	CH		43
c.6184C>T	p.Arg2062Trp	31			U	non	CH		
c.6276G>A	p.Glu2092Glu	31	rs2068096	0.1184	+	non,fs	CH	12	17,20,26
c.6282A>G	p.Gly2094Gly	31	rs41312172	0.0032	U	non	CH	1	20,26
c.6304G>A	p.Val2102Ile	31			U		CH		37
c.6335C>T	p.Thr2112Met	31			+		CLP		37
c.6352A>G	p.Asn2118Asp	31			+		CH		
c.6478G>A	p.Ala2160Thr	31	rs61753399	0.0014	U	mis	CH		26,60
c.6513C>T	p.Ala2171Ala	31			U		CH		
c.6672C>T	p.Gly2224Gly	31			U		CLP	1	37
c.6673G>A	p.Ala2225Thr	31			U		CH		26
c.6738G>A	p.Glu2246Glu	31	rs61729627	0.0632	U		CH		26,60
c.6822T>C	p.Ala2274Ala	32	rs61743849		U		CH		26
c.6843C>G	p.Asp2281Glu	32			U		CH		60
c.6924G>A	p.Ser2308Ser	32	rs61733338	0.0086	+	non	CH		54
c.6936+29G>T	p.?	intron 32	rs78582735		U		U		
c.6936+35C>T	p.?	intron 32	rs112592072		U		U		
c.6989G>C	p.Gly2330Ala	33	rs77704609		U		CH		26
c.6990C>T	p.Gly2330Gly	33	rs116523071		U		CH		
c.7107C>T	p.Val2369Val	33			U		C	1	37
c.7209G>A	p.Arg2403Arg	34	rs61746518		U		U		

c.7243G>T	p.Als2415Ser	34	rs41315633		U		U		
c.7278G>A	p.Gln2426Gln	34	rs115546145		U		U		
c.7356A>G	p.Thr2452Thr	34	rs2272727	0.0382	+	non, fs, del	CH		17,20,26
c.7471C>T	p.Arg2491Cys	34			U	spl	CH		
c.7579A>C	p.Met2527Leu	34			+		CH		26
c.7590A>G	p.Lys2530Lys	34	rs61742801	0.0086	U		CH		26
c.7830+44A>G	p.?	intron 35	rs115428154	0.0143	U		U		
c.7958G>A	p.Arg2653Gln	36			+	fs	CH		
c.8173A>G	p.Ile2725Val	38	rs113877656		U		U		
c.8199T>G	p.Ala2733Ala	38			U		CH		
c.8250T>G	p.Phe2750Leu	38	rs3750308		U		U		
c.8339C>T	p.Ala2780Val	38			+		CH		
c.8355C>T	p.Ala2785Ala	38			U	mis	CH		26
c.8365G>A	p.Ala2789Thr	38			U		IHH		20
c.8416C>G	p.Leu2806Val	38	rs45521933	0.0045	+		CH		26,60
c.8569T>G	p.Ser2857Ala	38			U		CH		26
c.8661G>A	p.Pro2887Pro	38			U	fs	CH		63
c.8790C>T	p.Ala2930Ala	38	rs61736186		U		CH		
c.8950C>T	p.Leu2984Phe	38			+	fs	CH		13,26

[a] Benign variants in the coding region and in the first or last 50 nucleotides of an intron of *CHD7* that have either been published, or found by the RUNMC or ICMM, or published with frequency data in the NCBI SNP database

[b] Minor allele frequency from NCBI SNP database (May 25th 2011) or the literature

[c] U, unknown;+, found in the parent or in one or more unaffected sibs of at least one index patient

[d] The type of pathogenic mutation that was found in combination with the benign variant in at least one index patient: fs, frameshift mutation; mis, missense mutation; hom, variant present in homozygous state; non, nonsense mutation; spl, splice site mutation

[e] C, control; CH, CHARGE syndrome; CHD, congenital heart defect; CLP, cleft lip and/or palate; IHH, idiopathic hypogonadotropic hypogonadism; SC, scoliosis patient or parent; U, unknown

[f] Variant found in controls that were published in literature: SC, scoliosis patients or parents; CLP, cleft lip and/or palate cohort.

Supplementary Methods

DNA was isolated according to standard procedures. The 37 coding exons of *CHD7* (exons 2–38, RefSeq NM_017780.02) and their flanking intron sequences were amplified by PCR and sequenced as described before.[13] Whole gene deletions and duplications were excluded by multiplex ligation-dependent probe amplification (MLPA) using a commercially available set of probes, the SALSA P201 kit (MRC-Holland, Amsterdam, the Netherlands; http://www.mrc-holland.com).

CHAPTER 2.2

A novel classification system to predict the pathogenic effects of *CHD7* missense variants in CHARGE syndrome

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Submitted

ABSTRACT

CHARGE syndrome is characterized by the variable occurrence of multi-sensory impairment, congenital anomalies and developmental delay, and is caused by heterozygous mutations in the *CHD7* gene. Correct interpretation of *CHD7* variants is essential for genetic counselling. This is particularly difficult for missense variants, because most variants in the *CHD7* gene are private and a functional assay is not yet available. We have therefore developed a novel classification system to predict the pathogenic effects of *CHD7* missense variants that can be used in a diagnostic setting. Our classification system combines the results from three computational algorithms (SIFT, PolyPhen-2 and Align-GVGD) with segregation and phenotypic data. The combination of different variables will lead to a more confident prediction of pathogenicity than was previously possible. Our classification was confirmed by studies using a structural model of the chromo- and helicase domains of CHD7. We have used our system to classify 145 *CHD7* missense variants. Our data show that pathogenic mutations are mainly present in the middle of the *CHD7* gene, whereas benign variants are clustered in the 5' and 3' regions. Finally, we show that *CHD7* missense mutations are in general associated with a milder phenotype than truncating mutations.

INTRODUCTION

CHARGE syndrome is a clinically heterogeneous syndrome that is characterized by the occurrence of ocular coloboma, heart defects, atresia of choanae, retardation of growth and/or development, genital anomalies, and ear anomalies often combined with deafness.[1-4] It is inherited in an autosomal dominant fashion (OMIM #214800). Most cases are sporadic due to *de novo* mutations but familial recurrence has also been described.[1] Previous studies have estimated that CHARGE syndrome has an incidence of 1/10,000 newborns,[5,6] but we estimate that the incidence is lower, between 1/15,000 and 1/17,000, based on the number of *CHD7* mutations found in the Netherlands between 2006 and 2009 (Janssen *et al.*, submitted). The major gene involved in CHARGE syndrome is *CHD7* and heterozygous *CHD7* mutations are found in more than 90% of the patients with typical CHARGE syndrome based on the clinical diagnostic criteria.[5,7-9] Nonsense and frameshift mutations are the most prevalent variants in this syndrome with a frequency of 43% and 34%, respectively (Janssen *et al.*, submitted). Splice site mutations are found in 11% of patients, while missense mutations are present in 9% of patients. Deletions and genomic rearrangements are rare events (3%). Although missense mutations in the *CHD7* gene are found in only a minority of patients, they can cause serious problems in genetic counselling.

Variants that are expected to lead to a truncated protein (nonsense and frameshift mutations and deletions) are considered to be pathogenic (disease causing), because they are highly likely to result in haploinsufficiency. The interpretation of missense variants, however, can be difficult, as they can be either pathogenic or benign. The interpretation of the consequences of a missense variant is especially difficult in rare diseases, like CHARGE syndrome, in which most mutations are private. A functional assay would be very helpful in the classification of missense variants, but is not available for CHD7. In order to analyze the consequences of missense variants, computational algorithms have been developed.[10] These are mostly based on multiple sequence alignments of a protein across species, with mutations at conserved positions being more likely to disrupt protein function, and/or on the nature of the specific amino acids involved. Each algorithm has its unique strengths and weaknesses. Therefore, it is worthwhile to combine different algorithms to increase the accuracy of the prediction.[11,12] In addition, structural models, when available, can help in predicting the effect of a certain variant on the structural and binding properties of the protein.[13-17] Apart from these tools, segregation analysis can supply crucial information for classifying missense variants.[18,19]

In this study, we present a novel classification system for *CHD7* missense variants that combines the results of three computational algorithms; SIFT,[20,21] PolyPhen-2,[22,23] and Align-GVGD[24,25] with segregation and phenotypic data. We have classified all the *CHD7* missense variants known to us (n=145). To study the reliability of our classification, we constructed a structural model of the CHD7 chromo- and helicase domains and studied the effects of missense variants located in these domains on protein stability. Furthermore, we compared the clinical features of patients with a missense variant that we classified as 'probably pathogenic' with the features of patients with a truncating mutation in order to test our hypothesis that missense mutations are associated with a less severe phenotype than truncating mutations.

PATIENTS AND METHODS

Patients and *CHD7* analysis

For the classification, we included all *CHD7* missense variants reported in the literature before June 15th 2011[1,7,9,14,26-43] and the variants that were reported in the NCBI Single Nucleotide Polymorphism database (http://www.ncbi.nlm.niv.gov/SNP, dbSNP build 132) with frequency data (n=104, Supplementary Table S1). In addition, we included all unpublished missense variants that were found in the DNA diagnostic laboratories of the Radboud University Nijmegen Medical Centre (RUNMC), Nijmegen, the Netherlands and the Department of Cellular and Molecular Medicine (ICMM), the Panum Institute, University of Copenhagen, Denmark (n=41). *CHD7* analysis was performed as previously described[7] and multiplex ligation-dependent probe amplification (MLPA) was performed if *CHD7* sequence analysis did not identify a mutation.[44] Segregation of the *CHD7* variant was studied whenever possible.

Genotype-phenotype correlation

For the phenotypic comparison of patients with a *CHD7* missense mutation with those carrying a truncating mutation, we only included the patients who were analyzed at the RUNMC and the ICMM. In total, we compared the clinical features of 39 patients with a missense variant that we had classified as 'probably pathogenic' with the features of 315 patients with a truncating mutation (5 patients with a deletion, 145 patients with a frameshift mutation and 165 patients with a nonsense mutation). Clinical data were gathered through questionnaires and/or retrospective chart review. χ^2-test and Fisher's exact test were performed to identify significant differences between the two groups of patients (significance level $p<0.05$).

Assessment of possible splice effects

We first screened all *CHD7* missense variants for possible splice effects using the splicing module of Alamut version 1.5. This module contains four splice prediction programs; SpliceSiteFinder-Like,[45] MaxEntScan,[46] NNSPLICE,[47] and GeneSplicer.[48]

Classification system for *CHD7* missense variants

Missense variants in the *CHD7* gene were analyzed with three computational algorithms that predict whether a variant is deleterious; SIFT, PolyPhen-2 and Align-GVGD (for further description, see below). The outcome of each of these three algorithms was scored as 0 (benign), +0.5 (possibly pathogenic), or +1 (probably pathogenic). The scores of the three algorithms were then summed as previously suggested by McGee *et al.*[12]

In addition to the score of the algorithms (varying between 0 and +3), we integrated data from segregation analysis in our classification system (Table 1). If the variant of interest had occurred *de novo* in one patient with features of CHARGE syndrome, 3 points were added. If a certain variant had occurred *de novo* twice, or more often, in patients with features of CHARGE syndrome, 4 points were added. If the variant was found in a first-degree relative of the index patient who also had features of CHARGE syndrome, 1 extra point was added. In contrast, 2 points were subtracted if the variant was found in one or more clinically well characterized persons without features of CHARGE syndrome, or if the variant was found in two or more persons reported to be normal, but for whom

Table 1. Novel classification system for *CHD7* missense variants

Computational algorithms (summed score between 0 and +3)
SIFT: tolerated = 0, not tolerated = +1
Polyphen: benign = 0, possibly damaging = +0.5, probably damaging = +1
Align-GVGD: C0 = 0, C15/C25/C35 = +0.5 and C45/C55/C65 = +1
Segregation analysis (summed score between -10 and +5)
Variant occurred *de novo* in one patient with features of CHARGE syndrome = +3 Variant occurred *de novo* twice, or more often, in patients with features of CHARGE syndrome = +4
Variant present in a first-degree relative with features of CHARGE syndrome = +1
Asymptomatic carrier of the variant* = -2
Variant found in a homozygous state = -5
Variant found in combination with a pathogenic *CHD7* mutation# = -3
Prediction based on total summed score (total score between -10 and +8)
Probably benign: total score 0 or less
Unknown: total score between 0 and +4
Probably pathogenic: total score +4 or more

* this category includes variants that are present in one or more clinically well characterized persons without features of CHARGE syndrome, or variants that are found in two or more persons reported to be normal, but for whom no detailed clinical information was available (e.g. controls reported in the NCBI SNP database or not thoroughly investigated family members)

a pathogenic *CHD7* mutation is defined as a truncating, missense or splice site mutation in the *CHD7* gene that is clearly pathogenic (this category does not include unclassified variants or benign variants)

no detailed clinical information was available (e.g. controls reported in the NCBI SNP database or not thoroughly investigated family members). Five points were subtracted if the variant was found in a homozygous state (this because homozygous mutations are presumed to be lethal). Three points were subtracted if the missense variant was found in combination with a clearly pathogenic *CHD7* mutation, i.e. a truncating, missense or splice site mutation.

Total scores could vary between -10 and +8. Variants with a negative score or 0 were classified as 'probably benign', those with a score between 0 and +4 were classified as 'unclassified variants (UV)', and those with scores of +4 and higher were classified as 'probably pathogenic' (Table 1).

In our classification system, absence of the missense variant of interest in control chromosomes is not integrated because the variant should be absent in a very large number of controls (>1000), before the variant can be classified as probably pathogenic. This is an effect of the individual rarity of *CHD7* missense variations.[18]

SIFT

The SIFT algorithm, Sorting Intolerant From Tolerant, is available at http://sift.jcvi.org. SIFT uses the PSI-BLAST algorithm to find functionally related protein sequences and then creates a protein sequence alignment of multiple species.[20,21] Prediction is based on the evolutionary conservation of the affected residue and the type of amino acid substitution. The SIFT score is calculated with position-specific scoring matrices with Dirichlet priors and ranges between 0 and 1. SIFT scores less

than 0.05 are predicted to be deleterious and scores greater or equal to 0.05 are predicted to be tolerated. For our classification system, we scored an output of 'tolerated' as 0 and an output of 'not tolerated' as +1.

PolyPhen

PolyPhen-2, Polymorphism Phenotyping program version 2, is available at http://genetics.bwh.harvard.edu/pph2/. PolyPhen-2 is an update from PolyPhen[23] and relies on sequence-based and structure-based features.[22] For this study, version 2.2.0 (r364) of PolyPhen-2 was used. The source of the sequence and structure information were UniProtKB/UniRef100 release April 5th 2011 and PDB/DSSP Snapshot April 6th 2011, respectively. HumVar-trained PolyPhen-2 was developed for diagnostic work in Mendelian diseases. PolyPhen-2 calculates the Naive Bayes posterior probability that a certain variant is damaging and gives estimations of the false-positive and true-positive rates. Based on the model's false-positive rate, a quantitative classification (benign, possibly damaging, or probably damaging) is given. If data are lacking, the PolyPhen-2 outcome is reported as 'unknown'. For our classification system, we assigned a score of 0 to variants that were predicted to be 'benign', a score of +0.5 for variants that were 'possibly damaging' and a score of +1 for variants that were 'probably damaging'. None of the *CHD7* missense variants that we entered in PolyPhen-2 had 'unknown' as an outcome.

Align-GVGD

Align-GVGD is available at http://agvgd.iarc.fr/agvgd_input.php. Align-GVGD combines protein sequence alignments of multiple species with the biophysical characteristics of amino acids to calculate the range of biochemical variation among amino acids found at a given position in the alignment (Grantham variation). In addition, the biochemical distance of the mutant amino acid from the observed amino acids at a particular position in different species is calculated (Grantham deviation).[24,25] A grade, varying from C0 to C65, is given to estimate the probability that a certain variant is pathogenic. For our classification system, C0 was scored as 0, C15, C25 and C35 were scored as +0.5, and C45, C55 and C65 were scored as +1.

Structural model of the CHD7 chromo- and helicase domains

No experimentally derived structures of the CHD7 chromo- and helicase domains are available as yet and we therefore constructed a structural model for these domains. We did not perform structural analysis of the SANT and BRK domains of CHD7, because only three of the 145 missense variants were identified in these domains. Template structures for the homology modelling of the CHD7 chromo- and helicase domains were selected from the protein database using BLAST (Supplementary Table S2).[49-54] We used the X-ray structure of the yeast chromatin remodeler Chd1 (3MWY) as a basis for our structural model and for all subsequent analyses, because it shows the chromo- and helicase domains in a single structure.[52] Multiple sequence alignments and structural alignments of the CHD7 target structure and the template structures were performed using Expresso/T-Coffee.[55,56] The homology models of the CHD7 protein were constructed using YASARA Structure version 11.4.18 using standard settings. A short combined steepest descent and simulated annealing minimization using constraints on aligned backbone atoms was performed, followed by

a full unrestrained simulated annealing minimization for the entire model using the YASARA2 force field.[57-59] Modelling of the *CHD7* variants and the assessment of the effect on CHD7 stability was performed using the FoldX protein design algorithm,[60,61] (Stricher F *et al.*, manuscript in preparation) as described previously.[13,15-17]

RESULTS

Classification of *CHD7* missense variants

A complete overview of all 145 missense variants in the *CHD7* gene is supplied in Supplementary Table S1. As a first screen, we ran the splice prediction programs and determined that 12 of the 145 missense variants might have an effect on splicing. However, as we were unable to confirm the splice prediction with RNA studies, we classified these variants with our scoring system to see whether the amino acid substitution had a pathogenic effect. Using our scoring system (see methods section and Table 1), 43 variants had a score ≥4 and were classified as 'probably pathogenic' (30%, with five variants possibly affecting splicing), 69 variants had a score between 0 and 4 and were classified as 'UV' (48%, with seven variants possibly affecting splicing), and 33 variants had a score ≤0 and were classified as 'probably benign' (23%) (Supplementary Table S1). Our classification agreed well with most of the classifications of the 104 previously reported variants (Table 2). In fact, it was only discordant for two variants, p.Val2102Ile and p.Ala2789Thr. We had classified both variants as 'probably benign', but they had previously been reported as pathogenic[14,32] (Supplementary Table S1).

Table 2. Our classification of 104 *CHD7* missense variants versus the classification as reported in the literature

		Classification as reported in the literature				
		Pathogenic	UV	dbSNP	Benign	Total
Our classification	Pathogenic	30	1	0	0	31
	UV	10	29	7	4	50
	Benign	2	5	2	14	23
	Total	42	35	9	18	104

dbSNP, variant solely reported in the NCBI SNP database; UV, unclassified variant

Most *CHD7* missense variants were identified in only one person, but 38 variants were recurrent (26%). Of these recurrent variants, 15/38 were classified as 'probably pathogenic' (including four variants possibly affecting splicing), 9/38 as 'UV' (including two variants possibly affecting splicing) and 14/38 as 'probably benign'. Four benign variants (p.Ser103Thr, p.Met340Val, p.Gly522Val and p.Phe2750Leu) were found in many persons (n>15). Two of these variants (p.Ser103Thr and p.Gly522Val) were found in homozygous states and are therefore surely benign (mice with homozygous *Chd7* mutations die in early embryogenesis[62]). The four most frequently occurring pathogenic missense mutations were each found in more than three index patients (p.Ile1028Val, p.Gln1214Arg, p.Gly2108Arg, and p.Arg2319Cys). Five pathogenic missense mutations were possibly implicated in familial CHARGE syndrome: p.Ser834Phe,[31] p.Leu2074Pro (the *CHD7* mutation was identified in the current study, but the clinical features of the affected siblings were previously published[63]), p.His2096Arg,[33] p.Gly2108Arg,[37] and p.Arg2319Cys.[28,33]

The 145 *CHD7* missense variants were distributed throughout the entire coding region of the *CHD7* gene, as shown in Figure 1. The variants that we classified as 'probably benign' were predominantly located in the 5' and 3' regions of the *CHD7* gene and those classified as 'probably pathogenic' were found in the middle of the gene. Forty-five variants were located in, or were in very close proximity to, functional domains of CHD7; 11 in the chromodomains, 31 in the helicase domain, and only three in the SANT and BRK domains.

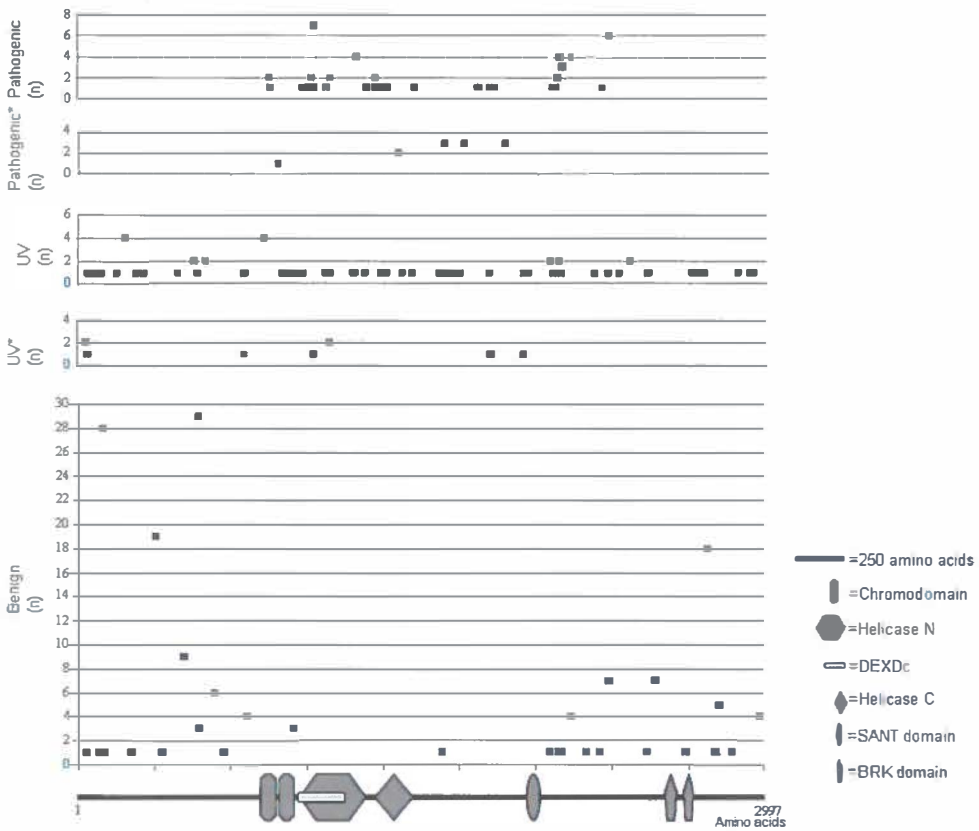

Figure 1. The distribution of 145 different missense variants over the coding region of the *CHD7* gene (the variants were found in 322 index patients or controls). The missense variants are divided into five categories: 'probably pathogenic', 'probably pathogenic with a possible effect on splicing', 'UV', 'UV with a possible effect on splicing' and 'probably benign'. The variants are scattered over the entire *CHD7* gene, but the 'probably pathogenic' variants are located in the middle, whereas the 'probably benign' variants are predominantly present in the 5' and 3' regions of the *CHD7* gene.
n, number of times that a variant was found in an index patient or control; *, variant possibly affecting splicing.

Structural modelling of *CHD7* variants

Modelling of the CHD7 chromo- and helicase domains

We constructed a structural model of the CHD7 chromo- and helicase domains using the template structures that are shown in Supplementary Table S2. One X-ray structure of the yeast chromatin remodeler Chd1 (3MWY) contains both chromo- and helicase domains in a single structure.[52] We

used this X-ray structure as the basis for our structural model and for all subsequent analyses (Figure 2). Additional models were built using the other templates mentioned in Supplementary Table S2 to aid or confirm the sequence alignment, to aid in side chain placement, and to explore possible structural variants. Our structural model shows the two chromodomains capping the DNA-binding cleft between the N-terminal and C-terminal lobe of the helicase domain and has the two helicase lobes relatively spaced far apart with residues of the C-terminal lobe not making any direct contact with ATP (Figure 2). Like the yeast Chd1 structure, the CHD7 model shows an acidic helix connecting chromodomain 1 and 2 that interacts with a basic patch on the C-terminal helicase lobe. This suggests that CHD7 might employ a similar mechanism to discriminate between nucleosome-DNA substrates as that proposed for the yeast Chd1 structure.[52]

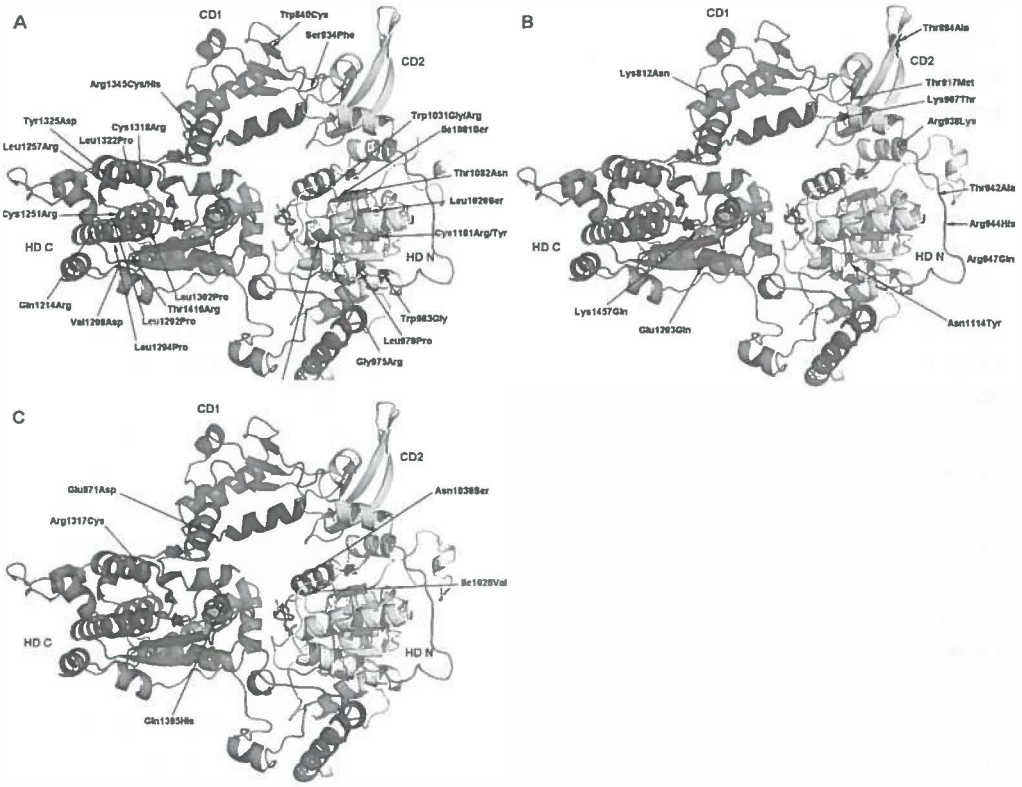

Figure 2. Structural model of the CHD7 chromo- and helicase domains (amino acids 799 – 1464) based on the 3MWY X-ray structure of yeast chromatin remodeler Chd1[52] and additional template structures (Supplementary Table S2). Our structural model shows the two chromodomains capping the DNA-binding cleft between the N-terminal and C-terminal lobe of the helicase domain and has the two helicase lobes relatively spaced far apart with residues of the C-terminal lobe not making any direct contact with ATP. Chromodomain 1 (CD1) is blue, chromodomain 2 (CD2) is green, the helicase N-lobe (HD N) is yellow, the helicase C-lobe (HD C) is red and the linkers between the domains are grey. The ATP-binding domain is depicted in cyan (located in the helicase N-lobe) and the missense variants are shown in purple. **A:** Missense variants that were predicted to have a deleterious effect on protein stability. Many are located in the core of the protein. **B:** Missense variants that were predicted to have a minor effect on protein stability. Many are located at the surface of the protein or in the linker between chromodomain 2 and the helicase N-lobe. **C:** p.Asn1030Ser and p.Gln1395His: possible effect on ATP-binding; p.Glu871Asp, p.Ile1028Val and p.Arg1317Cys: undetermined effect on protein stability. (see color image on page 234-235)

Structural assessment of the effect of *CHD7* variants

Based on the "wild-type" model described above, FoldX was used to create structural models of the different *CHD7* variants and estimate their effect on the structural stability of the CHD7 chromo- and helicase domains. The effect of the different missense variants was classified either as likely to have a minor effect, or likely to have a detrimental effect on the structural stability of the protein. Mutations increasing the calculated Gibbs free energy with more than 1 kcal/mol were considered to be potentially 'detrimental'. For the calculation, the main focus was on the terms that describe increases in energy due to Van der Waals clashes (mutation to a larger residue in the protein core), or to a loss in Van der Waals energy (mutation to a smaller residue in the protein core), or unfavourable solvation (mutation from a hydrophobic residue to a hydrophilic residue in the protein core). An increase in energy due to the loss of a hydrogen bond was ignored, due to the high dependence on accurate atom positions of the hydrogen-bond donor and acceptor. Apart from effects on structural stability, the position of the mutation in the structure was also taken into account: was it in close proximity to the ATP binding site or was a known interaction motive altered?

The prediction based on our classification system was largely confirmed by the predicted effects of the *CHD7* variants on protein stability in our structural model. Of the 42 *CHD7* variants that were located in the chromo- or helicase domains, 21 were labelled as 'probably pathogenic' by our classification system (Supplementary Table S1, Figure 2). Of these 21 variants, 19 were found to have a likely detrimental effect; on protein stability (n=17) or on ATP binding (n=2, p.Asn1030Ser and p.Gln1395His). p.Asn1030Ser was found to be in close proximity to the ATP binding site in the N-lobe of the helicase domain and p.Gln1395His was found on the opposite side in the C-lobe (Figure 2). Although not in direct contact with ATP, the latter variant is located next to p.Arg1399, which in homologous structures is considered to function as one of the Arginine finger residues involved in stabilizing the transition state of ATP. Therefore, these two variants could influence the ATPase activity of the helicase domain and are predicted to be 'detrimental'. The effect on protein stability of the remaining two variants that we classified as 'probably pathogenic' could not be determined (p.Ile1028Val and p.Glu871Asp). No direct effects on phosphorylation or interaction motives were found for any of the variants. The variants that we classified as 'probably pathogenic' were very often located in the core of the CHD7 protein (17/21 variants). We classified only one variant (p.Arg944His), located in the chromodomain, as 'probably benign' and our structural model also predicted it would have a minor effect on protein stability. The remaining twenty variants in the chromo- and helicase domains were classified as 'UV', because they lacked segregation or phenotypic data. Structural modelling showed that nine of these variants were possibly detrimental, 10 variants had only a minor effect (4 of these variants were located in the linker between chromodomain 2 and the helicase N-lobe) and one variant had an undetermined effect on protein stability.

Genotype-phenotype correlation

The clinical data of the patients who had *CHD7* analysis done at the RUNMC or ICMM were used to compare the phenotype of patients with a missense variant that we classified as 'probably pathogenic' (n=39) with that of patients with a truncating mutation in the *CHD7* gene (n=315) (Table 3). The patients with a truncating mutation more often fulfilled the clinical criteria of Blake *et al*[5] and Verloes[6] (p=0.017 and p=0.031, respectively). In addition, cleft lip and/or palate (p=0.041),

choanal anomalies (p=0.009) and congenital heart defects (p<0.001) were present significantly more often in patients with a truncating mutation compared to those with a missense mutation. Furthermore, tracheo-oesophageal anomalies were borderline significant (p=0.052), with more frequent occurrence in the patients with a truncating mutation. The other clinical features were not significantly different between the two groups. In conclusion, missense mutations were in general found to be associated with a milder phenotype compared to truncating mutations.

Table 3. Phenotypic comparison of patients carrying a *CHD7* missense mutation with patients carrying a *CHD7* truncating mutation

	Patients with a *CHD7* missense mutation (n=39)*	**Patients with a *CHD7* truncating mutation (n=315)^**	**Comparison p-value**
Blake criteria	57.1% (8/14)#	85.5% (106/124)#	*0.017*
Verloes criteria	71.4% (10/14)	92.5% (111/120)	*0.031*
Cleft lip and/or palate	30.4% (7/23)	55.6% (80/144)	*0.041*
Choanal anomaly	28.6% (6/21)	60.4% (110/182)	*0.009*
Heart defect	50.0% (16/32)	82.5% (212/257)	*<0.001*
Tracheo-oesophageal anomaly	13.0% (3/23)	33.6% (43/128)	0.052
Coloboma and/or microphthalmia	75.8% (25/33)	86.9% (199/229)	0.110
Cranial nerve dysfunction	81.0% (17/21)	90.8% (119/131)	0.240
Semicircular canal anomaly and/ or balance disturbance	95.0% (19/20)	100% (121/121)	0.142
External ear anomaly	96.6% (28/29)	98.2% (217/221)	0.463
Kidney anomaly	26.1% (6/23)	37.6% (44/117)	0.348

* 39 missense variants that were classified as 'probably pathogenic'

^ 315 truncating mutations; 5 deletions, 145 frameshift mutations, 165 nonsense mutations

Due to lacking clinical data, the number of patients is lower than the total number of patients

DISCUSSION

A novel classification system for *CHD7* missense variants

It is important to classify missense variants accurately as either benign or pathogenic, because of the clinical implications. Patients harbouring a pathogenic *CHD7* mutation should be screened for additional features of CHARGE syndrome, for example, hypogonadotropic hypogonadism, heart and kidney defects (see surveillance scheme in [1]). Early detection and treatment of hypogonadotropic hypogonadism is important, because this will reduce the risk of osteoporosis.[29] In addition, genetic counselling is indicated to inform the patient and the parents about reproductive options. Furthermore, correct classification of *CHD7* missense variants can contribute to the knowledge

about CHD7 function. A good classification of *CHD7* missense variants, however, is difficult. The absence of a particular missense variant in the control population is often used for confirmation of pathogenicity. However, most *CHD7* missense variants are found only once, even in cohorts of more than 1000 patients, and therefore very large numbers of controls should be screened before the missense variant can be classified as 'probably pathogenic'.[18] A control group of that size is currently not available, but the 1000 genome project and the Dutch genome project will likely supply useful data. In addition, a validated functional model can be very helpful in the classification, but such a model is currently not available, due to the complexity of CHD7 function. To overcome these problems, we have developed a novel classification system that can be used in a diagnostic setting. Our system combines different variables: the results of three computational algorithms, segregation and phenotypic data (Table 1). It will therefore increase the reliability of predictions. We used our system to classify all known missense variants (n=145) and were able to classify 43 variants as 'probably pathogenic', 69 as 'unknown', and 33 as 'probably benign'. The specificity and sensitivity of our classification is not known, because a gold standard does not exist. However, combining the output of different algorithms is known to increase the predictive value[64] and segregation data are widely accepted as a valuable source of information for the classification of missense variants.[18] In addition, our classification was largely in agreement with the predicted effects on protein stability in the structural model of the CHD7 chromo- and helicase domains that we constructed and was also frequently in agreement with predictions from the literature (Table 2).

According to our classification system, a variant is considered 'probably pathogenic' when it has occurred *de novo* twice, or more often, in patients with features of CHARGE syndrome, or when the variant has occurred *de novo* in one patient with features of CHARGE syndrome and is predicted to be deleterious according to at least one computational algorithm. A variant that has a deleterious effect according to all three computational algorithms and is present in a patient and a first-degree relative with features of CHARGE syndrome is also predicted to be 'probably pathogenic'. Considering that many diagnostic laboratories conclude that every *de novo* variant is pathogenic, we are confident that a variant classified as 'probably pathogenic' with our more conservative approach is very likely to be a true pathogenic mutation. We classify a variant as 'probably benign' when there are no clues to suggest pathogenicity from either the computational algorithms or segregation data or when the segregation data suggest that the variant is probably benign. This means that there is a chance that a variant that we classified as 'probably benign', might later receive the label 'probably pathogenic', if future studies show that the variant has occurred *de novo* twice, or more often, in patients with features of CHARGE syndrome. Two variants that we classified as 'probably benign', p.Val2102Ile and p.Ala2789Thr, were previously reported as pathogenic missense mutations.[14,32] Both variants were found in an index patient with few features of CHARGE syndrome; p.Val2102Ile was found in a patient with a cleft palate and a congenital heart defect, and p.Ala2789Thr was found in a patient with hypogonadotropic hypogonadism and myopia. Because both variants were predicted to be 'benign' by the three computational algorithms and segregation data were not available, the total summed score was 0, leading to our classification of 'probably benign'. Neither variant was located in the chromo- or helicase domain and therefore structural modelling was not performed by us. However, Ala2789Thr was predicted to be deleterious according to a structural model of CHD7 that was constructed by Kim *et al*.[14]

Unfortunately, we had to classify many variants as 'UV' (69/145=48%), due to a lack of segregation data (n=41) or of phenotypic data of the carrier parent (n=23). For five variants, these data were available, but we still had to classify the variant as 'UV'. Four of these five variants were predicted to be pathogenic according to the computational algorithms, but segregation data suggested that the variants were benign. Additional segregation and phenotypic data from patients and/or controls can ultimately lead to a correct classification of all missense variants. The locus-specific *CHD7* mutation database (available at www.CHD7.org[65]) provides a valuable source of information, as it contains both segregation and clinical data. Clinical data are very important, because the phenotype of patients who undergo *CHD7* analysis in a clinical diagnostic laboratory is not always highly suggestive of CHARGE syndrome.[28] On the contrary, many patients have only a few features of CHARGE syndrome and *CHD7* analysis is performed to exclude a diagnosis of CHARGE syndrome. The prior chance of finding a pathogenic *CHD7* mutation in this group is therefore much lower than in the group of patients with typical CHARGE syndrome.

The computational algorithms gave discordant predictions in 34% (50/145 variants, Supplementary Table S1). However, when all three computational algorithms gave the same prediction, this is usually, but not always, in agreement with our classification: 11 variants were predicted to be 'benign' by the three algorithms and also by our classification, but of the 58 variants that were considered 'probably pathogenic' by the three algorithms, we classified 32 as 'probably pathogenic', 25 as 'UV' and one as 'probably benign'. The variant that we classified as 'probably benign', p.Arg2062Trp, was found in combination with a nonsense mutation. It is possible that the missense variant and the nonsense mutation had both occurred on the same allele and therefore both could be pathogenic, although this is highly unlikely. Unfortunately, segregation data were not available for this patient and we could therefore not establish whether the missense variant was *in trans* with the pathogenic mutation. Based on our findings, complete reliance on the computational algorithms seems unadvisable.

Segregation data in combination with phenotypic data are reasonably reliable, but one should be aware that a variant that segregates with the disease is not always pathogenic, because the missense variant may be in linkage disequilibrium with an unidentified pathogenic mutation. When interpreting segregation data, the possibility of phenocopies, variable expressivity and non-paternity should be considered. The presence of a *CHD7* variant in the NCBI SNP database does not necessarily mean that the variant is benign, because there is always a chance that a mildly affected patient with CHARGE syndrome could have been included in the NCBI SNP cohorts.[18]

Our system mainly classifies missense variants according to the predicted effect of the amino acid substitution. However, missense variants, as well as synonymous changes, can also have a deleterious effect on splicing, because the variant can be located in, or close to, a splice site, or it can create a novel splice site. Of the 145 missense variants that were assessed in this study, 12 were predicted to have a possible effect on splicing according to the splice prediction programs (12/145=8%). RNA studies should be performed to confirm the splice effects. As *CHD7* is hardly expressed in leucocytes or fibroblasts, confirmation of the splice prediction can best be performed in an exon trap assay.

Distribution of *CHD7* missense variants

The *CHD7* missense variants were present in the entire coding region of the *CHD7* gene (Figure 1, Supplementary Table S1). The variants that we classified as 'probably pathogenic' were all located in the middle of the *CHD7* gene. Those that we classified as 'probably benign' were predominantly located at the 5' and 3' ends of the *CHD7* gene: 25/33 'probably benign' variants were found in amino acids 1-750 and 2320-2997 (Figure 1). The 5' end of the *CHD7* gene is only weakly to moderately conserved among species and both the 5' and 3' ends of the CHD7 protein do not contain functionally important domains.

Structural model of chromo- and helicase domains

We constructed a structural model of the chromo- and helicase domains of the CHD7 protein, based on different template structures (Figure 2, Supplementary Table S2). Unfortunately, there was a low percentage sequence identity (approximately 30%) between the target sequence and most of the template sequences. This increases the risk of alignment errors, resulting in the construction of faulty structural models. However, a structural superposition of structures of several chromo- or helicase domains derived from distantly related organisms showed that many structural features of these domains are particularly well conserved despite remote ancestry and divergent functionality. For example, the N- and C-terminal lobe of the zebra fish Rad54 SWI2/SNF2 chromatin-remodelling domain can be superimposed with less than 2-2.5 Å root mean square deviation over 120-200 C-alpha atoms on the equivalent domains of yeast chromatin remodeler Chd1 and on the SWI2/SNF2 ATPase core domain of the Archaea *Sulfolobus solfataricus*. For the chromodomains, the same trends were observed upon structural superimposition. This indicates that sufficiently accurate models can be constructed of the conserved regions and that the location of many of the *CHD7* variants can be predicted with reasonable accuracy. FoldX was used to create structural models of the different *CHD7* variants and estimate their effect on the structural stability of the CHD7 chromo- and helicase domains. Because the accuracy of the energy prediction by FoldX depends on the exact position of the amino acid atoms in a structure, the accuracy of our prediction is more limited than in previous works,[13,15,16] due to the use of models based on low sequence identity between target and template, and the low resolution of the available template structures.

Because of the somewhat limited predictive accuracy of our structural model, we used it solely for confirming the predictions made by our classification system. The predictions from the structural model were largely in agreement with our classification. The variants that we had classified as 'probably pathogenic' had a likely detrimental effect on protein stability and were frequently located in the core of the CHD7 protein. In addition, the variants that were predicted to have a minor effect on protein stability were frequently located at the surface of the CHD7 protein. A previous study constructed a structural model of the C-terminal part of the CHD7 protein and concluded that variants in the loop regions were likely detrimental, because of their possible effects on the structural and binding properties of the CHD7 protein.[14] This is in contrast to our model, where five *CHD7* missense variants located in loop regions were all predicted to have a likely minor effect on protein stability (Supplementary Table S1). No direct effects on interaction motives or phosphorylation could be identified.

Genotype-phenotype correlation

CHARGE syndrome is extremely variable and the phenotype cannot be predicted from the genotype. However, when comparing the clinical features of patients with a *CHD7* missense mutation with patients with a truncating mutation, we have shown that missense mutations are, in general, associated with a milder phenotype (Table 3). This association is also seen in other syndromes, e.g., Rett syndrome.[66] Three features were found significantly more often in the patients with a truncating mutation: cleft lip/palate, choanal anomalies, and congenital heart defects. The features that are almost always present in CHARGE syndrome (external ear anomalies, cranial nerve dysfunction and balance disturbance caused by semicircular canal anomalies[1]), do not occur significantly more often in patients with a truncating mutation. This was to be expected, because these features are frequently seen in very mildly affected patients.[1,31,37,39,67]

CONCLUSION

We have developed a novel classification system to predict the pathogenic effects of *CHD7* missense variants that can be used in a diagnostic setting. In our classification system we have combined the outcome of SIFT, PolyPhen-2 and Align-GVGD with segregation data and phenotypic data of carriers of a *CHD7* missense variant. The combination of different variables will lead to a more confident prediction of pathogenicity than was previously possible. Our classification was confirmed by studies in a structural model of the chromo- and helicase domains, which showed that pathogenic mutations were frequently located in the protein core and were predicted to reduce the stability of the CHD7 protein. We have used our system to classify 145 *CHD7* missense variants and have made our data available in the locus-specific *CHD7* mutation database (www.CHD7.org). Ongoing submission of new segregation and phenotypic data will contribute to a better classification, in particular for those *CHD7* missense variants that we have classified as UV. *CHD7* missense variants were found scattered throughout the entire coding region of the *CHD7* gene, with pathogenic mutations found in the middle of the *CHD7* gene and the benign variants clustered in the 5' and 3' regions. Finally, we showed that *CHD7* missense mutations are, in general, associated with a milder phenotype than truncating mutations.

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REFERENCES

1 Bergman JE, Janssen N, Hoefsloot LH, Jongmans MC, Hofstra RM, van Ravenswaaij-Arts CM. *CHD7* mutations and CHARGE syndrome: the clinical implications of an expanding phenotype. *J Med Genet* 2011;48(5):334-342.

2 Pagon RA, Graham JM, Jr., Zonana J, Yong SL. Coloboma, congenital heart disease, and choanal atresia with multiple anomalies: CHARGE association. *J Pediatr* 1981;99(2):223-227.

3 Sanlaville D, Verloes A. CHARGE syndrome: an update. *Eur J Hum Genet* 2007;15(4):389-399.

4 Zentner GE, Layman WS, Martin DM, Scacheri PC. Molecular and phenotypic aspects of *CHD7* mutation in CHARGE syndrome. *Am J Med Genet A* 2010;152A(3):674-686.

5 Blake KD, Davenport SL, Hall BD, Hefner MA, Pagon RA, Williams MS, et al. CHARGE association: an update and review for the primary pediatrician. *Clin Pediatr (Phila)* 1998;37(3):159-173.

6 Issekutz KA, Graham JM, Jr., Prasad C, Smith IM, Blake KD. An epidemiological analysis of CHARGE syndrome: preliminary results from a Canadian study. *Am J Med Genet A* 2005;133(3):309-317.

7 Jongmans MC, Admiraal RJ, van der Donk KP, Vissers LE, Baas AF, Kapusta L, et al. CHARGE syndrome: the phenotypic spectrum of mutations in the *CHD7* gene. *J Med Genet* 2006;43(4):306-314.

8 Verloes A. Updated diagnostic criteria for CHARGE syndrome: a proposal. *Am J Med Genet A* 2005;133(3):306-308.

9 Vissers LE, van Ravenswaaij CM, Admiraal R, Hurst JA, De Vries BB, Janssen IM, et al. Mutations in a new member of the chromodomain gene family cause CHARGE syndrome. *Nat Genet* 2004;36(9):955-957.

10 Tavtigian SV, Greenblatt MS, Lesueur F, Byrnes GB, IARC Unclassified Genetic Variants Working Group. In silico analysis of missense substitutions using sequence-alignment based methods. *Hum Mutat* 2008;29(11):1327-1336.

11 Frederic MY, Lalande M, Boileau C, Hamroun D, Claustres M, Beroud C, et al. UMD-predictor, a new prediction tool for nucleotide substitution pathogenicity – application to four genes: *FBN1*, *FBN2*, *TGFBR1*, and *TGFBR2*. *Hum Mutat* 2009;30(6):952-959.

12 McGee TL, Seyedahmadi BJ, Sweeney MO, Dryja TP, Berson EL. Novel mutations in the long isoform of the *USH2A* gene in patients with Usher syndrome type II or non-syndromic retinitis pigmentosa. *J Med Genet* 2010;47(7):499-506.

13 Alibes A, Nadra AD, De Masi F, Bulyk ML, Serrano L, Stricher F. Using protein design algorithms to understand the molecular basis of disease caused by protein-DNA interactions: the Pax6 example. *Nucleic Acids Res* 2010;38(21):7422-7431.

14 Kim HG, Kurth I, Lan F, Meliciani I, Wenzel W, Eom SH, et al. Mutations in *CHD7*, encoding a chromatin-remodeling protein, cause idiopathic hypogonadotropic hypogonadism and Kallmann syndrome. *Am J Hum Genet* 2008;83(4):511-519.

15 Pey AL, Stricher F, Serrano L, Martinez A. Predicted effects of missense mutations on native-state stability account for phenotypic outcome in phenylketonuria, a paradigm of misfolding diseases. *Am J Hum Genet* 2007;81(5):1006-1024.

16 Rakoczy EP, Kiel C, McKeone R, Stricher F, Serrano L. Analysis of disease-linked rhodopsin mutations based on structure, function, and protein stability calculations. *J Mol Biol* 2011;405(2):584-606.

17 van der Sloot AM, Tur V, Szegezdi E, Mullally MM, Cool RH, Samali A, et al. Designed tumor necrosis factor-related apoptosis-inducing ligand variants initiating apoptosis exclusively via the DR5 receptor. *Proc Natl Acad Sci U S A* 2006;103(23):8634-8639.

18 Bell J, Bodmer D, Sistermans E, Ramsden SC. Practice guidelines for the interpretation and reporting of unclassified variants (UVs) in clinical molecular genetics. Clinical Molecular Genetics Society 2007. http://www.cmgs.org/BPGs/pdfs%20current%20bpgs/UV%20GUIDELINES%20ratified.pdf .

19 Zanetti A, Ferraresi E, Picci L, Filocamo M, Parini R, Rosano C, et al. Segregation analysis in a family at risk for the Maroteaux-Lamy syndrome conclusively reveals c.1151G>A (p.S384N) as to be a polymorphism. *Eur J*

Hum Genet 2009;17(9):1160-1164.

20 Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc* 2009;4(7):1073-1081.

21 Ng PC, Henikoff S. Predicting the effects of amino acid substitutions on protein function. *Annu Rev Genomics Hum Genet* 2006;7:61-80.

22 Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. *Nat Methods* 2010;7(4):248-249.

23 Ramensky V, Bork P, Sunyaev S. Human non-synonymous SNPs: server and survey. *Nucleic Acids Res* 2002;30(17):3894-3900.

24 Mathe E, Olivier M, Kato S, Ishioka C, Hainaut P, Tavtigian SV. Computational approaches for predicting the biological effect of *p53* missense mutations: a comparison of three sequence analysis based methods. *Nucleic Acids Res* 2006;34(5):1317-1325.

25 Tavtigian SV, Deffenbaugh AM, Yin L, Judkins T, Scholl T, Samollow PB, et al. Comprehensive statistical study of 452 *BRCA1* missense substitutions with classification of eight recurrent substitutions as neutral. *J Med Genet* 2006;43(4):295-305.

26 De Arriba Munoz A, Monge Galindo L, Lopez Pison J, Lafuente Hidalgo M, Perez Delgado R, Dominguez Cajal M, et al. CHARGE syndrome and *CHD7* gene mutation. *Neurologia* 2011;26(4):255.

27 Asakura Y, Toyota Y, Muroya K, Kurosawa K, Fujita K, Aida N, et al. Endocrine and radiological studies in patients with molecularly confirmed CHARGE syndrome. *J Clin Endocrinol Metab* 2008;93(3):920-924.

28 Bartels CF, Scacheri C, White L, Scacheri PC, Bale S. Mutations in the *CHD7* gene: the experience of a commercial laboratory. *Genet Test Mol Biomarkers* 2010;14(6):881-891.

29 Bergman JE, Bocca G, Hoefsloot LH, Meiners LC, Ravenswaaij-Arts CM. Anosmia predicts hypogonadotropic hypogonadism in CHARGE syndrome. *J Pediatr* 2011;158(3):474-479.

30 Dauber A, Hirschhorn JN, Picker J, Maher TA, Milunsky A. Delayed puberty due to a novel mutation in *CHD7* causing CHARGE syndrome. *Pediatrics* 2010;126(6):e1594-e1598.

31 Delahaye A, Sznajer Y, Lyonnet S, Elmaleh-Berges M, Delpierre I, Audollent S, et al. Familial CHARGE syndrome because of *CHD7* mutation: clinical intra- and interfamilial variability. *Clin Genet* 2007;72(2):112-121.

32 Felix TM, Hanshaw BC, Mueller R, Bitoun P, Murray JC. *CHD7* gene and non-syndromic cleft lip and palate. *Am J Med Genet A* 2006;140(19):2110-2114.

33 Feret H, McDonald-McGinn DM, Santani A, Zackai EH. Expanding the phenotypic overlap between CHARGE and Kallmann syndromes due to *CHD7* mutations. 2010;Abstract of poster presented at the 60th Annual ASHG Meeting, Washington DC, 2-6 Nov. 2010. No.1671:www.ashg.org/cgi-bin/2010/ashg10s.

34 Fujita K, Aida N, Asakura Y, Kurosawa K, Niwa T, Muroya K, et al. Abnormal basiocciput development in CHARGE syndrome. *Am J Neuroradiol* 2009;30(3):629-634.

35 Gao X, Gordon D, Zhang D, Browne R, Helms C, Gillum J, et al. *CHD7* gene polymorphisms are associated with susceptibility to idiopathic scoliosis. *Am J Hum Genet* 2007;80(5):957-965.

36 Holak HM, Kohlhase J, Holak SA, Holak NH. New recognized ophthalmic morphologic anomalies in CHARGE syndrome caused by the R2319C mutation in the *CHD7* gene. *Ophthalmic Genet* 2008;29(2):79-84.

37 Jongmans MC, Hoefsloot LH, van der Donk KP, Admiraal RJ, Magee A, van de Laar I, et al. Familial CHARGE syndrome and the *CHD7* gene: a recurrent missense mutation, intrafamilial recurrence and variability. *Am J Med Genet A* 2008;146(1):43-50.

38 Jongmans MC, Ravenswaaij-Arts CM, Pitteloud N, Ogata T, Sato N, Claahsen-van der Grinten HL, et al. *CHD7* mutations in patients initially diagnosed with Kallmann syndrome -- the clinical overlap with CHARGE syndrome. *Clin Genet* 2009;75(1):65-71.

39 Lalani SR, Safiullah AM, Fernbach SD, Harutyunyan KG, Thaller C, Peterson LE, et al. Spectrum of *CHD7*

mutations in 110 individuals with CHARGE syndrome and genotype-phenotype correlation. *Am J Hum Genet* 2006;78(2):303-314.

40 Pauli S, von Velsen N, Burfeind P, Steckel M, Manz J, Buchholz A, et al. *CHD7* mutations causing CHARGE syndrome are predominantly of paternal origin. *Clin Genet* 2011;Epub ahead of print, doi: 10.1111/j.1399-0004.2011.01701.x.

41 Vuorela P, la-Mello S, Saloranta C, Penttinen M, Poyhonen M, Huoponen K, et al. Molecular analysis of the *CHD7* gene in CHARGE syndrome: identification of 22 novel mutations and evidence for a low contribution of large *CHD7* deletions. *Genet Med* 2007;9(10):690-694.

42 Wessels K, Bohnhorst B, Luhmer I, Morlot S, Bohring A, Jonasson J, et al. Novel *CHD7* mutations contributing to the mutation spectrum in patients with CHARGE syndrome. *Eur J Med Genet* 2010;53(5):280-285.

43 Wincent J, Holmberg E, Stromland K, Soller M, Mirzaei L, Djureinovic T, et al. *CHD7* mutation spectrum in 28 Swedish patients diagnosed with CHARGE syndrome. *Clin Genet* 2008;74(1):31-38.

44 Bergman JE, de Wijs I, Jongmans MC, Admiraal RJ, Hoefsloot LH, Ravenswaaij-Arts CM. Exon copy number alterations of the *CHD7* gene are not a major cause of CHARGE and CHARGE-like syndrome. *Eur J Med Genet* 2008;51(5):417-425.

45 Zhang MQ. Statistical features of human exons and their flanking regions. *Hum Mol Genet* 1998;7(5):919-932.

46 Yeo G, Burge CB. Maximum entropy modeling of short sequence motifs with applications to RNA splicing signals. *J Comput Biol* 2004;11(2-3):377-394.

47 Reese MG, Eeckman FH, Kulp D, Haussler D. Improved splice site detection in Genie. *J Comput Biol* 1997;4(3):311-323.

48 Pertea M, Lin X, Salzberg SL. GeneSplicer: a new computational method for splice site prediction. *Nucleic Acids Res* 2001;29(5):1185-1190.

49 Durr H, Korner C, Muller M, Hickmann V, Hopfner KP. X-ray structures of the Sulfolobus solfataricus SWI2/SNF2 ATPase core and its complex with DNA. *Cell* 2005;121(3):363-373.

50 Flanagan JF, Mi LZ, Chruszcz M, Cymborowski M, Clines KL, Kim Y, et al. Double chromodomains cooperate to recognize the methylated histone H3 tail. *Nature* 2005;438(7071):1181-1185.

51 Flanagan JF, Blus BJ, Kim D, Clines KL, Rastinejad F, Khorasanizadeh S. Molecular implications of evolutionary differences in CHD double chromodomains. *J Mol Biol* 2007;369(2):334-342.

52 Hauk G, McKnight JN, Nodelman IM, Bowman GD. The chromodomains of the Chd1 chromatin remodeler regulate DNA access to the ATPase motor. *Mol Cell* 2010;39(5):711-723.

53 Okuda M, Horikoshi M, Nishimura Y. Structural polymorphism of chromodomains in Chd1. *J Mol Biol* 2007;365(4):1047-1062.

54 Thoma NH, Czyzewski BK, Alexeev AA, Mazin AV, Kowalczykowski SC, Pavletich NP. Structure of the SWI2/SNF2 chromatin-remodeling domain of eukaryotic Rad54. *Nat Struct Mol Biol* 2005;12(4):350-356.

55 Armougom F, Moretti S, Poirot O, Audic S, Dumas P, Schaeli B, et al. Expresso: automatic incorporation of structural information in multiple sequence alignments using 3D-Coffee. *Nucleic Acids Res* 2006;34(Web Server issue):W604-8.

56 Notredame C, Higgins DG, Heringa J. T-Coffee: A novel method for fast and accurate multiple sequence alignment. *J Mol Biol* 2000;302(1):205-217.

57 Krieger E, Koraimann G, Vriend G. Increasing the precision of comparative models with YASARA NOVA -- a self-parameterizing force field. *Proteins* 2002;47(3):393-402.

58 Krieger E, Darden T, Nabuurs SB, Finkelstein A, Vriend G. Making optimal use of empirical energy functions: force-field parameterization in crystal space. *Proteins* 2004;57(4):678-683.

59 Krieger E, Joo K, Lee J, Lee J, Raman S, Thompson J, et al. Improving physical realism, stereochemistry, and side-chain accuracy in homology modeling: Four approaches that performed well in CASP8. *Proteins*

2009;77 (Suppl 9):114-122.

60 Guerois R, Nielsen JE, Serrano L. Predicting changes in the stability of proteins and protein complexes: a study of more than 1000 mutations. *J Mol Biol* 2002;320(2):369-387.

61 Schymkowitz JW, Rousseau F, Martins IC, Ferkinghoff-Borg J, Stricher F, Serrano L. Prediction of water and metal binding sites and their affinities by using the Fold-X force field. *Proc Natl Acad Sci U S A* 2005;102(29):10147-10152.

62 Bosman EA, Penn AC, Ambrose JC, Kettleborough R, Stemple DL, Steel KP. Multiple mutations in mouse *Chd7* provide models for CHARGE syndrome. *Hum Mol Genet* 2005;14(22):3463-3476.

63 Levy CM, Knudtzon J. Kallmann syndrome in two sisters with other developmental anomalies also affecting their father. *Clin Genet* 1993;43(1):51-53.

64 Chan PA, Duraisamy S, Miller PJ, Newell JA, McBride C, Bond JP, et al. Interpreting missense variants: comparing computational methods in human disease genes *CDKN2A*, *MLH1*, *MSH2*, *MECP2*, and tyrosinase (*TYR*). *Hum Mutat* 2007;28(7):683-693.

65 Swertz MA, Dijkstra M, Adamusiak T, van der Velde JK, Kanterakis A, Roos ET, et al. The MOLGENIS toolkit: rapid prototyping of biosoftware at the push of a button. *BMC Bioinformatics* 2010;11 Suppl 12:S12.

66 Cheadle JP, Gill H, Fleming N, Maynard J, Kerr A, Leonard H, et al. Long-read sequence analysis of the *MECP2* gene in Rett syndrome patients: correlation of disease severity with mutation type and location. *Hum Mol Genet* 2000;9(7):1119-1129.

67 Vuorela PE, Penttinen MT, Hietala MH, Laine JO, Huoponen KA, Kaariainen HA. A familial CHARGE syndrome with a *CHD7* nonsense mutation and new clinical features. *Clin Dysmorphol* 2008;17(4):249-253.

Supplementary Table S1. Classification of 145 missense variants in the *CHD7* gene

CHD7 variant	Domain[a]	SIFT[b]	Poly Phen-2[c]	Align GVGD	Score algorithms[d]	# controls[e]	# index patients[f]	Segregation[g]	Other mutation found in index patient[h]	Total summed score ; classification[i]	Effect on the stability of the CHD7 protein[j]	References[k]
c.109A>T p.Met37Leu	-	tolerated/0.23	benign /0.001	C0	0	0	1 CH	unaffected parent carrier	-	-2 ; benign		CS
c.123G>A p.Met41Ile	-	not tolerated	benign /0.002	C0	1	0	1 CH	U	-	1 ; UV		[28]
c.164A>G p.HisSSArg	-	not tolerated	benign /0.023	C0	1	0	1 KS	U	-	1 ; UV		[14]
c.215A>G p.Tyr72Cys	-	not tolerated	benign /0.247	C0	1	0	1 CH	U	-	1 ; UV		CS
c.257C>G p.Pro86Arg	-	not tolerated	poss /0.253	C0	1.5	0	1 CH	parent carrier	-	1.5 ; UV		[28]
c.277A>G p.Thr93Ala	-	tolerated/1.0	benign /0	C0	0	0	1 CH	parent carrier	-	0 ; benign		CS
c.295G>C p.Ala99Pro	-	not tolerated	benign /0.156	C0	1	0	1 CH	parent carrier	-	1 ; UV		CS
c.307T>A p.Ser103Thr	-	not tolerated	benign /0.013	C0	1	15	10 CH, 3 KS/HH	parent carrier	+, homo	-9 ; benign		[14,28,43], dbSNP, CS
c.350G>A p.Gly117Asp	-	not tolerated	poss /0.545	C0	1.5	0	1 CH	unaffected parent carrier	+	-3.5 ; benign		[43]
c.S00C>T p.Pro167Leu	-	not tolerated	benign /0	C0	1	1	0	U	-	1 ; UV		dbSNP
c.602A>G p.Gln201Arg	-	not tolerated	benign /0	C0	1	0	4 CH	parent carrier	-	1 ; UV		[28]
c.712G>A p.Val238Met	-	tolerated/0.25	benign /0.002	C0	0	0	1 CH	U	-	0 ; benign		[28]
c.712G>C p.Val238Leu	-	tolerated/0.16	benign /0	C0	0	0	1 CH	U	+	-3 ; benign		CS
c.715C>G p.Pro239Ala	-	not tolerated	benign /0.066	C0	1	0	1 CH	U	+	-2 ; benign		CS
c.760C>G p.Gln254Glu	-	not tolerated	benign /0.004	C0	1	0	1 CH	U	-	1 ; UV		CS
c.856A>G p.Arg286Gly	-	not tolerated	benign /0.043	C0	1	1	0	U	-	1 ; UV		dbSNP
c.1018A>G p.Met340Val	-	not tolerated	benign /0	C0	1	5	11 CH, 1 HH/KS, 2 SC/C	poss unaffected parent carrier	+	-4 ; benign		[14,28,35,41], dbSNP, CS

c.1105C>G p.Pro369Ala	-	tolerated/0.13	benign /0.066	C0	0	0	1 CH	3 family members carrier	-	-2 ; benign		[28]
c.1315C>T p.Pro439Ser	-	not tolerated	benign /0.112	C0	1	0	1 CH	U	-	1 ; UV		CS
c.1397C>T p.Ser466Leu	-	not tolerated	benign /0.001	C0	1	4	3 CH, 1 CLP, 1 KS/HH	parent carrier	-	-1 ; benign		[14,28,31], CS
c.1531T>G p.Leu511Val	-	not tolerated	benign /0.052	C0	1	1	1 CLP	U	-	1 ; UV		[32]
c.1565G>T p.Gly522Val	-	not tolerated	poss /0.622	C0	1.5	13	3 CH, 13 CLP	U	-, homo	-5.5 ; benign		[28,32], CS
c.1571A>C p.His524Pro	-	not tolerated	benign /0.045	C0	1	1	0	U	-	1 ; UV		dbSNP
c.1579T>G p.Ser527Ala	-	not tolerated	benign /0.008	C0	1	2	1 CLP	U	-	-1 ; benign		[32]
c.1672C>G p.Pro558Ala	-	not tolerated	benign /0.066	C0	1	0	2 CH	U	-	1 ; UV		[28], CS
c.1786C>A p.Gln596Lys	-	not tolerated	benign /0.008	C0	1	6	0	U	-	-1 ; benign		dbSNP
c.1907G>T p.Gly636Val	-	tolerated/0.40	poss /0.622	C0	0.5	0	1 CH	U	+	-2.5 ; benign		[26]
c.2095A>G p.Ser699Gly	-	not tolerated	benign /0.013	C0	1	0	2 CH	U	-	1 ; UV		CS
c.2096G>C p.Ser699Thr	-	not tolerated	benign /0.013	C0	1	0	1 CH	U	-	1 ; UV		[28]
c.2182G>A p.Asp728Asn	-	not tolerated	benign /0.139	C0	1	0	1 CH	U	-	1 ; UV		[28]
c.2194C>G p.Pro732Ala	-	not tolerated	benign /0.136	C0	1	0	1 CH	parent carrier	-	1 ; UV		[7]
c.2230G>A p.Gly7445er	-	not tolerated	prob /0.968	C0	2	0	4 CH	poss unaffected parent carrier	poss	0 or -1 ; benign		[26, 41]
c.2436A>T p.Lys812Asn	CD1 loop	not tolerated	prob /0.988	C65	3	2	4 CH	unaffected parent carrier	-	1 ; UV	minor	dbSNP, CS
c.2501C>T p.Ser834Phe	CD1 core	not tolerated	prob /0.998	C65	3	0	1 CH, 1 HH	de novo in affected parent	-	6 ; pathogenic	detrimental	[14,31]
c.2520G>C p.Trp840Cys	CD1 core	not tolerated	prob /0.999	C65	3	0	1 CH	de novo	-	6 ; pathogenic	detrimental	CS
c.2613G>T p.Glu871Asp	CD1 surface*	not tolerated	poss /0.560	C35	2	0	1 CH	de novo	-	5 ; pathogenic	undeterm	[28]
c.2680A>G p.Thr894Ala	CD2 surface	not tolerated	poss /0.850	C55	2.5	0	1 CH	parent carrier	-	2.5 ; UV	minor	[28]

c.2720A>C p.Lys907Thr	CD2 surface*	not tolerated	prob /0.999	C65	3	0	1 CH	U	-	3 ; UV	minor	28
c.2750C>T p.Thr917Met	CD2 core	not tolerated	prob /1.000	C65	3	0	1 CH	parent carrier	-	3 ; UV	minor	28
c.2813G>A p.Arg938Lys	CD2 linker surface*	not tolerated	benign /0.211	C25	1.5	0	1 CH	U	-	1.5 ; UV	minor	28
c.2824A>G p.Thr942Ala	CD2 linker loop surface	not tolerated /0.04	benign /0.021	C0	1	0	1 CH	parent carrier	-	1 ; UV	minor	CS
c.2831G>A p.Arg944His	CD2 linker loop surface	not tolerated	benign /0.185	C25	1.5	2	1 CH	parent carrier	-	-0.5 ; benign	minor	28, dbSNP
c.2840G>A p.Arg947Gln	CD2 linker loop surface	not tolerated	prob /0.986	C35	2.5	0	1 CH	parent carrier	-	2.5 ; UV	minor	28
c.2923G>A p.Gly975Arg	HD N core	not tolerated	prob /1.000	C65	3	0	1 CH	U	-	3 ; UV	detrimental	CS
c.2936T>C p.Leu979Pro	HD N core	not tolerated	prob /0.994	C65	3	0	1 CH	de novo	-	6 ; pathogenic	detrimental	42
c.2947T>G p.Trp983Gly	HD N core	not tolerated	prob /0.968	C65	3	0	1 CH	U	-	3 ; UV	detrimental	41
c.3005A>G p.Gln1002Arg	HD N core	not tolerated	prob /0.990	C35	2.5	0	1 CH	de novo	-	5.5 ; pathogenic	detrimental	28
c.3059T>C p.Leu1020Ser	HD N core	not tolerated	prob /0.991	C65	3	0	2 CH	de novo	-	6 ; pathogenic	detrimental	40, CS
c.3082A>G p.Ile1028Val	HD N core	not tolerated	prob /0.981	C25	2.5	0	7 CH	>1 de novo	-	6.5 ; pathogenic	undeterm	28,41,7/9/29 (same patient), CS
c.3089A>G p.Asn1030Ser	HD N ATP binding cleft	not tolerated	prob /0.995	C45	3	0	1 CH	de novo	-	6 ; pathogenic	ATP binding	30
c.3091T>C p.Trp1031Arg	HD N core	not tolerated	prob /0.999	C65	3	0	1 CH	de novo	-	6 ; pathogenic	detrimental	CS
c.3091T>G p.Trp1031Gly	HD N core	not tolerated	prob /0.998	C65	3	0	1 CH	de novo	-	6 ; pathogenic	detrimental	39
c.3242T>G p.Ile1081Ser	HD N core	not tolerated	prob /0.998	C65	3	0	1 CH	U	-	3 ; UV	detrimental	CS
c.3245C>A p.Thr1082Asn	HD N core	not tolerated	prob /0.999	C55	3	0	1 CH	de novo	-	6 ; pathogenic	detrimental	CS
c.3301T>C p.Cys1101Arg	HD N core	not tolerated	prob /0.998	C65	3	0	2 CH	>1 de novo	-	7 ; pathogenic	detrimental	29

c.3302G>A p.Cys1101Tyr	HD N core	not tolerated	prob /0.998	C65	3	0	1 CH	U	-	3 ; UV	detrimental	[7,29] (same patient)
c.3340A>T p.Asn1114Tyr	HD N loop surface	not tolerated	prob /0.999	C65	3	0	1 CH	U	-	3 ; UV	minor	[29]
c.3607G>C p.Glu1203Gln	HD C core	not tolerated	prob /0.998	C25	2.5	0	1 CH	U	-	2.5 ; UV	minor	[28]
c.3623T>A p.Val1208Asp	HD C core	not tolerated	prob /0.995	C65	3	0	1 CH	U	-	3 ; UV	detrimental	[28]
c.3641A>G p.Gln1214Arg	HD C core	not tolerated	prob /0.959	C35	2.5	0	4 CH	>1 de novo	-	6.5 ; pathogenic	detrimental	[39,41], CS
c.3751T>C p.Cys1251Arg	HD C core	not tolerated	prob /0.998	C65	3	0	1 CH	U	-	3 ; UV	detrimental	CS
c.3770T>G p.Leu1257Arg	HD C core	not tolerated	prob /0.997	C65	3	0	1 CH	de novo	-	6 ; pathogenic	detrimental	[7,9,29] (same patient)
c.3875T>C p.Leu1292Pro	HD C core	not tolerated	prob /0.997	C65	3	0	1 CH	de novo	-	6 ; pathogenic	detrimental	CS
c.3881T>C p.Leu1294Pro	HD C core	not tolerated	prob /0.997	C65	3	0	2 CH	de novo	-	6 ; pathogenic	detrimental	[28,39]
c.3905T>C p.Leu1302Pro	HD C core	not tolerated	prob /0.962	C65	3	0	1 CH	de novo	-	6 ; pathogenic	detrimental	[43]
c.3949C>T p.Arg1317Cys	HD C surface	not tolerated	prob /0.978	C65	3	0	1 CH	parent carrier	-	3 ; UV	undeterm	CS
c.3952T>C p.Cys1318Arg	HD C core	not tolerated	prob /0.951	C65	3	0	1 CH	de novo	-	6 ; pathogenic	detrimental	CS
c.3965T>C p.Leu1322Pro	HD C core	not tolerated	prob /0.976	C65	3	0	1 CH	U	-	3 ; UV	detrimental	[29]
c.3973T>G p.Tyr1325Asp	HD C core	not tolerated	prob /0.942	C65	3	0	1 CH	de novo	-	6 ; pathogenic	detrimental	[29]
c.4033C>T p.Arg1345Cys	HD C surface*	not tolerated	prob /0.999	C65	3	0	1 CH	parent carrier	-	3 ; UV	detrimental	[28]
c.4034G>A p.Arg1345His	HD C surface*	not tolerated	prob /0.999	C25	2.5	0	1 CH	de novo	-	5.5 ; pathogenic	detrimental	CS
c.4185G>C p.Gln1395His	HD C ATP binding cleft	not tolerated	prob /0.997	C15	2.5	0	2 CH	>1 de novo	-	6.5 ; pathogenic	ATP binding	[28,41]
c.4247C>G p.Thr1416Arg	HD C core	not tolerated	prob /0.965	C65	3	0	1 CH	U	-	3 ; UV	detrimental	[28]
c.4369A>C p.Lys1457Gln	HD C surface	not tolerated	prob /0.993	C45	3	0	1 CH	parent carrier	-	3 ; UV	minor	[28]
c.4406A>G p.Tyr1469Cys	-	not tolerated	prob /0.996	C65	3	0	1 CH	de novo	-	6 ; pathogenic		[7,29] (same patient)

c.4529C>G p.Ala1510Gly	-	not tolerated	poss /0.901	C55	2.5	0	1 CH	U	-	2.5 ; UV	[37]
c.4727T>G p.Phe1576Cys	-	not tolerated	benign /0.106	C65	2	0	1 CH	U	-	2 ; UV	[28]
c.4774C>T p.Arg1592Trp	-	not tolerated	prob /0.974	C65	3	0	1 CH	parent carrier	-	3 ; UV	[43]
c.4780C>T p.Pro1594Ser	-	not tolerated	poss /0.757	C65	2.5	0	1 CH	U	+	-0.5 ; benign	CS
c.4787A>G p.Asp1596Gly	-	not tolerated	prob /0.941	C65	3	0	3 CH	de novo	-	6 ; pathogenic	[26,41,7/29] (same patient)
c.4849G>A p.Gly1617Ser	-	not tolerated	prob /0.969	C55	3	0	1 CH	U	-	3 ; UV	[28]
c.4850G>A p.Gly1617Asp	-	not tolerated	prob /0.992	C65	3	0	2 CH	U	-	3 ; UV	CS
c.4856G>T p.Gly1619Val	-	not tolerated	prob /0.972	C65	3	0	1 CH	U	-	3 ; UV	CS
c.4929C>G p.Cys1643Trp	-	not tolerated	prob /0.992	C65	3	0	1 CH	U	-	3 ; UV	[41]
c.5015C>T p.Ala1672Val	-	not tolerated	benign /0.004	C65	2	1	0	U	-	2 ; UV	dbSNP
c.5050G>A p.Gly1684Ser	-	not tolerated	prob /0.969	C55	3	0	3 CH	>1 de novo	-	7 ; pathogenic	[27,34], CS
c.5216T>G p.Leu1739Arg	-	not tolerated	benign /0.463	C65	2	0	1 CH	de novo	-	5 ; pathogenic	[28]
c.5222G>C p.Arg1741Pro	-	not tolerated	prob /0.972	C65	3	0	1 CH	de novo	-	6; pathogenic	[29]
c.5225T>A p.Val1742Asp	-	not tolerated	prob /0.962	C65	3	0	1 CH	de novo	-	6 ; pathogenic	[43]
c.5234T>C p.Leu1745Pro	-	not tolerated	prob /0.960	C65	3	0	1 CH	de novo	-	6 ; pathogenic	[32]
c.5373C>A p.Asp1791Glu	-	not tolerated	prob /0.969	C35	2.5	0	1 CH	parent carrier	-	2.5 ; UV	[28]
c.5390G>T p.Gly1797Val	-	not tolerated	prob /0.998	C65	3	0	1 CH	de novo	-	6 ; pathogenic	CS
c.5402A>C p.His1801Pro	-	not tolerated	prob /0.935	C65	3	0	1 CH	de novo	-	6 ; pathogenic	[7/29] (same patient)
c.5405G>A p.Gly1802Asp	-	not tolerated	prob /0.990	C65	3	0	1 CH	de novo	-	6 ; pathogenic	[40]
c.5434G>C p.Asp1812His	-	not tolerated	prob /0.997	C65	3	0	1 CH	de novo	-	6 ; pathogenic	CS
c.5435A>G p.Asp1812Gly	-	not tolerated	prob /0.992	C65	3	0	1 CH	de novo	-	6 ; pathogenic	CS

c.5436C>A p.Asp1812Glu	-	not tolerated	prob /0.978	C35	2.5	0	1 CH	de novo	-	5.5 ; pathogenic	[7]
c.5444T>C p.Leu1815Pro	-	not tolerated	prob /0.984	C65	3	0	2 CH	de novo	-	6 ; pathogenic	[39], CS
c.5597A>G p.Asp1866Gly	-	not tolerated	poss /0.856	C0	1.5	0	3 CH	de novo	-	4.5 ; pathogenic	[28], CS
c.5848G>A p.Ala1950Thr	-	not tolerated	benign /0233	C55	2	0	1 CH	parent carrier	-	2 ; UV	[28]
c.5915C>G p.Ala1972Gly	SANT	not tolerated	poss /0.840	C55	2.5	0	1 CH	unaffected parent carrier	-	0.5 ; UV	CS
c.6184C>T p.Arg2062Trp	-	not tolerated	prob /0.990	C65	3	0	1 CH	U	+	0 ; benign	CS
c.6194G>A p.Arg2065His	-	not tolerated	prob /0.995	C25	2.5	0	2 CH	parent carrier	-	2.5 ; UV	[26]
c.6221T>C p.Leu2074Pro	-	not tolerated	prob /0.997	C65	3	0	1 KS/CH	affected sib carrier	-	4 ; pathogenic	[63], CS
c.6230G>A p.Arg2077Lys	-	not tolerated	poss /0.794	C25	2	0	1 CLP	parent carrier	-	2 ; UV	[32]
c.6250A>G p.Ser2084Gly	-	not tolerated	benign /0.032	C55	2	0	1 CH	U	-	2 ; UV	[28]
c.6271T>C p.Trp2091Arg	-	not tolerated	prob /0.997	C65	3	0	1 CH	de novo	-	6 ; pathogenic	CS
c.6287A>G p.His2096Arg	-	not tolerated	prob /0.982	C25	2.5	0	2 CH	de novo	-	5.5 ; pathogenic	[33,39]
c.6290A>G p.Asp2097Gly	-	not tolerated	prob /0.992	C65	3	0	1 CH	U	-	3 ; UV	CS
c.6304G>A p.Val2102Ile	-	tolerated/0.18	benign /0.005	C0	0	0	1 CH	U	-	0 ; benign	[32]
c.6308G>A p.Gly2103Asp	-	not tolerated	prob /0.992	C65	3	0	2 CH	U	-	3 ; UV	[26]
c.6322G>A p.Gly2108Arg	-	not tolerated	prob /0.999	C65	3	0	4 CH	>1 de novo	-	7 ; pathogenic	[37.1/29], (same patient), CS
c.6335C>T p.Thr2112Met	-	not tolerated	prob /0.998	C65	3	0	1 CLP	parent carrier	-	3 ; UV	[32]
c.6347T>A p.Ile2116Asn	-	not tolerated	prob /0.993	C65	3	0	1 KS/CH, 2 CH	>1 de novo	-	7 ; pathogenic	[20,36]
c.6352A>G p.Asn2118Asp	-	not tolerated	poss /0.560	C15	2	0	1 CH	2 family members carrier	-	0 ; benign	CS
c.6478G>A p.Ala2160Thr	-	tolerated/0.48	benign /0.003	C0	0	1	3 CH	poss unaffected parent carrier	poss, homo	-7 or -8 ; benign	[28,41], dbSNP

c.6673G>A p.Ala2225Thr	-	tolerated/0.33	benign /0	C0	0	0	1 CH	U	-	0 ; benign	[28]
c.6775G>A p.Ala2259Thr	-	not tolerated /0.02	benign /0.001	C0	1	0	1 CH	parent carrier	-	1 ; UV	CS
c.6843T>G p.Asp2281Glu	-	not tolerated	benign /0.106	C35	1.5	0	1 CH	poss unaffected parent carrier	poss	-0.5 or -1.5 ; benign	[41]
c.6857G>C p.Gly2286Ala	-	not tolerated	benign /0.095	C55	2	0	1 CH	de novo	-	5 ; pathogenic	CS
c.6935A>C p.Lys2312Thr	-	not tolerated	poss /0.826	C65	2.5	0	1 CH	U	-	2.5 ; UV	CS
c.6955C>A p.Arg2319Ser	-	not tolerated	poss /0.794	C65	2.5	0	1 CH	affected parent carrier	-	3.5 ; UV	[39]
c.6955C>T p.Arg2319Cys	-	not tolerated	prob /0.930	C65	3	0	6 CH	affected parent and sib carrier	-	4 ; pathogenic	[7,28,32,33,38,41]
c.6989G>C p.Gly2330Ala	-	tolerated/0.09	prob /0.935	C0	1	6	1 CH	U	-	-1 ; benign	[28], dbSNP
c.7097T>G p.Leu2366Arg	-	not tolerated	prob /0.923	C65	3	0	1 CH	U	-	3 ; UV	CS
c.7243G>T p.Ala2415Ser	-	not tolerated	poss /0.645	C65	2.5	2	0	U	-	0.5 ; UV	dbSNP
c.7390A>G p.Lys2464Glu	-	not tolerated	benign /0.015	C55	2	0	1 CH	parent carrier	-	2 ; UV	CS
c.7463G>A p.Gly2488Asp	-	not tolerated	poss /0.611	C65	2.5	0	1 CH	2 family members carrier	-	0.5 ; UV	CS
c.7471C>T p.Arg2491Cys	-	not tolerated	benign /0	C65	2	0	1 CH	U	+	-1 ; benign	CS
c.7485G>T p.Arg2495Ser	-	not tolerated	benign /0.108	C65	2	0	1 CH	parent carrier	-	2 ; UV	[28]
c.7579A>C p.Met2527Leu	-	tolerated/0.15	benign /0.064	C0	0	0	7 CH	parent carrier	-	0 ; benign	[28],CS
c.7958G>A p.Arg2653Gln	BRK2	not tolerated	prob /0.946	C35	2.5	0	1 CH	parent carrier	+	-0.5 ; benign	CS
c.8047C>T p.Pro2683Ser	BRK2	not tolerated	prob /0.985	C0	2	0	1 CH	parent carrier	-	2 ; UV	[28]
c.8104C>T p.Arg2702Cys	-	not tolerated	prob /0.951	C65	3	0	1 CH	parent carrier	-	3 ; UV	[28]
c.8173A>G p.Ile2725Val	-	not tolerated	benign /0.421	C0	1	1	0	U	-	1 ; UV	dbSNP
c.8197G>A p.Ala2733Thr	-	not tolerated	poss /0.824	C0	1.5	0	1 CH	U	-	1.5 ; UV	[28]

c.8250T>G p.Phe2750Leu	-	not tolerated	poss /0.623	C15	2	18	0	U	-	0 ; benign	dbSNP
c.8339C>T p.Ala2780Val	-	not tolerated	benign /0.095	C65	2	0	1 CH	3 family members carrier	-	0; benign	CS
c.8365G>A p.Ala2789Thr	-	tolerated/0.26	benign /0.082	C0	0	0	1 HH	U	-	0 ; benign	[14]
c.8416C>G p.Leu2806Val	-	not tolerated	poss /0.726	C25	2	0	5 CH	poss unaffected parent carrier	poss	0 or -1 ; benign	[28,41], CS
c.8569T>G p.Ser2857Ala	-	tolerated/0.13	benign /0.014	C0	0	0	1 CH	U	-	0 ; benign	[28]
c.8639C>T p.Pro2880Leu	-	not tolerated /0.02	benign /0.015	C15	1.5	0	1 HH	U	-	1.5 ; UV	[14]
c.8791G>A p.Val2931Met	-	not tolerated	benign /0.342	C0	1	0	1 CH	parent carrier	-	1 ; UV	[28]
c.8842A>G p.Lys2948Glu	-	not tolerated /0.05	benign /0.004	C0	1	0	1 KS	U	-	1 ; UV	[16]
c.8950C>T p.Leu2984Phe	-	not tolerated	benign /0.314	C0	1	0	4 CH	unaffected parent carrier	+	-4 ; benign	[28,7,29] (same patient)

Gray rows, possible effect on splicing

[a] -, not in domain; CD, chromodomain; *, interface between chromo- and helicase domain; HD N, helicase domain N-lobe; HD C, helicase domain C-lobe

[b] SIFT output: tolerated, SIFT score ≥0.05; not tolerated, SIFT score <0.05; 'not tolerated' is 0, unless otherwise indicated

[c] Polymorphism Phenotyping (PolyPhen) output: benign; poss, possibly damaging; prob, probably damaging; naïve Bayes posterior probability that a certain variant is damaging

[d] summed score of computational algorithms (Table 1)

[e] # controls, number of controls carrying the missense variant

[f] # index patients, number of index patients carrying the missense variant; CH, CHARGE syndrome; KS, Kallmann syndrome; HH, hypogonadotropic hypogonadism; SC/C, scoliosis or control (variants published by Gao *et al*[35]); CLP, cleft lip and/or palate

[g] U, unknown; parent carrier, unknown whether the carrier parent has features of CHARGE syndrome; poss unaffected parent carrier, the missense variant was found in the unaffected parent OR the missense variant was found in combination with a pathogenic *CHD7* mutation in the index patient (variants published by Vuorela *et al*[41])

[h] -, no other *CHD7* mutation found in the index patient; +, missense variant found in combination with a clearly pathogenic *CHD7* mutation in the index patient; homo, missense variant found in the homozygous state

[i] total summed score and classification according to our classification system (Table 1)

[j] undeterm, undetermined

[k] CS, current study (variant was not previously published); dbSNP, variant present in NCBI SNP database

Supplementary Table S2. Template structures that were used for homology modeling of the CHD7 chromo- and helicase domains

Template structure	Domain	PDB ID	%Seq ID	Resolution (Å)	Reference
Human CHD6 chromodomain 2	CD2	2EPB	68%	NMR	10.2210/pdb2epb/pdb
Yeast Chd1 tandem chromodomains	CD1&2	2H1E	26%	2.2	[51]
Human CHD1 tandem chromodomains	CD1&2	2B2T	28%	2.45	[50]
Yeast Chd1 chromodomain	CD2	2DY8	36%	NMR	[53]
Human CHD4 chromodomain	CD2	2EE1	32%	NMR	10.2210/pdb2ee1/pdb
Zebrafish Rad54 SWI2/SNF2 chromatin-remodeling domain	helicase	1Z3I	30%	3	[54]
Sulfolobus solfataricus SWI2/SNF2 ATPase domain	helicase	1Z6A	29%	3	[49]
Yeast Chd1 chromatin remodeler	CD1&2, helicase	3MWY	39%	3.7	[52]

CD, chromodomain; %Seq ID, percentage sequence identity; NMR, nuclear magnetic resonance; PDB, protein database

CHAPTER 2.3

Exon copy number alterations of the *CHD7* gene are not a major cause of CHARGE and CHARGE-like syndrome

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ABSTRACT

CHARGE syndrome is a multiple congenital anomaly syndrome caused by mutations in the *CHD7* gene. Mutations in this gene are found in 60-70% of patients suspected of having CHARGE syndrome. However, if only typical CHARGE patients are taken into account, mutations in the *CHD7* gene are found in over 90% of cases. The remaining 10% might be caused by hitherto undetected alterations of the *CHD7* gene, including whole exon duplications and deletions that are missed by the currently used diagnostic procedures. Therefore we looked for these kinds of alterations by multiplex ligation-dependent probe amplification in 54 patients suspected of having CHARGE syndrome without a *CHD7* mutation. In one patient a partial deletion of the *CHD7* gene (exon 13-38) was identified, while in the other patients no abnormalities were found. The frequency of exon deletions in our cohort was 1.9% (1/54) and 5.6% (1/18) in all patients and in typical CHARGE patients, respectively. We conclude that exon copy number alterations of the *CHD7* gene are not a major cause of CHARGE and CHARGE-like syndrome.

INTRODUCTION

In 2004 the underlying gene defect for CHARGE syndrome (the *CHD7* gene, OMIM #214800) was identified on chromosome 8 (8q12.1).[1] This multiple congenital anomaly syndrome was originally described as a combination of coloboma, heart defects, atresia of choanae, retardation of growth and/or development, genital hypoplasia and ear anomalies and/or deafness.[2] Later, additional congenital malformations were recognised, of which agenesis of the semicircular canals and arhinencephaly were found to be present in nearly all patients.[3-5] The prevalence is approximately 1 in 10,000. CHARGE syndrome is diagnosed on clinical grounds according to two different sets of criteria as proposed by Blake *et al*[6] and Verloes,[7] and/or analysis of the *CHD7* (chromodomain helicase DNA binding protein 7) gene. Three large, independent studies found heterozygous mutations in the *CHD7* gene in 60-70% of patients suspected of having CHARGE syndrome.[8-10] However, if the clinical criteria for CHARGE syndrome[6,7] are strictly applied, mutations in *CHD7* are present in over 90% of the typical CHARGE patients.

The two sets of criteria used for diagnosing CHARGE syndrome, consist of phenotypic features that do not fully overlap (Table 1). Blake *et al* consider a patient as having typical CHARGE syndrome if four major, or three major and three minor, criteria are present.[6] Based on new diagnostic insights, Verloes refined the criteria and included abnormalities of the semicircular canals.[7] Besides, Verloes distinguished three categories of CHARGE syndrome patients: typical, partial and atypical. Verloes considered typical CHARGE patients to present with three major, or two major and two minor, criteria. Patients were considered to have partial CHARGE syndrome when two major and one minor criteria were present, while atypical CHARGE patients have two major and no minor criteria, or one major and three minor criteria.

Most of the *CHD7* mutations that have been described in CHARGE syndrome are truncating (nonsense and frameshift), but missense mutations have also been found. Most mutations are unique, although some recurrent *de novo* mutations have been found. There seem to be no real mutation hot spots, but some exons (2, 3, 31 and 34) are more frequently mutated than others (own unpublished data). The higher mutation frequency of these exons appears to be related to their size, with the largest exon 2 most frequently mutated. Until now, only one intragenic deletion has been reported in a CHARGE patient.[11] Whole gene deletions of *CHD7*, although present in the two patients who contributed to the discovery of the *CHD7* gene,[1] were not found in two large cohorts of CHARGE patients.[9,10] However, single exon deletions or duplications would have been missed with the techniques used in these studies. Since the underlying defect in the remaining 10% of typical CHARGE patients has not yet been discovered, we hypothesised that exon copy number alterations of the *CHD7* gene might contribute to CHARGE syndrome. We identified a cohort of 54 patients suspected of having CHARGE syndrome who did not have a *CHD7* mutation. We screened for single exon deletions or duplications in the *CHD7* gene using multiplex ligation-dependent probe amplification (MLPA).

Table 1. Clinical criteria for identifying CHARGE syndrome patients

	Major criteria	Minor criteria	Inclusion rule
Blake *et al*[6]	1. Ocular coloboma or microphthalmia 2. Choanal atresia or stenosis 3. Characteristic external ear anomaly, or middle ear malformations or mixed deafness 4. Cranial nerve dysfunction	1. Congenital cardiovascular malformations 2. Tracheo-oesophageal defect 3. Genital hypoplasia or delayed pubertal development 4. Cleft lip and/or palate 5. Developmental delay 6. Growth retardation 7. Characteristic face	**Typical CHARGE**: 4 majors, OR 3 majors + 3 minors
Verloes[7]	1. Ocular coloboma 2. Choanal atresia or stenosis 3. Hypoplasia of semicircular canals	1. Heart or oesophagus malformation 2. Malformation of the inner or external ear 3. Rhombencephalic dysfunction including sensorineural deafness 4. Hypothalamo-hypophyseal dysfunction 5. Mental retardation	**Typical CHARGE**: 3 majors, OR 2 majors + 2 minors **Partial CHARGE**: 2 majors + 1 minor **Atypical CHARGE**: 2 majors, but no minors, OR 1 major + 3 minors

MATERIALS AND METHODS

Fifty-four patients were selected from a group of patients who were referred for mutation analysis of *CHD7* because of clinical features suggestive of CHARGE syndrome. Mutation screening performed by polymerase chain reaction (PCR) followed by direct sequencing had not revealed any *CHD7* alterations in these patients.[9] The DNA samples were subsequently screened for exon deletions and/or duplications of the *CHD7* gene by MLPA analysis. Half of the patients (see Table 2) were analysed using home-designed synthetic oligonucleotides, covering all the coding exons of the *CHD7* gene in four probe sets as described before.[12] The other patients were screened with a commercially available set of probes, the SALSA P201 kit (MRC-Holland, Amsterdam, the Netherlands; http://www.mrc-holland.com). This set includes the usual control probes located on different chromosomes, together with probes for most exons of the *CHD7* gene (27 of 38 exons). When exons were located closely together, one representative exon was chosen for the region. Because probes for both the first non-coding exon (exon 1) and the last exon (exon 38, containing the stopcodon) were included, both kits would also have detected whole gene deletions.

The MLPA analysis as well as the statistical analysis was performed as described previously.[12] A patient with a known heterozygous deletion of the whole gene was used as a positive control and we included two negative controls in each analysis.

Clinical information on the 54 index patients was obtained from our investigations at the outpatient clinic for children with CHARGE syndrome or through the referring clinicians, by means

of a written questionnaire submitted prior to DNA analysis. Additional information was requested when necessary. We scored patients for CHARGE features according to the two sets of criteria (Blake *et al*[6] and Verloes[7]) as summarised in Table 1. Patients were classified as typical CHARGE syndrome when they fulfilled the criteria for typical CHARGE of at least one of the two scoring sets.

RESULTS

In our cohort of 54 patients we found a deletion of exon 13-38 in patient no. 1 (Figure 1). In all other patients no exon copy number changes in the *CHD7* gene were found.

An overview of the clinical features of patient no. 1 is given in Table 2 (see also Figure 2). The patient was born at 42 weeks of gestation with a birth weight of 3.685 kg. At birth right facial nerve palsy and dysmorphic ears were noted. In addition he had partial palsy of cranial nerves IX and X, for which he required tube feeding for one year. Choroid colobomas, hypoplasia of the pons and vermis of the cerebellum, severe bilateral mixed hearing loss, undescended testes and micropenis were present as well. At age 12 years, his height was 141.5 cm (-2 SD). He had a square and asymmetric face and proved to be anosmic by the University of Pennsylvania Smell Identification Test.[13] Unfortunately, imaging of the semicircular canals was not performed, but vestibular dysfunction was noted on physical examination.

Our cohort proved to be phenotypically heterogeneous, varying from typical CHARGE patients to partial and atypical patients and patients only suspected of having CHARGE syndrome. Table 2 gives an overview of their clinical features and scores according to Blake *et al*'s and Verloes' criteria. Eighteen patients had typical CHARGE syndrome (according to Blake *et al* (n=5), Verloes (n=7) or both (n=6)). Four patients had partial CHARGE, 26 had atypical CHARGE syndrome according to Verloes and six were suspected of having CHARGE syndrome but did not satisfy either set of criteria.

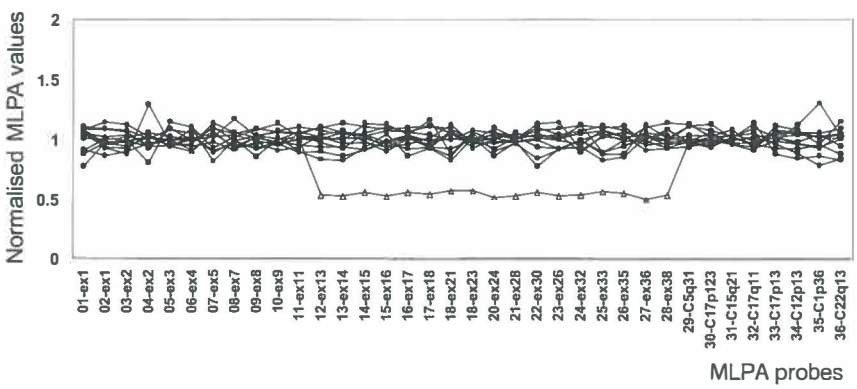

Figure 1. Example of an MLPA analysis (SALSA P201 kit) with 13 different DNA samples from (suspected) CHARGE patients. On the X-axis the probes of the different exons are named (01- till 28-, representing exon 1 till 38 of the *CHD7* gene, and 29- till 36-, control probes that have two genomic copies in the normal population). On the Y-axis are the normalised values (see Koolen *et al* for details[12]), 1 means that two copies are present, 0.5 means that only one copy is present. The sample marked with triangles is patient no. 1 with the partial deletion of the *CHD7* gene involving exon 13 till 38.

Table 2. Clinical features of 54 patients suspected of having CHARGE syndrome but with no mutation in the *CHD7* gene

Case	Sex[a]	Age[b]	Diagnostic criteria		Major criteria					Minor criteria[h]
		yr	Blake	Verloes[c]	Eye anomaly[d]	Atresia of choanae[e]	Cranial nerve dysfunction[f]	Ear anomaly	SCC[g]	
1	**M**	**12**	-	**+**	**C**	-	**VII, VIII, IX, X**	**+**	**+**	**AN, B, CF, GH**
2	M	2	+	+	C, Mi	- (CLP)	VIII	+	+	MR, S
3 *	F	10	+	+	C	S	VIII	-	-	H, MR, RG, S
4 *	F	21	+	+	C	A	VII, VIII	-	+	B, H, HHD, GH, MR, R
5 *	M	2 d	+	+	C, Mi	- (CLP)	U	+	U	CF, H, S, TE
6	F	3	+	+	C	S	VIII	+	U	MR
7	F	1	+	+	-	- (CP)	VIII	+	+	CF, H, RG
B	M	10	+	a	C	-	VIII	+	U	B, CF, GH, MR
9	F	7	+	a	Mi	A	VIII	+	U	B, CF, MR, S
10 *	F	14	+	a	-	A	VIII	+	U	H, MR, RG, S
11 *	M	3	+	a	-	A (CP)	VIII	+	U	B, H, MR
12 *	F	4	+	a	C	-	VI, VII, VIII, IX, XII	+	U	CF, GH, H, MR
13 *	F	29	-	+	C, Mi	A	-	+	U	MR
14 *	F	6	-	+	C	-	-	+	+	H, MR, R, S
15	M	1	-	+	C	A	U	+	U	H
16	F	2B	-	+	C, Mi	A	VIII	U	U	H, MR, S
17	F	½	-	+	C, Mi	- (CP)	U	+	U	TE
18 *	M	3	-	+	C, Mi	- (CP)	VIII	-	U	GH, MR
19	M	7	-	p	C, Mi	- (CP)	-	+	U	
20	F	4	-	p	Mi	S	VIII	-	+	B
21	F	16	-	p	C, Mi	- (CP)	U	U	U	H, RG
22	F	13	-	p	C, Mi	A	U	U	U	MR, RG
23 *	M	46	-	a	C	-	VIII	+	U	MR
24 *	M	1	-	a	-	A	-	+	U	B, GH, H, R, S
25 *	F	3	-	a	-	A	VIII	+	U	B, CF, MR
26 *	M	½	-	a	-	A	VIII	+	U	H, S
27 *	M	2	-	a	C	-	-	+	U	H, MR, RG
28 *	M	36	-	a	C	-	VIII	-	U	B, GH, H, HHD
29 *	F	11	-	a	C	-	-	-	U	H, MR, R, S
30	M	39	-	a	C, Mi	-	-	-	U	H, MR, RG, S
31	M	11 d	-	a	-	A	-	+	U	B, CF, H
32 *	F	2	-	a	Mi	- (CLP)	-	+	U	B, H
33 *	F	12	-	a	C, Mi	-	-	+	U	B, MR, RG
34 *	M	¼	-	a	-	A	-	+	U	B, H, R
35 *	F	29	-	a	-	A	VII, VIII	-	-	S, TE
36	F	4	-	a	-	A	-	+	U	B, MR, RG
37 *	M	2	-	a	C	-	-	-	-	H, MR

38 *	F	3	-	a	-	A	-	-	-	H, MR
39	M	4	-	a	-	A	-	+	U	H, MR, RG
40	M	3	-	a	C, Mi	-	VIII	-	U	B, CF, H, R, RG
41	F	5	-	a	C, Mi	-	VIII	-	U	GH, H, MR, S
42	F	1	-	a	C	-	VIII	-	U	H, MR, S
43	F	11	-	a	C, Mi	-	-	+	U	GH, H, R, RG
44	F	8	-	a	C	-	-	-	U	H, MR
45	F	3	-	a	-	A	VIII	+	-	H, MR, S
46	F	4	-	a	-	A (CP)	-	+	U	CF, H, MR, S
47	F	11		a	C	-	-	+	U	MR, RG
48	F	3	-	a	-	- (CP)	-	+	U	H
49 *	F	22	-	-	-	-	VIII	+	U	H, HHD, S
50 *	F	6	-	-	-	-	VII, VIII	+	-	B, H, MR, TE
51 *	M	8	-	-	C, Mi	-	-	-	U	H, R, RG
52 *	M	9	-	-	-	A	-	-	U	H
53	F	10	-	-	-	-	VIII	+	U	CF, MR, R, S, TE
54	M	4	-	-	-	- (CLP)	-	-	U	H, R

The patient with the partial *CHD7* deletion is depicted in bold.

*The patients that were screened with home-designed synthetic oligonucleotides are indicated with an asterisk. The other patients were analysed with the SALSA P201 MLPA kit.

[a] F, female; M, male. [b] d, day; yr, year. [c] a, atypical; p, partial. [d] C, coloboma; Mi, microphthalmia. [e] A, atresia of choanae; CLP, cleft lip and palate; CP, cleft palate; S, stenosis of choanae. Presence of CLP or CP was counted as a major criterion, because this rarely occurs together with atresia of choanae. [f] U, unknown. [g] SCC, semicircular canal hypoplasia or vestibular dysfunction [h] AN, anosmia; B, brain abnormalities; CF, characteristic face; H, heart defect; GH, genital hypoplasia; HHD, hypothalamo-hypophyseal dysfunction; MR, mental retardation; R, renal abnormality; RG, retarded growth; S, skeletal abnormality; TE, tracheo-oesophageal abnormalities.

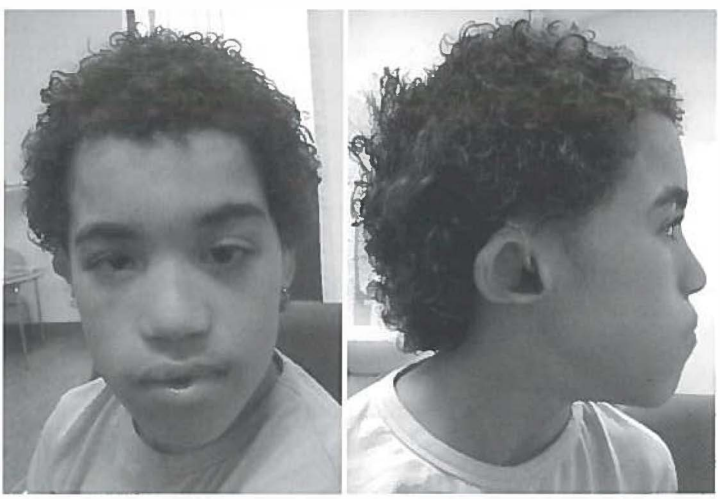

Figure 2. Photographs of patient no. 1 with a partial deletion of the *CHD7* gene at 12 years and 3 months of age. Note square face with facial palsy and microcornea (both right-sided), dysplastic ear with typical triangular concha and absence of the ear lobe and a flat midface.

DISCUSSION

A defect in the *CHD7* gene is found in 60-70% of all patients suspected of having CHARGE syndrome and in over 90% of typical CHARGE patients.[9] In the remaining 10% of typical patients the cause of CHARGE syndrome remains elusive. Genetic heterogeneity could be present, but the only other gene that has been implicated in CHARGE syndrome, the *SEMA3E* gene, was found to be mutated in only one CHARGE patient.[14] So far, mutations in this gene have not been reported in other CHARGE patients. It seems therefore more plausible to assume that *CHD7* is the major causative gene, considering the small percentage of typical patients in whom no mutation is found.

CHARGE patients normally undergo only a routine sequence analysis, which would miss any mutations located deep in the introns or in the promotor region of the *CHD7* gene. In addition, whole exon deletions or duplications would not be detected. Hitherto, only three CHARGE patients have been described with a genomic rearrangement leading to a deletion of the whole *CHD7* gene.[1,15,16] Whole gene duplications are not likely to cause CHARGE syndrome. Based on the mutations found so far (predominantly leading to a truncated protein) and on the presumed function of *CHD7*, the identified mutations most likely have a loss-of-function effect. This, however, does not rule out single exon duplications, which would result in a distortion of the reading frame, as a cause of CHARGE syndrome.

So far no intragenic duplications and only one patient with an intragenic deletion of exons 8-12 has been reported.[11] The described intragenic deletion was detected by multiplex PCR/liquid chromatography assay and appeared to have arisen through an *Alu*-mediated replacement event. This patient was one of a cohort of 13 typical CHARGE patients studied by Udaka *et al*, which leads to an exon deletion frequency of 7.7% (1/13). Another study, by Vuorela *et al*, did not find *CHD7* deletions with quantitative real-time PCR and MLPA analysis in 44 *CHD7* mutation negative patients.[17] Unfortunately they did not supply the clinical characteristics of their patients and therefore it is not known how many typical CHARGE patients were included in their cohort.

In our cohort of 54 patients suspected of having CHARGE syndrome without *CHD7* alterations on routine sequencing, we found one patient with a deletion of exons 13-38. In the other patients no exon copy number changes of the *CHD7* gene were found by MLPA analysis. Our patient with the partial *CHD7* deletion could clinically not be distinguished from the patients with normal MLPA results. One of the used MLPA kits (SALSA P201) did not cover all exons of the *CHD7* gene directly. However, exons missing in this MLPA kit are located close to exons that are represented in the kit. Very small deletions or duplications (within exons) will be missed using MLPA analysis in general, but bigger copy number variations are detectable with both MLPA kits.

The frequency of exon deletions in our cohort was 1.9% (1/54) and 5.6% (1/18) in all patients and in typical CHARGE patients, respectively. The latter figure is in accordance with the finding of Udaka *et al*. However, it should be noted that the classification of our CHARGE patients is based on the available clinical information. Unfortunately in 42 patients imaging of the semicircular canals has not been performed. This means that the 25 patients who satisfied the Verloes' criteria for

atypical or partial CHARGE could in theory have typical CHARGE in the presence of semicircular canal anomalies. If we take this precaution into account, the frequency of partial *CHD7* deletions in typical CHARGE patients without a mutation in *CHD7* is 2.3-5.6% (1/43-1/18) according to our study.

Since we did not detect any exon copy number alterations in the 36 non-typical patients, it seems that MLPA analysis of the *CHD7* gene does not significantly improve the mutation analysis for this group of patients. In typical CHARGE patients however, we do recommend MLPA analysis of the *CHD7* gene, even though the frequency of partial *CHD7* deletions is low. A thorough clinical work-up is essential (e.g. imaging of semicircular canals) in order to classify patients as typical or non-typical CHARGE patients.

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REFERENCES

1 Vissers LE, van Ravenswaaij CM, Admiraal R, Hurst JA, De Vries BB, Janssen IM, et al. Mutations in a new member of the chromodomain gene family cause CHARGE syndrome. *Nat Genet* 2004;36(9):955-957.

2 Pagon RA, Graham JM,Jr., Zonana J, Yong SL. Coloboma, congenital heart disease, and choanal atresia with multiple anomalies: CHARGE association. *J Pediatr* 1981;99(2):223-227.

3 Admiraal RJ, Joosten FB, Huygen PL. Temporal bone CT findings in the CHARGE association. *Int J Pediatr Otorhinolaryngol* 1998;45(2):151-162.

4 Amiel J, Attiee-Bitach T, Marianowski R, Cormier-Daire V, Abadie V, Bonnet D, et al. Temporal bone anomaly proposed as a major criteria for diagnosis of CHARGE syndrome. *Am J Med Genet* 2001;99(2):124-127.

5 Sanlaville D, Etchevers HC, Gonzales M, Martinovic J, Clement-Ziza M, Delezoide AL, et al. Phenotypic spectrum of CHARGE syndrome in fetuses with *CHD7* truncating mutations correlates with expression during human development. *J Med Genet* 2006;43(3):211-217.

6 Blake KD, Davenport SL, Hall BD, Hefner MA, Pagon RA, Williams MS, et al. CHARGE association: an update and review for the primary pediatrician. *Clin Pediatr (Phila)* 1998;37(3):159-173.

7 Verloes A. Updated diagnostic criteria for CHARGE syndrome: a proposal. *Am J Med Genet A* 2005;133(3):306-308.

8 Aramaki M, Udaka T, Kosaki R, Makita Y, Okamoto N, Yoshihashi H, et al. Phenotypic spectrum of CHARGE syndrome with *CHD7* mutations. *J Pediatr* 2006;148(3):410-414.

9 Jongmans MC, Admiraal RJ, van der Donk KP, Vissers LE, Baas AF, Kapusta L, et al. CHARGE syndrome: the phenotypic spectrum of mutations in the *CHD7* gene. *J Med Genet* 2006;43(4):306-314.

10 Lalani SR, Safiullah AM, Fernbach SD, Harutyunyan KG, Thaller C, Peterson LE, et al. Spectrum of *CHD7* mutations in 110 individuals with CHARGE syndrome and genotype-phenotype correlation. *Am J Hum Genet* 2006;78(2):303-314.

11 Udaka T, Okamoto N, Aramaki M, Torii C, Kosaki R, Hosokai N, et al. An Alu retrotransposition-mediated deletion of *CHD7* in a patient with CHARGE syndrome. *Am J Med Genet A* 2007;143(7):721-726.

12 Koolen DA, Nillesen WM, Versteeg MH, Merkx GF, Knoers NV, Kets M, et al. Screening for subtelomeric

rearrangements in 210 patients with unexplained mental retardation using multiplex ligation dependent probe amplification (MLPA). *J Med Genet* 2004;41(12):892-899.

13 Doty RL, Shaman P, Dann M. Development of the University of Pennsylvania Smell Identification Test: a standardized microencapsulated test of olfactory function. *Physiol Behav* 1984;32(3):489-502.

14 Lalani SR, Safiullah AM, Molinari LM, Fernbach SD, Martin DM, Belmont JW. *SEMA3E* mutation in a patient with CHARGE syndrome. *J Med Genet* 2004;41(7):e94.

15 Arrington CB, Cowley BC, Nightingale DR, Zhou H, Brothman AR, Viskochil DH. Interstitial deletion 8q11.2-q13 with congenital anomalies of CHARGE association. *Am J Med Genet A* 2005;133(3):326-330.

16 Hurst JA, Meinecke P, Baraitser M. Balanced t(6;8)(6p8p;6q8q) and the CHARGE association. *J Med Genet* 1991;28(1):54-55.

17 Vuorela P, la-Mello S, Saloranta C, Penttinen M, Poyhonen M, Huoponen K, et al. Molecular analysis of the *CHD7* gene in CHARGE syndrome: identification of 22 novel mutations and evidence for a low contribution of large *CHD7* deletions. *Genet Med* 2007;9(10):690-694.

CHAPTER 3.1

Study of smell and reproductive organs in a mouse model for CHARGE syndrome

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ABSTRACT

CHARGE syndrome is a multiple congenital anomaly syndrome characterised by coloboma, heart defects, atresia of choanae, retardation of growth and/or development, genital hypoplasia, and ear anomalies often associated with deafness. It is caused by heterozygous mutations in the *CHD7* gene and shows a highly variable phenotype. Anosmia and hypogonadotropic hypogonadism occur in the majority of CHARGE patients, but the underlying pathogenesis is unknown. Therefore, we studied the ability to smell and aspects of the reproductive system (reproductive performance, gonadotropin-releasing hormone (GnRH) neurons and anatomy of testes and uteri) in a mouse model for CHARGE syndrome, the whirligig mouse (*Chd7*$^{Whi/+}$). We showed that chromodomain helicase DNA-binding protein 7 (Chd7) is expressed in brain areas involved in olfaction and reproduction during embryonic development. We observed poorer performance in the smell test in adult *Chd7*$^{Whi/+}$ mice, secondary either to olfactory dysfunction or to balance disturbances. Olfactory bulb and reproductive organ abnormalities were observed in a proportion of *Chd7*$^{Whi/+}$ mice. Hypothalamic GnRH neurons were slightly reduced in *Chd7*$^{Whi/+}$ females and reproductive performance was slightly less in *Chd7*$^{Whi/+}$ mice. This study shows that the penetrance of anosmia and hypogonadotropic hypogonadism is lower in *Chd7*$^{Whi/+}$ mice than in CHARGE patients. Interestingly, many phenotypic features of the *Chd7* mutation showed incomplete penetrance in our model mice, despite the use of inbred, genetically identical mice. This supports the theory that the extreme variability of the CHARGE phenotype in both humans and mice might be attributed to variations in the foetal microenvironment or to purely stochastic events.

INTRODUCTION

CHARGE syndrome is a multiple congenital anomaly syndrome with variable occurrence of coloboma, heart defects, atresia of choanae, retardation of growth and/or development, genital hypoplasia, ear abnormalities and deafness.[1] More recently, semicircular canal hypoplasia and anosmia due to olfactory bulb aplasia were found to be part of the CHARGE spectrum.[2-5] Since the discovery that heterozygous mutations in the *CHD7* gene (OMIM *608892) are the major cause of CHARGE syndrome, the phenotypic spectrum has broadened.[6,7] Some very mild cases of CHARGE syndrome have been identified, some of which were originally diagnosed as Kallmann syndrome.[8] A recent study claimed that Kallmann syndrome might be a mild allelic variant of CHARGE syndrome.[9] However, it might also be that *CHD7* mutations can have a very mild presentation mimicking Kallmann syndrome.[8] Kallmann syndrome and CHARGE syndrome share many features, including not only anosmia and hypogonadotropic hypogonadism (owing to gonadotropin-releasing hormone (GnRH) deficiency), but also hearing impairment, cleft lip/palate and renal agenesis.[8] The underlying cause of this phenotypic overlap is uncertain. It might be that chromodomain helicase DNA-binding protein 7 (CHD7), a putative chromatin remodelling protein, could affect expression of one or several Kallmann syndrome genes (e.g. *KAL1*, *FGFR1*, *PROK2*, *PROKR2* and *FGF8*).[10] If suitable mouse models are available, mouse studies can provide insights into the underlying pathogenic pathways of anosmia and hypogonadotropic hypogonadism in CHARGE syndrome.

The whirligig mouse (*Chd7*$^{Whi/+}$) carries an ENU (*N*-ethyl-*N*-nitroso-urea)-induced nonsense mutation in the *Chd7* gene and shows a phenotype that is very similar to that of humans with CHARGE syndrome.[11] Features that are present in both mouse and human include fully penetrant semicircular canal defects and occasional heart defects, choanal atresia, cleft palate and eye defects.[11] However, olfaction and reproductive organs have not been extensively studied so far.

We performed a smell test and analysed olfactory bulb and reproductive organ anatomy in *Chd7*$^{Whi/+}$ mice. We also studied GnRH neurons in the hypothalamus of embryonic and adult *Chd7*$^{Whi/+}$ mice and analysed the expression of Chd7 during olfactory and GnRH neuron development. The overlap between CHARGE and Kallmann syndromes was further explored by crossing *Chd7*$^{Whi/+}$ mice with *Fgfr1*$^{Hspy/+}$ mice. *Fgfr1*$^{Hspy/+}$ mice have a missense mutation (W691R) in the conserved kinase domain of the *Fgfr1* gene (one of the genes involved in Kallmann syndrome).[12] This missense mutation causes a loss-of-receptor-function (Calvert J, Dedos S, Hawker K and Steel KP, manuscript in preparation).

METHODS

Mice and genotyping

Mutant mice were maintained as heterozygote by wild-type matings on a C3HeB/FeJ background.[11-13] Additional matings were set up between *Fgfr1*$^{Hspy/+}$ females and *Chd7*$^{Whi/+}$ males to create double heterozygous (*Hspy*/+;*Whi*/+) mice. The mouse housing and experiments complied with UK Home Office requirements.

Genomic DNA from ear clips was purified[14] and genotyping of *Fgfr1*$^{Hspy/+}$ (supplementary methods) and *Chd7*$^{Whi/+}$ mice was performed.[11] Wild-type littermates were used as controls.

Olfaction test

In the olfaction tests, we determined the ability of wild-type C3HeB/FeJ mice to discriminate between urine from various mouse strains (adapted from Brown *et al*[15] and Lee *et al*[16]). Urine samples from five adult BALB/c and five adult 129/S5 male mice was pooled, diluted with distilled water ([10^{-2}], [10^{-3}] and [10^{-4}]), aliquoted and frozen at -80°C until use. C3HeB/FeJ mice (8 males and 5 females) aged between 8 and 12 weeks were used for the habituation-dishabituation experiment. Clean plastic mouse cages (~ 31 x 12 x 13 cm) with a gridded lid were placed in a separate room away from the colony. The experiments were conducted in normal light, as C3HeB/FeJ mice are blind because of retinal degeneration from age three weeks onwards.[17] On day 1, the mice were allowed to become familiar with the test situation by placing them in a clean cage with bedding for 3 min, followed by a 2-min presentation of water (100 µl) on a cotton bud inserted through the lid. On day 2, seven mice were presented with 100 µl water, 3 x 100 µl of 129/S5 urine [10^{-4}] and 1 x 100 µl of BALB/c urine [10^{-4}] on cotton buds for 2 min each with 2 min between each presentation. The other six mice received 2-min sessions of 100 µl water, 3 x 100 µl of BALB/c urine [10^{-4}] and 1 x 100 µl of 129/S5 urine [10^{-4}]. On day 3 all urine concentrations were raised to [10^{-3}] and on day 4 to [10^{-2}]. During each 2-min session, the number of sniff bouts and their cumulative duration were recorded.

For the olfaction test, we used 21 *Chd7*$^{Whi/+}$ mice aged between 11 and 15 weeks (9 females and 12 males), and 19 age- and sex-matched wild-type littermates (8 females and 11 males). On day 1, all mice became familiar with the test situation as described above. On day 2, all mice were presented with 100 µl water followed by 100 µl of BALB/c urine [10^{-2}] for 2 min each, with 2 min between the presentations. During each 2-min session, the number of sniff bouts and their cumulative duration were recorded. The data of *Chd7*$^{Whi/+}$ and wild-type mice were compared using the Wilcoxon signed-rank test (as in Lee *et al*[16]).

Histopathological examination

Mice used for the smell test (n=40) were weighed, injected with pentobarbital sodium BP (Lethobarb, Fort Dodge Animal Health, Southhampton, UK) and transcardially perfused with ice-cold 10% formalin (Sigma, Gillingham, UK). Brain, testes, ovaries and uteri were dissected, weighed and preserved in 10% neutral buffered formalin (Sigma). Gross anatomy was viewed using a Leica stereomicroscope and digital photographs were taken with a Leica Dfc490 camera. The olfactory bulb and brain length, used to establish the olfactory bulb/brain ratio, were measured as depicted in Figure 3A. Testes were weighed and their length was measured. Uterine horns and ovaries were weighed together. Measurements of the *Chd7*$^{Whi/+}$ and wild-type brains and reproductive organs were analysed using the Student's t-test (or the Mann-Whitney test for small sample sizes, n<15). Brains were processed for paraffin embedding and cut in 8 µm coronal sections with every tenth

section mounted on a SuperFrost (VWR, Lutterworth, UK) glass slide. Olfactory bulb organisation was analysed in Nissl-stained sections.[18]

E12.5 and E16.5 wild-type, *Chd7*$^{Whi/+}$ and *Hspy/+;Whi/+* embryos were obtained from timed pregnancies, with E0.5 at noon on the day the vaginal plug was found. The embryos (n=28) were dissected in ice-cold PBS and yolk sacs were taken for genotyping.[11] Heads were fixed in 10% neutral-buffered formalin, dehydrated and embedded in paraffin. Sections of 8 µm thickness were stained for haematoxylin/eosin or processed for immunohistochemistry, for which the Ventana Discovery System (Ventana, Tucson, AZ, USA) and Ventana reagents (Ezprep, cat. no. 950-100), CC1 (cat. no. 950-124), LCS (cat. no. 50-010), Reaction buffer (cat. no. 950-300), DABMap Kit (cat. no. 760-124), and haematoxylin counterstain (cat. no. 760-2021) were used according to the manufacturer's instructions. Antibodies against GnRH1 (Chemicon, Watford, UK, cat. no. MAB5456) and CHD7 (a gift from Abcam, Cambridge, UK, cat. no. ab31824) were diluted 1:500 in Ventana buffer (cat. no. 251-018, Ventana). The respective secondary antibodies, biotinylated rabbit anti-mouse, and biotinylated donkey anti-rabbit, were diluted 1:100 in PBS containing 10% heat-inactivated foetal calf serum, 2% BSA, 0.1% Triton X-100 and 10mM sodiumazide. The sections were examined with a Zeiss Axioscope (Welwyn Garden City, UK) and digital images were taken using a Zeiss Axioscope HRc.

Breeding data analysis

The reproductive function of adult wild-type (mean age 2.2 months, SD 0.77) and *Chd7*$^{Whi/+}$ (mean age 3.2 months, SD 1.76) mice was assessed by the number of days required for production of the first litter after placing the test mutant with a wild-type mate. Differences were analysed with the Student's t-test (or the Mann-Whitney test for small samples).

RESULTS

Chd7 is present in brain areas involved in olfaction and reproduction

Immunohistochemistry tests in wild-type embryos showed decreasing amounts of Chd7 in the brain with increasing age (Figure 1). At E12.5 and E14.5, Chd7 protein was present in all layers of the olfactory epithelium and olfactory bulb (Figure 1A&B). At E16.5, the expression became more restricted, but Chd7 was detected in the vomeronasal organ, olfactory epithelium, olfactory bulb, hypothalamus and pituitary (Figure 1C-F). In the olfactory epithelium, Chd7 expression was highest in the inner basal layer, which contains horizontal and globose basal cells. Chd7 expression was also present in the nuclei of olfactory sensory neurons in the intermediate layer. The apical layer with supportive sustentacular cells showed the lowest expression of the Chd7 protein (Figure 1D).

***Chd7*$^{Whi/+}$ mice performed worse than wild-type mice on olfaction test**

During the habituation-dishabituation test, we found that wild-type C3HeB/FeJ mice showed no interest in 129/S5 urine, but did respond to BALB/c urine from 10^{-3} concentration onwards (data not shown). Therefore, BALB/c urine at 10^{-2} concentration was used to assess any difference in smell

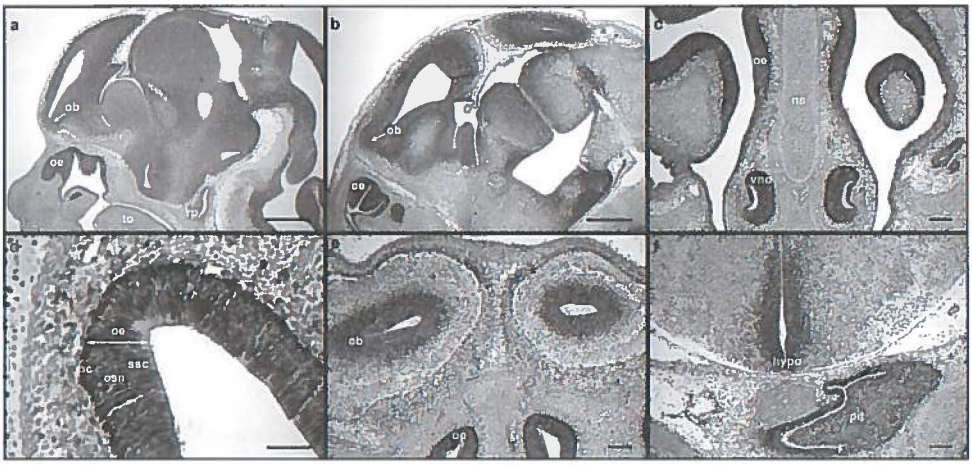

Figure 1. Immunohistochemistry showed that Chd7 protein is present in olfactory epithelium and in areas of the brain involved in olfaction and reproduction in wild-type C3HeB/FeJ mice. (**a**) At E12.5 and (**b**) E14.5, Chd7 is present in the olfactory epithelium and olfactory bulb (sagittal sections). (**c-f**) At E16.5, Chd7 is present in the vomeronasal organ and olfactory epithelium, olfactory bulb, hypothalamus and pituitary (coronal sections). (**d**) Chd7 expression is most prominent in the basal cells and olfactory sensory neurons. Scale bars=500 µm (**a&b**); 100 µm (**c**, **e&f**); 50 µm (**d**). bc, basal cells; hypo, hypothalamus; ns, nasal septum; ob, olfactory bulb; oe, olfactory epithelium; osn, olfactory sensory neurons; pit, pituitary; rp, Rathke's pouch; ssc, supportive sustentacular cells; to, tongue; vno, vomeronasal organ. (see color image on page 236)

behaviour between wild-type and *Chd7*$^{Whi/+}$ mice. Wild-type mice (n=19) showed an increased response to urine compared to water (P<0.01; Wilcoxon test; Figure 2). *Chd7*$^{Whi/+}$ mice (n=21) also showed an increased response to urine compared to water (P<0.05 for number of sniff bouts and P<0.01 for the cumulative duration of sniff bouts; Figure 2), but the response was less than that seen in wild-type mice (P<0.01; Figure 2). In addition, the number of non-responders, defined as those mice that did not explore the urine,[15] was greater in *Chd7*$^{Whi/+}$ mice (5/21) compared to wild-type mice (1/19).

Chd7$^{Whi/+}$ mice have mild olfactory bulb hypoplasia

Macroscopic examination of the *Chd7*$^{Whi/+}$ and wild-type brains was carried out to analyse the olfactory bulb/brain length ratio (Figure 3A-C, F). *Chd7*$^{Whi/+}$ mice (n=23) displayed a slightly decreased olfactory bulb/brain length ratio compared to wild-type controls (n=16) (P<0.01; Student's t-test; Figure 3F). Abnormal olfactory bulbs were seen in two *Chd7*$^{Whi/+}$ mice; one olfactory bulb consisted of two parts (Figure 3B) and in one case, asymmetrical olfactory bulb hypoplasia was observed (Figure 3C). Microscopic analysis of olfactory bulbs in *Chd7*$^{Whi/+}$ mice showed no abnormalities in the layered organisation of the olfactory bulb (Figure 3D&E).

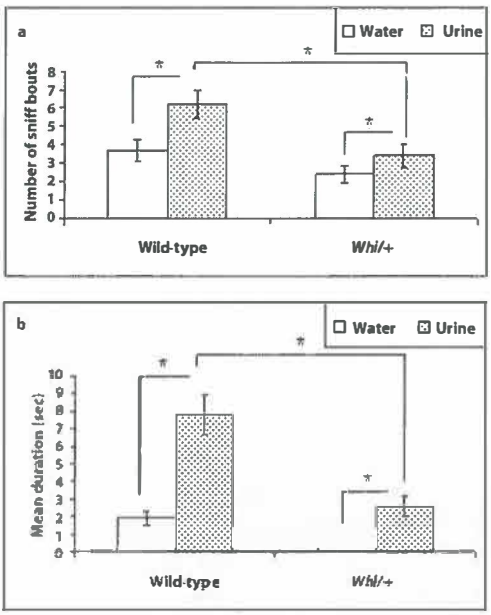

Figure 2. Olfaction test in wild-type and *Chd7*$^{Whi/+}$ mice. (**a**) Mean number of sniff bouts on presentation of water and urine. (**b**) Mean cumulative duration of sniff bouts. Error bars are means ± SEM. * P<0.05.

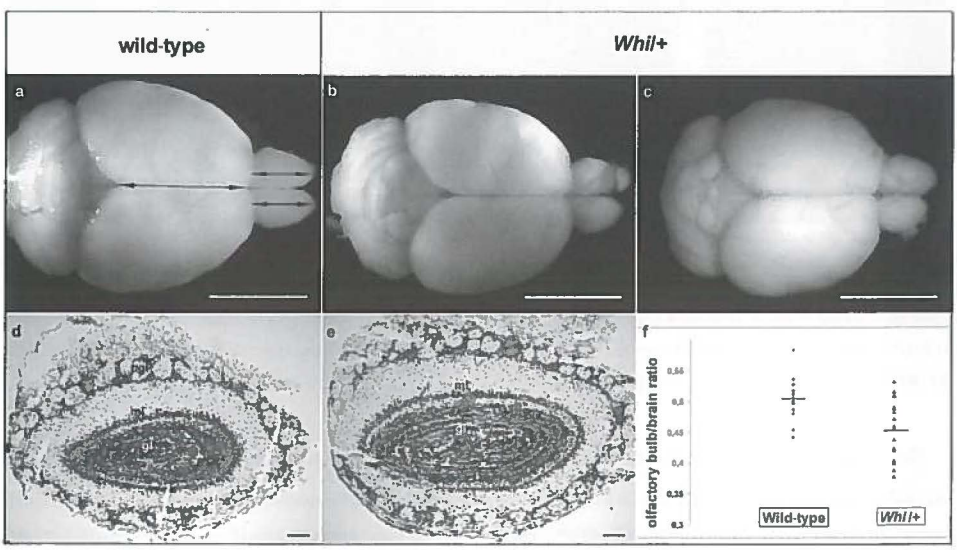

Figure 3. Macroscopic and microscopic analysis of olfactory bulbs from wild-type and *Chd7*$^{Whi/+}$ mice. (**a-c**) Macroscopic view of brains of adult wild-type and *Chd7*$^{Whi/+}$ mice, with olfactory bulb/brain length ratio measured as mean olfactory bulb length divided by length of cerebral hemispheres (arrows in **a**). (**b**) Left olfactory bulb consisting of two parts, (**c**) asymmetrical olfactory bulb hypoplasia. (**d&e**) Nissl-stained coronal sections of adult wild-type and *Chd7*$^{Whi/+}$ olfactory bulbs, showing no abnormalities. (**f**) Scatter plot of olfactory bulb/brain length ratio of adult wild-type and *Chd7*$^{Whi/+}$ mice. Scale bars=5 mm (**a-c**); 100 µm (**d&e**). gl, glomerular cell layer; ml, mitral cell layer; pgl, periglomerular layer. (see color image on page 236)

Abnormal reproductive system in *Chd7*$^{Whi/+}$ mice

Reproductive organs were examined at a macroscopic level (Figure 4). The mean testis weight of adult *Chd7*$^{Whi/+}$ males (n=12) was less than for adult wild-type males (n=11) (P≤0.05; Mann-Whitney test; Table 1). However, as *Chd7*$^{Whi/+}$ mice had a lower body weight, they had a significantly raised testis weight/body weight ratio (gonadosomatic index, GSI, P<0.05; Mann-Whitney test; Table 1, Figure 4C). Of twelve *Chd7*$^{Whi/+}$ males, two had severely hypoplastic testes with a decreased GSI (Figure 4B&C). The combined weights of uteri and ovaries did not differ between wild-type and *Chd7*$^{Whi/+}$ mice (Table 1), but abnormalities were present in all *Chd7*$^{Whi/+}$ females (n=9) (Figure 4E&F). We identified a cyst in one uterine horn (data not shown), one unilateral hypoplastic uterine horn (Figure 4E), and all other uteri were shorter and wider than in wild-type mice (Figure 4F).

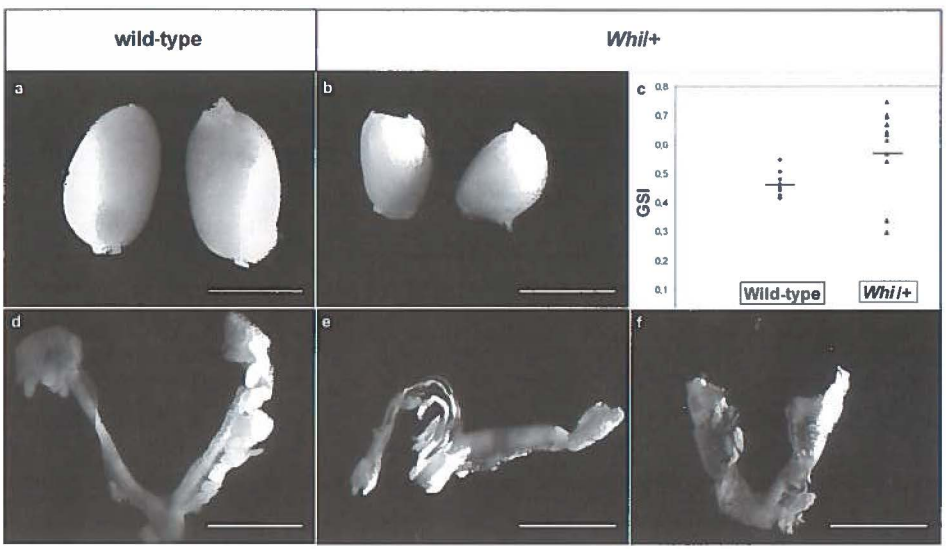

Figure 4. Macroscopic view of male and female reproductive organs of adult wild-type and *Chd7*$^{Whi/+}$ mice. (**a**) Normal testes in wild-type males. (**b**) Hypoplastic testes in some *Chd7*$^{Whi/+}$ males. (**c**) Scatter plot of gonadosomatic index (GSI) of adult wild-type and *Chd7*$^{Whi/+}$ males. (**d**) Normal uteri in wild-type females. (**e-f**) Abnormal uteri in all *Chd7*$^{Whi/+}$ females, (**e**) unilateral hypoplastic uterine horn and (**f**) wider than normal uterine horns. Scale bars=5 mm (**a&b**); 1 cm (**d-f**). (see color image on page 237)

The reproductive performance of *Chd7*$^{Whi/+}$ mice was slightly impaired. Maintaining the colony was not a major problem, but the number of days required to produce the first litter was significantly greater in both male and female *Chd7*$^{Whi/+}$ mice than in wild-type mice (P<0.05, Mann-Whitney or t-test, Table 1). The percentage of matings that did not lead to a litter two months after set-up was also counted (matings of mutant with wild-type compared to wild-type matings). In wild-type mice, the percentage of matings not producing a litter within two months after set-up was 6.1%. The percentage of *Chd7*$^{Whi/+}$ X wild-type matings that did not produce a litter after two months was not significantly different to that of wild-type X wild-type matings.

Table 1. Reproductive and physical parameters of wild-type and *Chd7*$^{Whi/+}$ mice

	Females		Males	
	Wild-type	***Chd7*$^{Whi/+}$**	**Wild-type**	***Chd7*$^{Whi/+}$**
Testes weight (g)	-	-	0.17 ± 0.002 (n=11)	0.14 ± 0.011* (n=12)
Body weight (g)	33.5 ± 2.3 (n=8)	21 ± 0.5* (n=9)	37.2 ± 0.9 (n=11)	23.9 ± 0.6* (n=12)
Gonadosomatic index	-	-	0.46 ± 0.01 (n=11)	0.58 ± 0.04* (n=12)
Combined weight of uterus and ovaries	0.25 ± 0.019 (n=8)	0.20 ± 0.082 (n=9)	-	-
Fertility (days/litter)	22.8 ± 0.3 (n_1=46)	27.4 ± 2.0* (n_1=7)	22.8 ± 0.3 (n_1=46)	24.3 ± 0.6* (n_1=54)

Values are expressed as mean ± SEM. * P≤0.05 compared to controls of the same sex. Gonadosomatic index: (testes weight in g/bodyweight in g) x 100. n = number of mice. n_1 = number of matings.

GnRH1 neurons in developing and adult *Chd7*$^{Whi/+}$ mice

An immunohistochemistry test with an anti-GnRH1 antibody was carried out to detect GnRH neurons in the adult brain. GnRH1 neurons were present in the organum vasculosum of the lamina terminalis and their fibres extended to the median eminence (ME) of adult wild-type and *Chd7*$^{Whi/+}$ mice (Figure 5). Representative photomicrographs were taken to analyse the GnRH neuron density, which appeared to be reduced in the ME of adult *Chd7*$^{Whi/+}$ females compared to wild-type females (Figure 5G&H).

No morphological abnormalities were found in the olfactory epithelia or olfactory bulbs of the *Chd7*$^{Whi/+}$ embryos at E16.5 (Supplementary Figure S1). GnRH1-positive cells were detected along their migration path, alongside the nasal septum, in the olfactory bulb and in the hypothalamus of the wild-type and *Chd7*$^{Whi/+}$ embryos (Supplementary Figure S1A-F). No visible difference in the GnRH1 neuron density between the wild-type and *Chd7*$^{Whi/+}$ embryos could be detected (Supplementary Figure S1).

Combining mutations in *Fgfr1* and *Chd7* is lethal

We collected offspring from matings between *Fgfr1*$^{Hspy/+}$ females and *Chd7*$^{Whi/+}$ males. No double heterozygous (*Hspy*/+;*Whi*/+) animals were recovered at weaning (0/55), showing a significant deviation from normal Mendelian ratios (χ^2-test P<0.01). Subsequently, we collected embryos at E12.5, E16.5 and P0 and found 8 out of 37 *Hspy*/+;*Whi*/+ mice, with 2 at P0 (one dead). This corresponds with the normal Mendelian ratios (χ^2-test P>0.9). This suggests that the combination of both heterozygous mutations results in perinatal or early postnatal death. No anatomical abnormalities were observed in E16.5 *Hspy*/+;*Whi*/+ embryos (n=5), other than the anomalies present in *Chd7*$^{Whi/+}$ mice with variable penetrance (cleft palate, choanal atresia, heart defect;

data not shown). Furthermore, a normal distribution of the GnRH1 neurons was present in E16.5 *Hspy/+;Whi/+* embryos (data not shown).

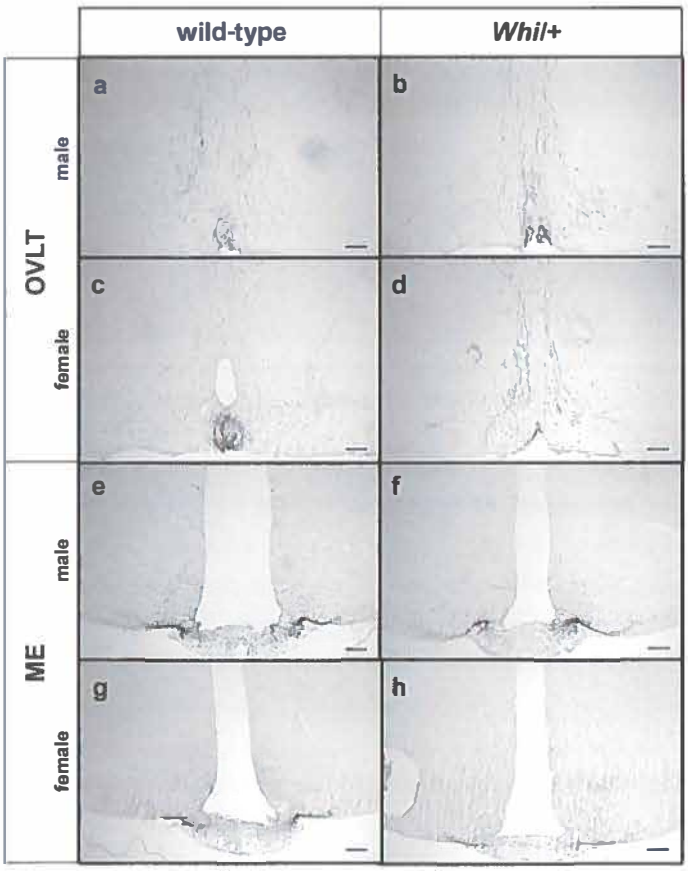

Figure 5. Representative photomicrographs of coronal brain sections of adult wild-type and *Chd7*$^{Wh/+}$ mice showing GnRH1-positive cells labelled by immunohistochemistry. (**a-d**) GnRH1 neurons in the organum vasculosum of the lamina terminalis (OVLT). (**e-h**) GnRH1 axon terminals in the median eminence (ME). Scale bars=100 µm. (see color image on page 238)

DISCUSSION

The Chd7 protein distribution described here is similar to the *Chd7* mRNA distribution previously described[5,9,11] and corresponds to the ß-galactosidase activity in mice carrying a *Chd7*^{Gt} allele.[19] In the olfactory epithelium, the highest levels of Chd7 were found in the basal cells and olfactory sensory neurons, suggesting a role for Chd7 in the development of these cell types. We also detected Chd7 in the developing olfactory bulb and the hypothalamus, pointing to Chd7 having a role in the development of these organs.

Sense of smell was evaluated with urine as an attractive odour, as previous studies had shown that mice are not interested in other odours.[16] Urine contains pheromones as well as volatile

constituents of urinary odour types and is detected by both the vomeronasal organ and the olfactory epithelium.[20-22] The smell test we used could therefore not distinguish between actions of the vomeronasal organ and the olfactory epithelium. As Chd7 is expressed in both organs, we expect that both could be affected by *Chd7* mutations. Overall, *Chd7*$^{Whi/+}$ mice performed worse than did wild-type mice, implying that they might have a smell deficit. However, within the mutant group, some mice performed well and seemed to have a normal sense of smell, whereas other mice did not explore the cotton bud dipped in urine (non-responders). The non-responders could be anosmic, but alternatively severe balance disturbances could also have contributed to their poor performance on the smell test. Our study design did not allow discrimination between olfactory and balance/neuromotor dysfunction. However, a concurrent study by Layman *et al*[23] has confirmed the presence of a smell deficit in *Chd7*-deficient mice (*Chd7*$^{Gt/+}$ mice), with an odour-evoked electro-olfactogram.

Pathological examination of the brains of the *Chd7*$^{Whi/+}$ mice showed mild hypoplasia of the olfactory bulbs, with only two mice (out of 23) having overt olfactory bulb anomalies. The organisation of the layers of the olfactory bulb was normal. These findings are in concordance with a concurrent study.[23] One could argue whether these mild olfactory bulb anomalies would have any effect on the ability to smell. It is also possible that a dysfunction of the olfactory epithelium causes the smell deficit, as was postulated by Layman *et al*.[23] The low incidence of olfactory bulb anomalies in *Chd7*$^{Whi/+}$ mice are in contrast to the previously described complete penetrance of olfactory bulb aplasia in humans.[3-5] However, we know from our own observations that some patients with a *CHD7* mutation have normosmia or slight hyposmia (5/24, Bergman JEH and van Ravenswaaij-Arts CMA, unpublished results).

Defects of reproductive organs were seen in all *Chd7*$^{Whi/+}$ females and some *Chd7*$^{Whi/+}$ males. Of 12 *Chd7*$^{Whi/+}$ males, 2 had severely hypoplastic testes and all nine *Chd7*$^{Whi/+}$ females had abnormal uterine horns. GnRH1 neurons were present in the hypothalamus of *Chd7*$^{Whi/+}$ mice, but were mildly decreased in female *Chd7*$^{Whi/+}$ mice. In contrast, no GnRH1 neuron abnormalities were observed in *Chd7*$^{Whi/+}$ embryos, suggesting that *Chd7* might have an effect on the GnRH1 neuron survival in a similar way to *Fgfr1*.[24] The effect of the reproductive organ anomalies and assumed reduction in the GnRH1-positive neurons on reproductive performance seemed minimal, as maintaining the colony did not present any major problems. This is to be expected, as there is a substantial redundancy known to be present in the GnRH neuronal population.[25] However, on further analysis, *Chd7*$^{Whi/+}$ mice were found to have a slightly lower reproductive performance (needing more days to produce the first litter compared to wild-type mice). Reproductive fitness could be affected by balance defects leading to abnormal head-bobbing and circling behaviour, by reduced body weight or by abnormal mating behaviour of *Chd7*$^{Whi/+}$ mice. Reduced body weight was previously correlated with reduced reproductive fitness in mice.[26] The cause of reduced body weight in *Chd7*$^{Whi/+}$ mice is unknown, but could be caused by increased activity, poor feeding (owing to cranial nerve anomalies or olfactory deficit) or growth hormone deficiency. Mating behaviour was not observed in this study, but it was

previously shown that anosmic mice can mate and are fertile.[27] However, other studies[21,28] claim that anosmia could lead to reduced reproductive fitness in mice. We were unable to differentiate between the different mechanisms that could influence reproductive fitness because some features that might affect reproductive fitness are fully penetrant in *Chd7*$^{Whi/+}$ mice (e.g. balance disturbance). The ability of *Chd7*$^{Whi/+}$ mice to reproduce corresponds with the situation in a minority of humans with CHARGE syndrome, in which some mildly affected patients are seen to reproduce normally.[29,30]

Anosmia and hypogonadotropic hypogonadism often occur together because of the interlinked migration process of olfactory neurons and GnRH neurons during embryonic development.[31] However, in our study, poor performance on the smell test did not correlate with hypoplasia of reproductive organs in *Chd7*$^{Whi/+}$ mice. Hypoplasia of reproductive organs was also not associated with a clearly reduced GnRH1 neuronal population in the hypothalamus, implying that gonadal anomalies in *Chd7*$^{Whi/+}$ mice might be caused by a local effect of Chd7.

The phenotypic overlap between CHARGE and Kallmann syndromes might be attributed to an interaction between the genes (or their encoded proteins) involved in both syndromes. Intercrosses between *Fgfr1*$^{Hspy/+}$ females and *Chd7*$^{Whi/+}$ males showed that combining mutations in both *Fgfr1* and *Chd7* leads to perinatal or early postnatal death. This is in contrast with the normal viability of *Fgfr1*$^{Hspy/+}$ mice and 50% lethality of *Chd7*$^{Whi/+}$ mice, suggesting that mutations in *Fgfr1* and *Chd7* interact synergistically, showing more than simply an additive effect on viability. As Chd7 is a member of the chromodomain helicase DNA-binding protein family,[32,33] one can hypothesise that reduced levels of Chd7 may decrease *Fgfr1* expression, leading to the Kallmann-like phenotype in CHARGE syndrome patients. However, the Kallmann phenotype (olfactory bulb defects and hypogonadotropic hypogonadism) was not observed in double heterozygote mice. This argues against genetic interaction between *Fgfr1* and *Chd7* in olfactory bulb and hypothalamus development.

CONCLUSIONS

Whirligig mice show a reduced penetrance of features associated with anosmia and hypogonadotropic hypogonadism compared to humans with CHARGE syndrome. The observation of incomplete penetrance in mice that have the same *Chd7* mutation on an identical genetic background is in line with the variable expression and reduced penetrance observed in humans with CHARGE syndrome. The reduced penetrance and variable expression in these mice may be attributed to purely stochastic events, or may be influenced by environmental differences between mice in their foetal microenvironment. In humans, the modifying effects of the varied genetic backgrounds of people with *CHD7* mutations is also likely to influence the penetrance and expression of each feature of the syndrome. The *Chd7*$^{Whi/+}$ mice may be useful in investigating the potential influence of epigenetic alterations on different aspects of the phenotypic spectrum in these syndromes.

ACKNOWLEDGEMENTS

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REFERENCES

1 Pagon RA, Graham JM, Jr., Zonana J, Yong SL. Coloboma, congenital heart disease, and choanal atresia with multiple anomalies: CHARGE association. *J Pediatr* 1981;99(2):223-227.

2 Amiel J, Attiee-Bitach T, Marianowski R, Cormier-Daire V, Abadie V, Bonnet D, et al. Temporal bone anomaly proposed as a major criteria for diagnosis of CHARGE syndrome. *Am J Med Genet* 2001;99(2):124-127.

3 Chalouhi C, Faulcon P, Le Bihan C, Hertz-Pannier L, Bonfils P, Abadie V. Olfactory evaluation in children: application to the CHARGE syndrome. *Pediatrics* 2005;116(1):e81-e88.

4 Pinto G, Abadie V, Mesnage R, Blustajn J, Cabrol S, Amiel J, et al. CHARGE syndrome includes hypogonadotropic hypogonadism and abnormal olfactory bulb development. *J Clin Endocrinol Metab* 2005;90(10):5621-5626.

5 Sanlaville D, Etchevers HC, Gonzales M, Martinovic J, Clement-Ziza M, Delezoide AL, et al. Phenotypic spectrum of CHARGE syndrome in fetuses with *CHD7* truncating mutations correlates with expression during human development. *J Med Genet* 2006;43(3):211-217.

6 Jongmans MC, Admiraal RJ, van der Donk KP, Vissers LE, Baas AF, Kapusta L, et al. CHARGE syndrome: the phenotypic spectrum of mutations in the *CHD7* gene. *J Med Genet* 2006;43(4):306-314.

7 Vissers LE, van Ravenswaaij CM, Admiraal R, Hurst JA, De Vries BB, Janssen IM, et al. Mutations in a new member of the chromodomain gene family cause CHARGE syndrome. *Nat Genet* 2004;36(9):955-957.

8 Jongmans MC, Ravenswaaij-Arts CM, Pitteloud N, Ogata T, Sato N, Claahsen-van der Grinten HL, et al. *CHD7* mutations in patients initially diagnosed with Kallmann syndrome--the clinical overlap with CHARGE syndrome. *Clin Genet* 2009;75(1):65-71.

9 Kim HG, Kurth I, Lan F, Meliciani I, Wenzel W, Eom SH, et al. Mutations in *CHD7*, encoding a chromatin-remodeling protein, cause idiopathic hypogonadotropic hypogonadism and Kallmann syndrome. *Am J Hum Genet* 2008;83(4):511-519.

10 Hardelin JP, Dode C. The complex genetics of Kallmann syndrome: *KAL1*, *FGFR1*, *FGF8*, *PROKR2*, *PROK2*, et al. *Sex Dev* 2008;2(4-5):181-193.

11 Bosman EA, Penn AC, Ambrose JC, Kettleborough R, Stemple DL, Steel KP. Multiple mutations in mouse *Chd7* provide models for CHARGE syndrome. *Hum Mol Genet* 2005;14(22):3463-3476.

12 Hawker K, Fuchs H, Angelis MH, Steel KP. Two new mouse mutants with vestibular defects that map to the highly mutable locus on chromosome 4. *Int J Audiol* 2005;44(3):171-177.

13 Pau H, Hawker K, Fuchs H, de Angelis MH, Steel KP. Characterization of a new mouse mutant, flouncer, with a balance defect and inner ear malformation. *Otol Neurotol* 2004;25(5):707-713.

14 Chang H, Huylebroeck D, Verschueren K, Guo Q, Matzuk MM, Zwijsen A. *Smad5* knockout mice die at mid-gestation due to multiple embryonic and extraembryonic defects. *Development* 1999;126(8):1631-1642.

15 Brown RE, Singh PB, Roser B. The major histocompatibility complex and the chemosensory recognition of

individuality in rats. *Physiol Behav* 1987;40(1):65-73.

16 Lee AW, Emsley JG, Brown RE, Hagg T. Marked differences in olfactory sensitivity and apparent speed of forebrain neuroblast migration in three inbred strains of mice. *Neuroscience* 2003;118(1):263-270.

17 Farber DB, Lolley RN. Cyclic guanosine monophosphate: elevation in degenerating photoreceptor cells of the C3H mouse retina. *Science* 1974;186(4162):449-451.

18 Kawamura S, Niimi K. Counterstaining of Nauta-Gygax impregnated sections with cresyl violet. *Stain Technol* 1972;47(1):1-6.

19 Hurd EA, Capers PL, Blauwkamp MN, Adams ME, Raphael Y, Poucher HK, et al. Loss of Chd7 function in gene-trapped reporter mice is embryonic lethal and associated with severe defects in multiple developing tissues. *Mamm Genome* 2007;18(2):94-104.

20 Boehm U. The vomeronasal system in mice: from the nose to the hypothalamus- and back! *Semin Cell Dev Biol* 2006;17(4):471-479.

21 Kelliher KR. The combined role of the main olfactory and vomeronasal systems in social communication in mammals. *Horm Behav* 2007;52(5):561-570.

22 Restrepo D, Arellano J, Oliva AM, Schaefer ML, Lin W. Emerging views on the distinct but related roles of the main and accessory olfactory systems in responsiveness to chemosensory signals in mice. *Horm Behav* 2004;46(3):247-256.

23 Layman WS, McEwen DP, Beyer LA, Lalani SR, Fernbach SD, Oh E, et al. Defects in neural stem cell proliferation and olfaction in *Chd7* deficient mice indicate a mechanism for hyposmia in human CHARGE syndrome. *Hum Mol Genet* 2009;18(11):1909-1923.

24 Tsai PS, Moenter SM, Postigo HR, El Majdoubi M, Pak TR, Gill JC, et al. Targeted expression of a dominant-negative fibroblast growth factor (FGF) receptor in gonadotropin-releasing hormone (GnRH) neurons reduces FGF responsiveness and the size of GnRH neuronal population. *Mol Endocrinol* 2005;19(1):225-236.

25 Herbison AE, Porteous R, Pape JR, Mora JM, Hurst PR. Gonadotropin-releasing hormone neuron requirements for puberty, ovulation, and fertility. *Endocrinology* 2008;149(2):597-604.

26 Bunger L, Lewis RM, Rothschild MF, Blasco A, Renne U, Simm G. Relationships between quantitative and reproductive fitness traits in animals. *Philos Trans R Soc Lond B Biol Sci* 2005;360(1459):1489-1502.

27 Belluscio L, Gold GH, Nemes A, Axel R. Mice deficient in G(olf) are anosmic. *Neuron* 1998;20(1):69-81.

28 Vandenbergh JG. Effects of central and peripheral anosmia on reproduction of female mice. *Physiol Behav* 1973;10(2):257-261.

29 Delahaye A, Sznajer Y, Lyonnet S, Elmaleh-Berges M, Delpierre I, Audollent S, et al. Familial CHARGE syndrome because of *CHD7* mutation: clinical intra- and interfamilial variability. *Clin Genet* 2007;72(2):112-121.

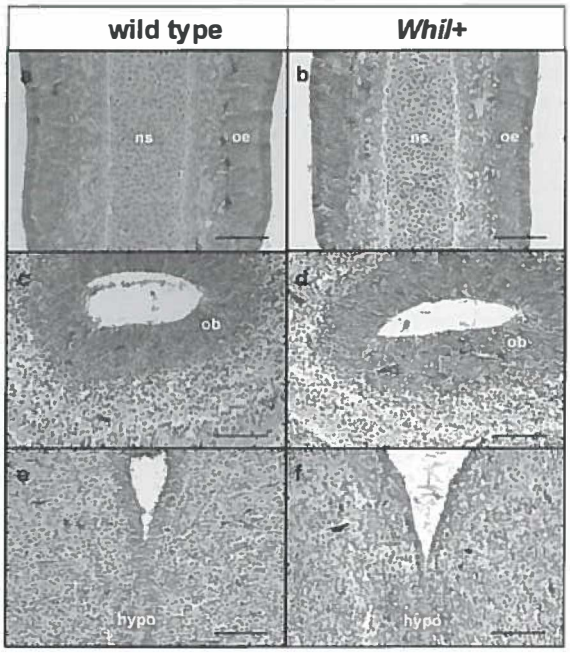
30 Jongmans MC, Hoefsloot LH, van der Donk KP, Admiraal RJ, Magee A, van de Laar I, et al. Familial CHARGE syndrome and the *CHD7* gene: a recurrent missense mutation, intrafamilial recurrence and variability. *Am J Med Genet A* 2008;146(1):43-50.

31 Cariboni A, Maggi R. Kallmann's syndrome, a neuronal migration defect. *Cell Mol Life Sci* 2006;63(21):2512-2526.

32 Hall JA, Georgel PT. CHD proteins: a diverse family with strong ties. *Biochem Cell Biol* 2007;85(4):463-476.

33 Marfella CG, Imbalzano AN. The Chd family of chromatin remodelers. *Mutat Res* 2007;618(1-2):30-40.

Supplementary information



Supplementary Figure S1. Representative photomicrographs of coronal brain sections of E16.5 wild type and *Chd7*$^{Whi/+}$ embryos showing GnRH1-positive cells labelled by immunohistochemistry. (**a&b**) GnRH1 neurons alongside the nasal septum, (**c&d**) in the olfactory bulb and (**e&f**) in the hypothalamus. Scale bars=100 µm. hypo, hypothalamus; ns, nasal septum; ob, olfactory bulb; oe, olfactory epithelium. (see color image on page 239)

Material and methods

Fgfr1$^{Hspy/+}$ genotyping

Fgfr1$^{Hspy/+}$mice were genotyped by a PCR with primer sets of Fw: 5'-GTGGTCTTTTGGAGTGCTCCTG-3' and Rv: 5'-GCTTGCCAGTGGTGGATCCAA-3' with ReddyMix (Abgene), followed by DdeI (100 U/ml) restriction digest overnight at 37°C. A DdeI recognition site is introduced when the mutant sequence is combined with the single base pair (bp) mismatch in the forward primer. Digesting PCR products with DdeI followed by agarose gel electrophoresis generates a single band of 208 bp for wild type, two bands of 208 and 184 bp for *Fgfr1*$^{Hspy/+}$ and one 184 bp band for *Fgfr1*$^{Hspy/Hspy}$ animals.

CHAPTER 3.2

Anosmia predicts hypogonadotropic hypogonadism in CHARGE syndrome

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ABSTRACT

Objective To test the hypothesis that a smell test could predict the occurrence of hypogonadotropic hypogonadism (HH) in patients with CHARGE syndrome, which is a variable combination of ocular coloboma, heart defects, choanal atresia, retardation of growth/development, genital hypoplasia and ear anomalies or hearing loss caused by mutations in the *CHD7* (chromodomain helicase DNA-binding protein 7) gene.

Study design We performed endocrine studies and smell testing (University of Pennsylvania Smell Identification Test) in 35 adolescent patients with molecularly confirmed CHARGE syndrome.

Results Complete data on smell and puberty were available for 15 patients; eleven patients had both anosmia and HH, whereas four patients had normosmia/hyposmia and spontaneous puberty. In addition, seven boys were highly suspected of having HH (they were too young for definite HH diagnosis, but all had cryptorchidism, micropenis, or both) and had anosmia. The type of *CHD7* mutation could not predict HH because a father and daughter with the same *CHD7* mutation were discordant for HH and anosmia.

Conclusion Anosmia and HH were highly correlated in our cohort and therefore smell testing seems to be an attractive method to predict the occurrence of HH in patients with CHARGE syndrome. The use of this test could prevent delay of hormonal pubertal induction, resulting in an age-appropriate puberty.

INTRODUCTION

CHARGE syndrome (OMIM #214800; 1/10,000 newborns[1]) is an autosomal dominant disorder characterized by a variable occurrence of multiple congenital anomalies.[2] Originally, ocular coloboma, heart defects, atresia of choanae, retardation of growth/development or both, genital hypoplasia and ear anomalies including hearing loss, were identified as core features of the syndrome.[3] More recently, semicircular canal anomalies, cranial nerve defects and arhinencephaly were found to be present in most patients.[4] The diagnostic criteria for CHARGE syndrome have been revised several times.[1,5] In 2004, the chromodomain helicase DNA-binding protein 7 (*CHD7*) gene was identified as the major gene involved in CHARGE syndrome[6] and *CHD7* mutations have been found in very mildly affected patients who do not fulfil the diagnostic criteria.[7] Olfactory bulb hypoplasia has long been recognized in CHARGE syndrome.[3] Arhinencephaly was reported to be fully penetrant in patients with CHARGE syndrome and it was suggested that this feature might be a new diagnostic criterion.[4,8-10] Impaired olfaction was found in most patients with smell testing.[9-11] Genital hypoplasia caused by hypogonadotropic hypogonadism (HH) is also frequently encountered in CHARGE syndrome.[12-14] In boys, micropenis and cryptorchidism can be early manifestations of HH. In girls, HH is often only diagnosed when puberty does not occur spontaneously. An earlier study found gonadotropin deficiency in approximately 85% of boys and 70% of girls with a *CHD7* mutation.[15] The co-occurrence of anosmia and HH is not unique for CHARGE syndrome, but is also seen in Kallmann syndrome. It is speculated that the embryonic migration process of gonadotropin-releasing hormone neurons (GnRH neurons) along the olfactory and vomeronasal nerve fibers is disturbed in Kallmann syndrome.[16] The same defect might underlie anosmia, olfactory bulb defects and HH in CHARGE syndrome. A recent study in *Chd7*-deficient mice suggested that the smell deficit in CHARGE syndrome is caused by a dysfunctional olfactory epithelium.[11] Our group identified smell deficits, genital anomalies and fewer GnRH neurons in a proportion of *Chd7*-mutated mice.[17] Unfortunately, hormone levels and thus HH were not studied in both mouse models. From expression studies, we know that *CHD7* is present in the olfactory epithelium, olfactory bulbs and pituitary gland during embryonic development.[4]

Because anosmia and HH often occur in association, we hypothesized that a smell test might be a means of predicting the occurrence of HH in patients with CHARGE syndrome and thus offer the opportunity to start hormone replacement therapy at a more appropriate age. To investigate this hypothesis, we studied smell and pubertal development in a large cohort (n=35) of adolescent patients with CHARGE syndrome.

METHODS

Patients

Patients with CHARGE syndrome (19 male, 16 female) aged ≥ 10 years were recruited through the Dutch national multidisciplinary CHARGE clinic. Inclusion criteria were a proven *CHD7* mutation or clinically confirmed CHARGE syndrome according to the criteria of Blake *et al* or Verloes.[1,5] All

patients underwent standardized physical examination by a clinical geneticist (Ravenswaaij-Arts CMA) and paediatric endocrinologist (Bocca G). The Tanner stages were scored in all patients and stretched penile length (SPL) and testicular volume were measured in boys. Micropenis was defined as an SPL <-2.5 SD score for a given age in a Dutch cohort.[18] Cryptorchidism was present when testes were not palpable in the scrotum at two consecutive examinations. Additional clinical information was obtained from retrospective chart review. Family history was taken for pubertal development, but none of the families showed evidence of constitutional delay of pubertal maturation.

Informed consent was obtained from the patients or their parents for the collection of medical data, including re-analysis of magnetic resonance imaging (MRI) scans, the olfaction test and molecular and endocrine studies in a clinical setting according to the guidelines of the local ethical board.

Molecular and endocrine studies

Molecular analysis of the *CHD7* gene was performed as previously described.[15] Multiplex ligation-dependent probe amplification (MLPA) was performed when no *CHD7* mutation was found with routine analysis (method as described previously[19]).

Baseline luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels were determined, as well as basal testosterone levels in boys and basal estradiol levels in girls. Because patients were recruited from all over the Netherlands, most of the hormone studies had already been done in different laboratories. FSH and LH were analyzed with Autodelfia (Perkin Elmer, Waltham, Massachusetts), Axsym (Abbott, Abbott Park, Illinois), DPC Immulite 2000 (Block Scientific, Holbrook, New York), Elecsys (Roche, Basle, Switzerland) or Modular E170 (Roche). Estradiol was measured with Autodelfia, Modular E170 or radioimmunoassay. Testosterone levels were analyzed with Modular E170 or radioimmunoassay. Growth hormone deficiency had been diagnosed in one of the patients (M13) and four patients (M2, M4, M14, M17) had undergone baseline analysis of LH, FSH and testosterone levels in the neonatal period because of the presence of a micropenis.

Olfaction test and magnetic resonance imaging brain scan evaluation

The third edition of the University of Pennsylvania Smell Identification Test (UPSIT, Sensonics Inc, Haddon Heights, New Jersey; www.sensonics.com) was used to assess olfactory function.[20] This test consists of 40 scratch-and-sniff odorants and is forced-choice (with four options for each odour). The UPSIT is suitable for children aged ≥5 years. UPSIT scores range between 0 and 40, with each correctly identified odorant scoring 1 point. Patients with CHARGE syndrome who had head colds were not tested because their sense of smell could be negatively affected. Patients with mental retardation (developmental age <5 years), uncorrected bilateral choanal atresia, or prolonged feeding difficulties necessitating tube feeding were excluded from the UPSIT. Patients with CHARGE syndrome who had communication difficulties were tested with a newly designed picture book for the Dutch population, with photographs representing the odour options (available on request). All

tests were conducted by one researcher (van Ravenswaaij-Arts CMA) in a separate room. Twelve healthy Dutch adults (six male, six female; age 20-26 years) took the same test to see whether the American UPSIT could be used for the Dutch population. The mean score of the male control subjects was 31.7 (range 29-35 or 73-88% correct answers; SD 2.7) and the mean score of the female control subjects was 32 (range 29-35 or 73-88%, SD 2.0). Five odours (fruit punch, cheddar cheese, turpentine, lime and lemon) were not correctly identified by at least half of the Dutch control subjects. When excluding these five odours, the mean percentage of correctly identified odours increased from 79% to 86% for male control subjects and from 80% to 89% for female control subjects. These five odours were also excluded in the test results of patients with CHARGE syndrome.

Cerebral MRI scans were available for ten patients with CHARGE syndrome and were retrospectively analyzed for olfactory bulb anomalies by an experienced neuroradiologist (Meiners L). The MRI scans were performed in different hospitals with widely different techniques, making them less optimal for evaluating the olfactory bulbs. As a result, analysis of MRI scans was possible for only three patients.

RESULTS

Clinical data and the results of molecular and endocrine studies, olfaction test and cerebral MRI scan are summarized in the Table. *CHD7* analysis showed mutations in 34 patients. In one patient (M5), *CHD7* analysis was not performed, but this patient fulfilled both Blake's and Verloes's clinical criteria for CHARGE syndrome.

Of nineteen male patients, two had no genital anomalies, one had a micropenis, seven had cryptorchidism and nine had both micropenis and cryptorchidism (Table). Only two of the eleven male patients aged ≥14 years had experienced spontaneous puberty. HH was diagnosed in the other nine patients on the basis of physical examination and prepubertal baseline levels of LH, FSH and testosterone. Six of these nine patients had started sex hormone replacement therapy (HRT). Four of the male patients (M2, M4, M14, M17) were treated with testosterone in the neonatal period. The eight boys between 10 and 14 years of age were too young for definite HH diagnosis, but none of them showed pubertal maturation yet and all eight had micropenis, cryptorchidism or both.

None of the 16 female patients had genital abnormalities. Four of the twelve female patients aged ≥13 years had experienced spontaneous puberty (Table). The other eight patients were prepubertal on physical examination and had prepubertal baseline levels of LH, FSH and estradiol. HH was diagnosed in all eight patients and six of them had started sex HRT. The four younger girls (aged 10-11 years) did not show any secondary sexual development.

Olfactory evaluation and MRI brain scan evaluation

The UPSIT was administered to 26 of 35 patients (12 male, 14 female). The scores were adapted for the Dutch population (see Methods section). There was a clear-cut difference between patients who were able to smell (n=5; UPSIT scores, 60%-89%) and patients who were anosmic (n=21; UPSIT

Table. Clinical data, results of molecular and endocrine studies, age at start of hormone replacement therapy, and olfaction test (University of Pennsylvania Smell Identification Test) in 35 patients with CHARGE syndrome divided in 3 groups: hypogonadotropic hypogonadism (HH), normal puberty and too young for definite HH diagnosis

	Age[a]	*CHD7* Mutation[b]	Blake / Verloes[c]	Tanner stage[d]	Genitals[e]	TV[f] (mL)	LH[g] (U/L)	FSH[g] (U/L)	T or E[g] (nmol/L or pmol/L)	Age at start HRT[h]	UPSIT%[i] (uncorrected)	UPSIT%[i] (corrected)
HH												
M1	14;0	Missense	- / a	G1P1	C, M	2	0.1	0.6	<0.1	14;1	22.5	25.7
M2	14;4	Missense	- / a	G1P2	C, M	2	0.05	0.22	1.6	14;5	10.0	8.6
M3	14;8	Frameshift	+ / +	G1P2	C	U	<0.2	0.2	U	14;9	U	U
M4	15;2	Frameshift	– / +	G1P1	C, M	2	0.03	0.29	1.3	15;6	32.5	37.1
M5	16;7	U	+ / +	G1P2	M	2	U	U	U	-	U	U
M6	18;9	Nonsense	+ / a*	G1P3	C, M	U	<1	0.4	2.4	18;10	25.0	25.7
M7	20;3	Frameshift	+ / +	G1P3	C, M	2	0.2	0.4	0.57	20;7	U	U
M8	20;10	Frameshift	+ / +	G1P2	C	U	0.01	0.16	2.7	-	U	U
M9	>40	Splice site	+ / a*	G1P2	C, M	U	U	U	U	-	U	U
F1	13;1	Frameshift	– / –	B1P1	-	-	0.10	1.36	30	13;6	15.0	17.1
F2	14;9	Nonsense	– / +	B1P2	-	-	<1.0	<1.0	42	-	U	U
F3	16;1	Nonsense	– / a*	B1P3	-	-	0.07	0.18	U	16;2	30.0^	34.3^
F4	16;6	Nonsense	+ / +	B1P1	-	-	<0.2	<0.2	24	19;1	30.0	34.3
F5	16;8	Missense	- / +	B1P2	-	-	<0.2	0.4	37	16;11	35.0	37.1
F6	17;1	Nonsense	+ / +	B1P2	-	-	0.06	0.38	40	17;4	22.5	25.7
F7	17;6	Frameshift	+ / +	B1P2	-	-	Low	Low	U	17;9	35.0	40.0
F8	18;0	Splice site	+ / +	B1P2	-	-	<1.0	<1.0	52	-	25.0	22.9
Normal puberty												
M10	14;10	Frameshift	– / –*	G4PS	-	5	4.78	6.37	9.9	-	U	U
M11	>30	Frameshift	– / –*	U	U	U	0.76	2.33	6.5	-	72.5	80.0
F9	14;8	Missense	+ / +	BSPS	-	-	5.98	3.52	380	-	60.0	62.9
F10	17;3	Missense	– / –	BS	-	-	8.49#	2.64#	140#	-	87.5	88.6
F11	17;9	Nonsense	- / +	BSPS	-	-	3.78	6.17	270	-	U	U
F12	20;7	Missense	- / +	BSP4	-	-	U	U	U	-	62.5	68.6

Too young for definite HH diagnosis												
M12	10;4	Frameshift	+ / +	G1P1	C	1	0.03	0.39	1.6	-	15.0	17.1
M13	10;11	Frameshift	- / -	G1P1	C	U	<0.15	0.38	0.2	-	30.0	31.4
M14	11;3	Missense	– / +	G1P2	C, M	1	0.04	0.19	1.9	-	32.5	31.4
M15	11;4	Frameshift	- / a	G1P1	C	1	0.07	0.13	1.8	-	7.5	8.6
M16	12;0	Splice site	+ / +	G1P1	C, M	U	0.05	0.26	1.4	-	U^	U^
M17	12;5	Deletion	– / a*	G1P2	C, M, UTA	1	<1.0	0.2	0.6	12;6	25.0	25.7
M18	12;7	Splice site	– / a	G1P1	C	1	0.05	0.16	1.9	-	25.0	28.6
M19	13;3	Nonsense	– / a	G1P1	C	U	<1	<1	0.4	13;4	35.0	40.0
F13	10;4	Missense	– / p	B1P1	-	-	U	U	U	-	57.5^	60.0^
F14	10;11	Missense	+ / +	B1P1	-	-	0.04	0.29	30	-	20.0	22.9
F15	11;3	Missense	+ / +	B1P1	-	-	0.09	0.44	20	-	27.5	28.6
F16	11;4	Splice site	- / a	B1P2	-	-	U	U	U	-	27.5	22.9

F, female; M, male. U, unknown.

[a] Age at evaluation (year;month). [b] *CHD7* mutations: deletion, deletion of exon 13-38; frameshift, frameshift mutations (c.3053_3054insA, p.Phe1019fs; c.5535-7G>A, p.Gly1801fs; c.5564dup, p.Glu1856fs; c.5768_5769del, p.Ser1923fs; c.6018dup, p.Ser2007fs; c.6835del, p.Ala2279fs; c.7400del, p.Leu2467fs; c.7650_7651del, p.Glu2550fs; c.7769del, p.Asp2590fs; c.8267del, p.Thr2756fs); missense, missense mutations (c.3082A>G, p.Ile1028Val; c.3301T>C, p.Cys1101Arg; c.3302G>A, p.Cys1101Tyr; c.3770T>G, p.Leu1257Arg; c.3973T>G, p.Tyr1325Asp; c.4406A>G, p.Tyr1469Cys; c.4787A>G, p.Asp1596Gly; c.5222G>C, p.Arg1741Pro; c.5402A>C, p.His1801Pro; c.6322G>A, p.Gly2108Arg); nonsense, nonsense mutations (c.1714C>T, p.Gln572X; c.1940C>G, p.Ser647X; c.3655C>T, p.Arg1219X; c.4015C>T, p.Arg1339X; c.4157C>G, p.Ser1386X; c.6070C>T, p.Arg2024X; c.7824T>A, p.Tyr2608X); splice site, splice site mutations (c.2238+1G>A; c.2443+5G>C; c.3340A>T; c.3990-1G>C; c.5405-17G>A, p.His1801_Gly1802insAspHisGlyThr). [c] Score on Blake and Verloes criteria: +, positive; -, negative; a, atypical; p, partial; *, semicircular canal imaging not performed. [d] Tanner stage: B, breast development; G, genital development; P, pubic hair. [e] Genitals: C, cryptorchidism; M, micropenis; UTA, unilateral testis agenesis. [f] TV, testes volume. [g] Basal values of LH, FSH, T, testosterone and E, estradiol; #, endocrine studies done while using an oral anti-conceptive; low, documented as prepubertal. [h] HRT, hormone replacement therapy. [i] UPSIT, University of Pennsylvania Smell Identification Test: uncorrected for the Dutch population (40 multiple choice questions); corrected for the Dutch population (5 odours that were not recognized by healthy Dutch controls were left out); ^, olfactory bulb anomalies on cerebral MRI scan (F3 and M16 aplasia of olfactory bulbs, F13 hypoplasia of olfactory bulbs).

scores, 9% - 40%; Figure;Table). This difference was also seen without correction of the UPSIT for the Dutch population (Table). One patient (M14) took the UPSIT twice at age 9 and 11 years, resulting in an UPSIT scores of 14/35 (40%) and 11/35 (31%), respectively. We could not administer the UPSIT to nine patients because they had either a developmental age <5 years (M3, M5, M7, M8, M9, M16, F2) or prolonged tube feeding (M10) or uncorrected bilateral choanal atresia (F11).

With cerebral MRI scans, aplasia of the olfactory bulbs was shown in two of three patients. One of these patients could not perform the UPSIT because of developmental delay (M16) and the other patient had anosmia (F3). The third cerebral MRI scan showed asymmetrical olfactory bulbs with aplasia of the right olfactory bulb and hypoplasia of the left olfactory bulb. This patient (F13) had an UPSIT score of 21/35 (60%) showing that olfaction was not severely impaired by the olfactory bulb anomalies.

Correlation between olfactory function and pubertal development

Both smell and pubertal development could be evaluated in fifteen patients (five boys aged ≥14 years and ten girls aged ≥13 years). Eleven of these patients had both anosmia (highest UPSIT score 14/35 or 40%) and HH, whereas four patients had spontaneous puberty and normosmia or hyposmia (lowest UPSIT score 22/35 or 63%; Table; Figure). An additional eight boys and four girls

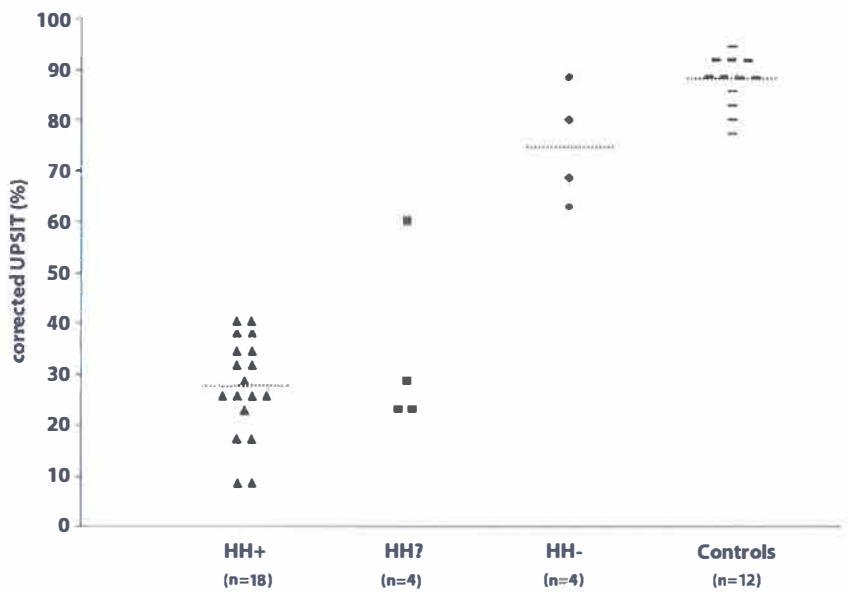

Figure. University of Pennsylvania Smell Identification Test (UPSIT) results corrected for the Dutch population (see Methods section) in 26 patients with CHARGE syndrome and 12 healthy Dutch controls. (▲) Patients with proven hypogonadotropic hypogonadism (HH, n =11) or highly suspected of HH (boys aged 10–13 years with cryptorchidism/micropenis and no pubertal maturation, n=7); (■) girls aged 10–13 years with unknown HH status (n=4); (♦) patients with normal pubertal development (n=4); and (—) Dutch control subjects (n=12). The mean is indicated by a dotted line (...) for each subgroup.

were too young for definite HH diagnosis. However, all eight boys had cryptorchidism and three boys also had a micropenis, suggesting HH. Seven of the boys (M12, M13, M14, M15, M17, M18, M19) had anosmia (UPSIT scores 3-14/35; 9-40%). In the eighth boy (M16), the UPSIT could not be performed, but the cerebral MRI scan showed olfactory bulb aplasia.

DISCUSSION

We show that the sense of smell is highly correlated with pubertal development in our patients (n=15) and we propose that smell testing can be used as a method of predicting HH in patients with CHARGE syndrome. Knowing the HH status is beneficial because HRT can be started in time and reduce socio-emotional problems and the risk of related osteoporosis and cardiovascular disease.

Anosmia can be reliably diagnosed with the UPSIT in most patients with CHARGE syndrome. Anosmia was present in 81% of our patients (21/26), whereas 19% of our patients were able to smell (5/26). Other studies have reported a similar distribution of anosmia/normosmia in patients with CHARGE syndrome by using the Biolfa test and the B-SIT.[9-11] There was a clear-cut difference between the scores of patients who were able to smell and the anosmic test results (Figure). It is important to inform patients with anosmia (or their caregivers) that they are not able to detect warning odours (e.g., gas, rotten food, or smoke) or foul body odours.

In this study, 15 patients showed 100% correlation between sense of smell and pubertal development. Eleven patients had anosmia with HH and four patients had both spontaneous pubertal development and normosmia/hyposmia. Officially, delayed puberty cannot be diagnosed in boys before the age of 14 years. However, when we extend our results with our findings in the seven boys aged 10 to 13 years who all had cryptorchidism, micropenis, or both, 22 patients with CHARGE syndrome showed concordance between the results on smell testing and HH or expected HH. In the four young girls no conclusion could be drawn about HH and a smell test could not be applied in the nine remaining patients (from the total cohort of 35). This high concordance between smell and puberty corresponds to two earlier reports that together describe six female patients with CHARGE syndrome with both HH and either anosmia or olfactory bulb anomalies.[10,21]

The combination of anosmia and pubertal delay is also seen in Kallmann syndrome. The clinical overlap between Kallmann and CHARGE syndrome might be explained by the presumed function of CHD7 as a regulator of gene expression. CHD7 is a member of the chromodomain helicase DNA binding protein family and is thought to regulate gene expression with its chromatin remodeling activity. We do not yet know which genes are regulated by CHD7,[22] but we speculate that one or more of the genes involved in Kallmann syndrome (*KAL1*, *FGFR1*, *PROK2*, *PROKR2* and *FGF8*) are targets of CHD7. The extremely high correlation between anosmia and HH in patients with *CHD7* mutations is in contrast with patients with *FGFR1* mutations who can present with anosmia alone, HH alone, or both. This suggests that the Kallmann phenotype in CHARGE syndrome is not caused by an effect of CHD7 on *FGFR1* expression, but could point to an influence on the *KAL1* gene (which always leads to the combination of anosmia and HH in boys). This is supported by the over-representation of

HH in boys (9/11, 82%) compared with girls (8/12, 67%) in our study, although a larger study group is needed to confirm this observation.

Two recent studies analyzed the *CHD7* gene in patients with Kallmann syndrome or normosmic HH. Jongmans *et al*[23] performed *CHD7* analysis in 36 patients and found three *CHD7* mutations in patients who had both anosmia and HH. Kim *et al*[24] analyzed *CHD7* in 197 patients and reported seven *CHD7* mutations: three in patients with both anosmia and HH, and four in patients with normosmic HH. This seems to contradict our observation that in CHARGE syndrome HH is always correlated with anosmia. There are two possible explanations: our patients were recruited from the CHARGE outpatient clinic so they might have a more complex phenotype, or there could be a difference in the clinical evaluation of the *CHD7*-positive patients in the different studies. In our study and that by Jongmans *et al*, the *CHD7*-positive patients were carefully examined (or re-examined) for CHARGE features and formal smell testing was performed. It is unclear whether Kim *et al* re-examined their *CHD7*-positive patients or used a formal smell test. In our experience, patients are often unaware of their smell deficit and smell testing therefore is crucial.

The presence of HH in CHARGE syndrome cannot be predicted by the type of *CHD7* mutation. This is illustrated by a family included in our study: patient F1 is the daughter of patient M11 and they share the same mutation. Father has a proven fertility and normosmia, whereas his daughter has HH and anosmia. Earlier studies have also shown great intrafamilial variability in CHARGE syndrome and a lack of phenotype-genotype correlations.[7,25] Truncating *CHD7* mutations are most frequently found, but missense mutations are seen in as much as 12% of patients.[2] In our cohort, missense mutations were over-represented (29%, 10/34). All ten missense mutations were considered to be pathogenic because all were *de novo*, located in a highly conserved region and predicted to be 'not tolerated' with SIFT (Sorting Intolerant From Tolerant). Five of the ten missense mutations were located in a functional domain of the CHD7 protein.

Because this study was conducted in an as minimally invasive way as possible, it had some weaknesses. First, the endocrine studies were done in different laboratories with different assays. However, there were no marginal results and the presence of HH was easily recognized by low levels of gonadotropins and sex steroids in the different assays. Second, only three of ten MRI scans were of sufficient quality for olfactory bulb analysis (a high-resolution MRI with ≤ 3 mm slices in the coronal plane is required). However, it has been shown that, apart from complete absence (bilateral aplasia), olfactory bulb anomalies do not correlate well with olfactory function[9,26] and thus the information that can be obtained from such scans is limited, as illustrated by patient F1 3.

HH is a frequent feature in CHARGE syndrome, but is often only established at a relatively advanced age when puberty fails to occur spontaneously. Diagnosing HH in childhood is impossible because levels of LH, FSH and sex steroids are physiologically low. However, in boys and girls a peak of FSH, LH and sex steroids is present between age 2 weeks and 6 months.[27] When CHARGE syndrome is suspected, HH can be diagnosed during this period. In addition, micropenis, cryptorchidism, or both are easily recognizable in boys (present in 17/19 male patients in our cohort) and suggest

the presence of HH. Only four male patients in our cohort were diagnosed with HH in the neonatal period, showing that HH is often overlooked in patients with CHARGE syndrome. In girls, there are no other features to suggest HH. Of the 17 patients with definite HH in this study, twelve were treated with sex HRT. Four patients aged ≥16 years had not yet been treated (M5, M8, M9, F8). They all had developmental delay and severe behavioural problems and adverse effects from sex HRT were anticipated. In male patients, testosterone treatment increases muscle mass and can aggravate behavioural problems, especially aggressiveness, whereas in female patients menstruation can be a burden. Another reason for postponing treatment could be to achieve an increase in final adult height in patients with extremely short stature (as is done in Turner syndrome). Arguments in favour of early HRT are the decreased risk of osteoporosis[13] and the possible lower risk of cardiovascular disease in men.[28] In addition, early HRT can have a positive effect on psychological well-being, as was shown for girls with Turner syndrome and in boys with constitutional delay of puberty.[29,30]

In conclusion, we propose that smell testing can be used to predict the occurrence of HH in patients with CHARGE syndrome. Early diagnosis of HH can prevent a delay in inducing puberty with HRT and thus results in an age-appropriate puberty. This may lead to increased socio-emotional well-being and a lower risk of osteoporosis and cardiovascular disease.

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REFERENCES

1 Blake KD, Davenport SL, Hall BD, Hefner MA, Pagon RA, Williams MS, et al. CHARGE association: an update and review for the primary pediatrician. *Clin Pediatr (Phila)* 1998;37(3):159-173.

2 Sanlaville D, Verloes A. CHARGE syndrome: an update. *Eur J Hum Genet* 2007;15(4):389-399.

3 Pagon RA, Graham JM,Jr., Zonana J, Yong SL. Coloboma, congenital heart disease, and choanal atresia with multiple anomalies: CHARGE association. *J Pediatr* 1981;99(2):223-227.

4 Sanlaville D, Etchevers HC, Gonzales M, Martinovic J, Clement-Ziza M, Delezoide AL, et al. Phenotypic spectrum of CHARGE syndrome in fetuses with *CHD7* truncating mutations correlates with expression during human development. *J Med Genet* 2006;43(3):211-217.

5 Verloes A. Updated diagnostic criteria for CHARGE syndrome: a proposal. *Am J Med Genet A* 2005;133(3):306-308.

6 Vissers LE, van Ravenswaaij CM, Admiraal R, Hurst JA, De Vries BB, Janssen IM, et al. Mutations in a new member of the chromodomain gene family cause CHARGE syndrome. *Nat Genet* 2004;36(9):955-957.

7 Jongmans MC, Hoefsloot LH, van der Donk KP, Admiraal RJ, Magee A, van de Laar I, et al. Familial CHARGE syndrome and the *CHD7* gene: a recurrent missense mutation, intrafamilial recurrence and variability. *Am J Med Genet A* 2008;146(1):43-50.

8 Asakura Y, Toyota Y, Muroya K, Kurosawa K, Fujita K, Aida N, et al. Endocrine and radiological studies in patients with molecularly confirmed CHARGE syndrome. *J Clin Endocrinol Metab* 2008;93(3):920-924.

9 Chalouhi C, Faulcon P, Le Bihan C, Hertz-Pannier L, Bonfils P, Abadie V. Olfactory evaluation in children: application to the CHARGE syndrome. *Pediatrics* 2005;116(1):e81-e88.

10 Pinto G, Abadie V, Mesnage R, Blustajn J, Cabrol S, Amiel J, et al. CHARGE syndrome includes hypogonadotropic hypogonadism and abnormal olfactory bulb development. *J Clin Endocrinol Metab* 2005;90(10):5621-5626.

11 Layman WS, McEwen DP, Beyer LA, Lalani SR, Fernbach SD, Oh E, et al. Defects in neural stem cell proliferation and olfaction in *Chd7* deficient mice indicate a mechanism for hyposmia in human CHARGE syndrome. *Hum Mol Genet* 2009;18(11):1909-1923.

12 Blake KD, Salem-Hartshorne N, Daoud MA, Gradstein J. Adolescent and adult issues in CHARGE syndrome. *Clin Pediatr (Phila)* 2005;44(2):151-159.

13 Forward KE, Cummings EA, Blake KD. Risk factors for poor bone health in adolescents and adults with CHARGE syndrome. *Am J Med Genet A* 2007;143(8):839-845.

14 Wheeler PG, Quigley CA, Sadeghi-Nejad A, Weaver DD. Hypogonadism and CHARGE association. *Am J Med Genet* 2000;94(3):228-231.

15 Jongmans MC, Admiraal RJ, van der Donk KP, Vissers LE, Baas AF, Kapusta L, et al. CHARGE syndrome: the phenotypic spectrum of mutations in the *CHD7* gene. *J Med Genet* 2006;43(4):306-314.

16 Cariboni A, Maggi R. Kallmann's syndrome, a neuronal migration defect. *Cell Mol Life Sci* 2006;63(21):2512-2526.

17 Bergman JE, Bosman EA, Ravenswaaij-Arts CM, Steel KP. Study of smell and reproductive organs in a mouse model for CHARGE syndrome. *Eur J Hum Genet* 2010;18(2):171-177.

18 Massa GG, Langenhorst V, Oostdijk W, Wit JM. [Micropenis in children: etiology, diagnosis and therapy]. *Ned Tijdschr Geneeskd* 1997;141(11):511-515.

19 Bergman JE, de Wijs I, Jongmans MC, Admiraal RJ, Hoefsloot LH, Ravenswaaij-Arts CM. Exon copy number alterations of the *CHD7* gene are not a major cause of CHARGE and CHARGE-like syndrome. *Eur J Med Genet* 2008;51(5):417-425.

20 Doty RL, Shaman P, Dann M. Development of the University of Pennsylvania Smell Identification Test: a standardized microencapsulated test of olfactory function. *Physiol Behav* 1984;32(3):489-502.

21 Ogata T, Fujiwara I, Ogawa E, Sato N, Udaka T, Kosaki K. Kallmann syndrome phenotype in a female patient with CHARGE syndrome and *CHD7* mutation. *Endocr J* 2006;53(6):741-743.

22 Schnetz MP, Bartels CF, Shastri K, Balasubramanian D, Zentner GE, Balaji R, et al. Genomic distribution of CHD7 on chromatin tracks H3K4 methylation patterns. *Genome Res* 2009;19(4):590-601.

23 Jongmans MC, Ravenswaaij-Arts CM, Pitteloud N, Ogata T, Sato N, Claahsen-van der Grinten HL, et al. *CHD7* mutations in patients initially diagnosed with Kallmann syndrome--the clinical overlap with CHARGE syndrome. *Clin Genet* 2009;75(1):65-71.

24 Kim HG, Kurth I, Lan F, Meliciani I, Wenzel W, Eom SH, et al. Mutations in *CHD7*, encoding a chromatin-remodeling protein, cause idiopathic hypogonadotropic hypogonadism and Kallmann syndrome. *Am J Hum Genet* 2008;83(4):511-519.

25 Delahaye A, Sznajer Y, Lyonnet S, Elmaleh-Berges M, Delpierre I, Audollent S, et al. Familial CHARGE syndrome because of *CHD7* mutation: clinical intra- and interfamilial variability. *Clin Genet* 2007;72(2):112-121.

26 Yousem DM, Geckle RJ, Bilker W, McKeown DA, Doty RL. MR evaluation of patients with congenital hyposmia or anosmia. *Am J Roentgenol* 1996;166(2):439-443.

27 Grumbach, Styne DM. Puberty: ontogeny, neuroendocrinology, physiology, and disorders. In: Larsen PR, Kronenberg HM, Melmed S, Polonsky KS, editors. Williams Textbook of Endocrinology, Philadelphia: WB Saunders, 2003:1115-1286.

28 Traish AM, Saad F, Feeley RJ, Guay A. The dark side of testosterone deficiency: III. Cardiovascular disease. *J Androl* 2009;30(5):477-494.

29 Ross JL, McCauley E, Roeltgen D, Long L, Kushner H, Feuillan P., et al. Self-concept and behavior in adolescent girls with Turner syndrome: potential estrogen effects. *J Clin Endocrinol Metab* 1996;81(3):926-931.

30 Albanese A, Stanhope R. Investigation of delayed puberty. *Clin Endocrinol (Oxf)* 1995;43(1):105-110.

CHAPTER 3.3

Death in CHARGE syndrome after the neonatal period

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ABSTRACT

CHARGE syndrome is a multiple congenital anomaly syndrome that can be life-threatening in the neonatal period. Complex heart defects, bilateral choanal atresia, oesophageal atresia, severe T-cell deficiency, and brain anomalies can cause neonatal death. As little is known about the causes of death in childhood and adolescence, we studied post-neonatal death in patients with CHARGE syndrome. We collected medical data on three deceased children from a follow-up cohort of 48 CHARGE patients and retrospectively on an additional four deceased patients (age at death 11 months to 22 years). We analyzed the factors that had contributed to their death. In five patients respiratory aspiration had most likely contributed to premature death, one died of post-operative complications, and another choked during eating. From our findings and a literature review, we suggest that swallowing problems, gastro-oesophageal reflux disease, respiratory aspiration and post-operative airway events are important contributors to post-neonatal death in CHARGE syndrome. Cranial nerve dysfunction is proposed as the underlying pathogenic mechanism. We recommend every CHARGE patient with feeding difficulties to be assessed by a multidisciplinary team to evaluate cranial nerve function and swallowing. Timely treatment of swallowing problems and gastro-oesophageal reflux disease is important. Surgical procedures on these patients should be combined whenever possible because of their increased risk of post-operative complications and intubation problems. Finally, we recommend performing autopsy in deceased CHARGE patients in order to gain more insight into causes of death.

INTRODUCTION

CHARGE syndrome is a congenital malformation syndrome that occurs in approximately 1/10,000 live births.[1,2] CHARGE (OMIM 214800) is an acronym for coloboma, heart defects, atresia of choanae, retardation of growth and development, genital abnormalities, and ear anomalies (including deafness).[3] Arhinencephaly, hypoplasia of semicircular canals, and cranial nerve (CN) palsies are also present in the majority of patients.[2,4-6] Occasional features include oesophageal atresia, cleft lip/palate, renal abnormalities, and hypogonadotropic hypogonadism.[2] The genetic aetiology of CHARGE syndrome was clarified in 2004, when mutations in the Chromodomain Helicase DNA binding protein 7 (*CHD7*) gene were described.[7]

Some of the congenital abnormalities present in CHARGE syndrome can lead to premature death. Abnormalities that can be life-threatening in the neonatal period are complex heart defects, bilateral choanal atresia, oesophageal atresia, severe T-cell deficiency and brain anomalies.[1,8-11] However, from clinical practice, we know that a significant percentage of children with CHARGE syndrome die after the neonatal period (with post-neonatal period defined as 28 days or older). Death is sometimes related to surgical procedures or major congenital abnormalities, but occasionally it occurs suddenly and unexpectedly. Previous studies have described the clinical features in large series of patients with CHARGE syndrome, but did not give details on causes of death.[1,8,9,12,13] We have, therefore, focused on causes of death in patients with CHARGE syndrome that died after the neonatal period. We collected medical data on three deceased children from a follow-up cohort of 48 CHARGE patients and retrospectively on an additional four deceased patients. Based on our findings we propose recommendations to prevent premature death in patients with CHARGE syndrome.

3.3

PATIENTS AND METHODS

We studied a cohort of 48 patients with CHARGE syndrome who were seen at the multidisciplinary CHARGE outpatient clinic in the Netherlands between 2005 and 2009. The mean age at first admission to the CHARGE outpatient clinic was 11 years 8 months (range 4 months – 49 years). All 48 patients (21 females, 27 males) had definite CHARGE syndrome, 46 had a *CHD7* mutation (*CHD7* analysis as described in Jongmans *et al*[12] and the other two patients fulfilled the clinical criteria described by Blake or Verloes.[14,15] Three of the patients in this cohort died at age 11.5 months, 8 years and 22 years. These data were used to produce a Kaplan-Meier actuarial survival curve.

In addition, we collected the medical data of a further four patients with CHARGE syndrome who had died after 11 months of age. These patients were identified through the Dutch patient organisation (n=1), through the database of the Department of Genetics, University Medical Centre Groningen (n=1), and through the Canadian database of CHARGE patients (Prof. K. Blake, n=2). All patients either fulfilled the clinical criteria for CHARGE syndrome postulated by Blake or Verloes[14,15] and/or had a pathogenic *CHD7* mutation. The 22q11 deletions were excluded in all patients by fluorescent *in situ* hybridization (FISH) analysis and all karyotypes were normal. The parents of

the Dutch patients gave informed consent to release the medical records and for publishing photographs. Ethical consent was granted by the IWK Health Centre for chart review of the Canadian patients.

In the surviving patients of ten years and older from the CHARGE outpatient clinic (n=25), we scored the clinical features that were present in their first 10 years of life. We compared these features with the features of the six patients that died before 10 years of age (two patients from the CHARGE outpatient clinic and four patients from other centres). We tried to identify possible risk factors for premature death. One of the factors we scored was feeding difficulties, which we defined as feeding problems necessitating tube feeding (at present or in the past). Breathing difficulties were scored if patients had had a tracheotomy or needed extra oxygen. A χ^2-test was performed to identify significant differences between the two groups (significance level 0.05).

RESULTS

The demographics, results of *CHD7* analysis, scores on Blake's and Verloes' criteria, and the clinical details of the seven deceased patients with CHARGE syndrome are summarized in Table 1. A Kaplan-Meier actuarial survival curve is presented in Figure 1, showing the incidence of premature death among patients with CHARGE syndrome, who were seen in the multidisciplinary CHARGE outpatient clinic in the Netherlands. Three of these patients died at the ages of 11.5 months, 8 years and 22 years, respectively, giving an actuarial post-neonatal survival of 98% at 1 year of age, 95% at 10 years of age and 76% at 25 years of age. We would like to stress that this survival curve only applies to patients, who survived the neonatal period, as that is the population of the CHARGE outpatient clinic.

Patient A

Patient A (Figure 2A) had oesophageal atresia, ventricular and atrial septal defects and brain anomalies (hypoplasia of cerebrum/cerebellum, small pons). He received tube feeding until he was two years old and underwent fundoplication for gastro-oesophageal reflux disease (GERD). He had recurrent aspiration pneumonias. He ingested solid food rapidly but experienced problems with swallowing liquids. His parents described him as a voracious eater. A barium swallow was advised, but before this could take place he died unexpectedly while eating when he was 8 years old. His mother heard laboured breathing and saw he was choking. She was not able to free the airway; Heimlich manoeuvre and resuscitation were not successful. Intubation by the emergency team failed. In the hospital (90 min later), secondary respiratory aspiration was seen. Because of the deteriorated respiratory and neurological condition, resuscitation was stopped. The cause of death was fatal choking on food. No autopsy was performed.

Patient B

Patient B (Figure 2B) had neonatal convulsions. He suffered from recurrent aspiration pneumonias

Table 1. Clinical features of seven deceased patients with CHARGE syndrome

Child	Sex[a]	Age at death[b]	CHD7 mutation[c]	Clinical criteria[d]		CHARGE features[e]	Cranial nerve dysfunction[f]	Features contributing to premature death[g]	Cause of death
				Blake	Verloes				
A	M	8 y	+ (fs)	-	-*	H, G, E	III, VII, VIII, IX-X	FD, GERD, RA, SGS, TE	Fatal choking on food
B	M	22 y	+ (fs)	+	+	C, R, G, E	VII, VIII, IX-X	CP, FD, GERD, RA	Diagnosed with pneumonia: respiratory aspiration or circulatory arrest?
C	M	11.5 m	+ (fs)	+	+	C, A, G, E	VII, IX-X	FD, GERD, LM	Diagnosed with viral respiratory tract infection: respiratory aspiration or circulatory arrest?
D	M	14 m	U	+	a*	C, H, R, G, E	VIII, IX-X	FD, GERD, RA, TE	Respiratory aspiration or circulatory arrest?
E	F	27 m	U	+	+	C, H, A, R, E	IX-X	FD, GERD	Respiratory aspiration?
F	M	9 y	U	+	+	C, H, A, R, G, E	V, VII, VIII, IX-X	CLP, FD, GERD	Respiratory aspiration?
G	M	14 m	+ (non)	+	+	C, H, A, R, G, E	IX-X	CP, FD, GERD, RA, SGS	Postoperative complications

Cases were identified through the CHARGE outpatient clinic (cases A-C), through the database of the Department of Genetics, University Medical Centre Groningen (case D), through the Canadian database (cases E and F) and through the Dutch patient organisation (case G).

[a] F, female; M, male. [b] m, months; y, years. [c] fs, frameshift mutation (c.1820_1821insTTGT, p.Ala608fs; c.4779del, p.Gln1595fs; c.6835del, p.Ala2279fs); non, nonsense mutation (c.934C>T, p.Arg312X); U, unknown. [d] +, positive, or -, negative for clinical criteria; a, atypical CHARGE patient; * semicircular canal imaging not performed. [e] C, coloboma; H, heart defect; A, atresia or stenosis of choanae; R, retardation of growth and/or development; G, genital hypoplasia; E, ear anomalies or deafness. [f] III, oculomotor nerve palsy; V, trigeminal nerve palsy; VII, facial nerve palsy; VIII, sensorineural hearing loss; IX-X, swallowing problems. [g] CLP, cleft lip and palate; CP, cleft soft palate; FD, feeding difficulties; GERD, gastro-oesophageal reflux disease; LM, laryngomalacia; RA, respiratory aspiration; SGS, subglottic stenosis; TE, tracheo-oesophageal problems.

and respiratory insufficiency, which necessitated a tracheotomy until he was ten years old. He was fed by a G-tube and fundoplication was performed because of GERD. The day before his death, at age 22 years, he was diagnosed with pneumonia and given antibiotic treatment. The next evening, his condition seemed to have improved, but he died unexpectedly while lying on his bed. Although some vomit was found, his parents did not have the impression that he had aspirated. Autopsy was not performed and cause of death is unknown. He may have died of respiratory aspiration or cardiac arrest.

Patient C

Patient C (Figure 2C) had neonatal convulsions and brain anomalies (enlarged ventricles, abnormal pons). For several days a Mayo tube was needed because of respiratory problems (unilateral choanal atresia and laryngomalacia). He was fed by a G-tube and had GERD. A barium swallow was performed a few days before his death, showing abnormal swallowing movements and nasal regurgitation. At age 11.5 months, a viral upper respiratory tract infection was diagnosed and within 48 hours he died unexpectedly during the night. When his parents found him, he had a pink colour and was still warm; resuscitation was unsuccessful. No autopsy was performed and the cause of death is unknown. It was speculated that he died from respiratory aspiration or circulatory arrest (myocarditis or arrhythmia).

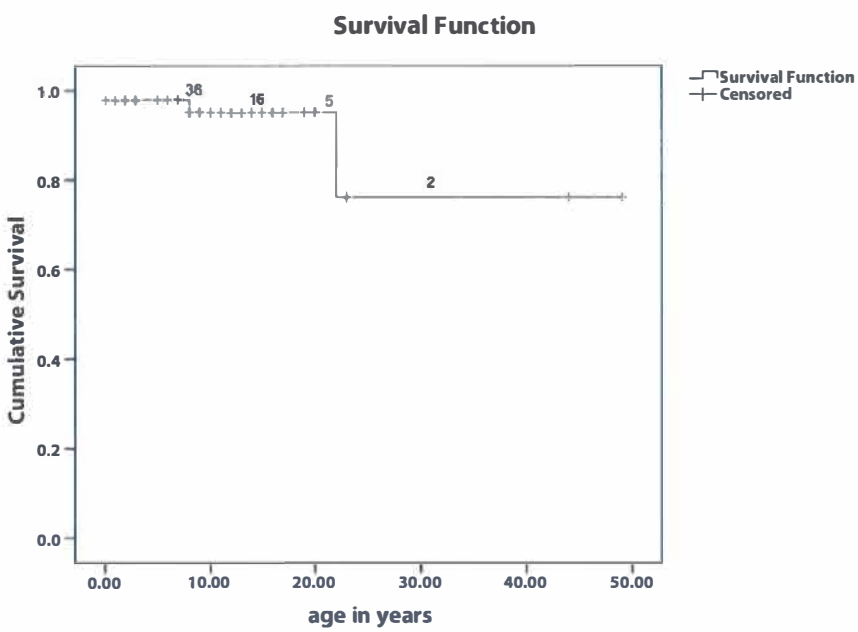

Figure 1. Kaplan-Meier curve. Kaplan-Meier post-neonatal actuarial survival of 48 patients with CHARGE syndrome. Along the curve is the number of patients that were still being followed up at the Dutch CHARGE outpatient clinic.

Patient D

Patient D (Figure 2D) had oesophageal atresia, a congenital heart defect (Fallot's tetralogy, aberrant course of subclavian artery and aorta and a dysplastic pulmonary valve) and brain anomalies (polymicrogyria and abnormal cerebellum). In addition, on ECG examination supraventricular tachycardias were recorded. He suffered from recurrent aspiration pneumonias and received tube feeding. Fundoplication was performed for GERD. When he was 13 months old, surgical correction of the Fallot's tetralogy was performed. The surgery and the post-operative stay in the hospital were uneventful and after four weeks he was discharged in good condition. A few days later, his parents found him dead in his bed at night (age at death 14 months). No autopsy was performed and the cause of death is unknown. He may have had an arrhythmic event, but this remains speculative without monitoring. In general, supraventricular arrhythmias do not cause sudden death at this age. It is also possible he died from respiratory aspiration.

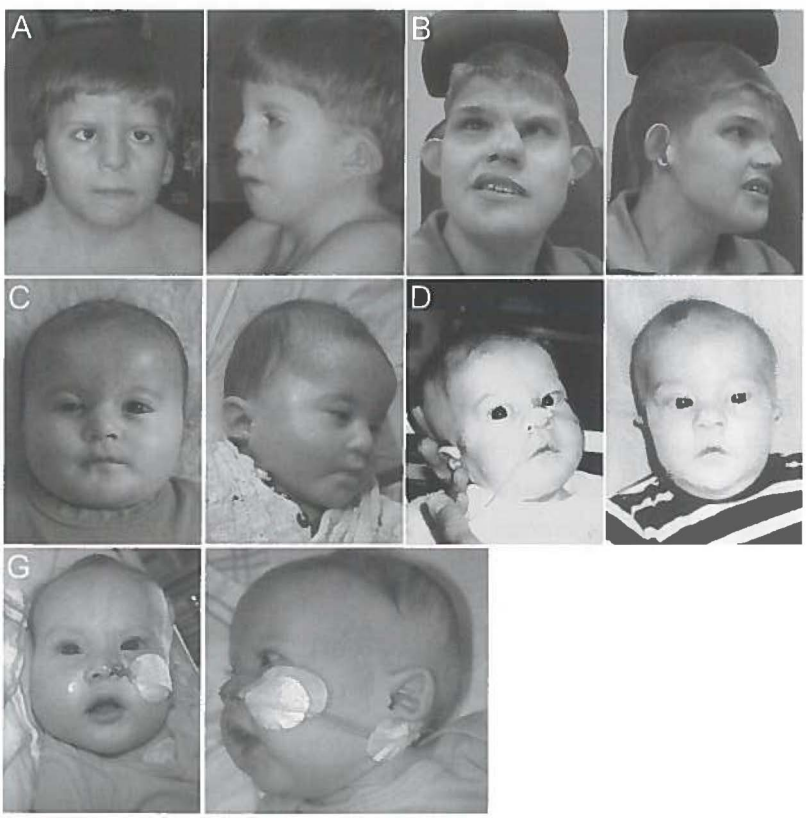

Figure 2. Photographs of deceased patients with CHARGE syndrome. (**A**) Patient A at age 7 years 6 months, with strabismus, broad short neck and low set dysmorphic ears with triangular conchae and absent ear lobes. (**B**) Patient B at age 20 years 10 months, with strabismus, left facial palsy, deviated nose and bilateral cup ears. (**C**) Child C at 3 months of age, showing a square face with small chin and low set dysmorphic ears without lobes. (**D**) Child D at 1 month (left) and 6 months (right) of age showing strabismus and dysmorphic ears. (**G**) Child G at 3 months of age, showing a square face with small chin and dysmorphic ears.

Patient E

Patient E had Fallot's tetralogy and bilateral choanal atresia. She had a G-tube for severe feeding problems and GERD. She died at age 27 months. The cause of death is unknown, but it was thought that severe GERD led to respiratory aspiration and therefore played a major role in her death. No autopsy was performed.

Patient F

Patient F had seizures and a complex heart defect (double outlet right ventricle, sub-aortic stenosis and ventricular septal defect). A G-tube was inserted at the age of 15 months because of feeding problems and withdrawn when he was 5 years old. The presence of GERD led to a fundoplication procedure. He suffered from recurrent bowel obstructions and sepsis, which was also the reason for his final admission to the hospital. After recovery from the bowel sepsis, he died suddenly during hospitalization at age 9 years. No autopsy was performed; as the bowel sepsis was no longer a problem at the time of death, other issues such as respiratory aspiration, were cited as contributing to his death.

Patient G

Patient G (Figure 2G) was born with bilateral choanal atresia, a complex heart defect (double outlet right ventricle, pulmonary valve stenosis and ventricular septal defect) and brain abnormalities (hypoplasia of the vermis, enlarged ventricles). Feeding difficulties and recurrent aspiration pneumonias required insertion of a G-tube. He underwent fundoplication surgery for GERD. At the age of 13 months, he had cardiovascular surgery complicated by post-operative respiratory problems, propofol intoxication, hypertriglyceridaemia, liver and kidney function disturbances, sepsis and hypotension. He developed a severe posthypoxic encephalopathy with cerebral atrophy. Contact was no longer possible and from then on he received only palliative treatment. He died at age 14 months. No autopsy was performed.

We compared the features of the survivors aged 10 years and older (n=25) with the patients who died before they were 10 years old (n=6) (Table 2). The most striking difference was seen for GERD, which was present in all of the deceased patients, but only in 25% of survivors before the age of 10 years ($p=0.002$, χ^2-test, univariate analysis). The combination of breathing and feeding difficulties with GERD was also significantly more often present in the deceased patients ($p=0.029$). Apart from congenital heart defects ($p=0.022$), none of the other clinical features was significantly over-represented in the deceased patients.

DISCUSSION

Post-neonatal demise is an under-estimated complication in patients with CHARGE syndrome. In our prospective cohort of 48 patients with CHARGE syndrome, three patients died after 11 months

of age, resulting in an actuarial post-neonatal survival of 95% and 76% at the ages of 10 and 25 years, respectively (Figure 1). This percentage is in line with previous studies that reported mortality rates between 7.5% and 30% in CHARGE patients older than one year.[8,11,16,17] It must be noted that our prospective cohort (from the Dutch CHARGE outpatient clinic) is biased. Very severely or very mildly affected patients are often under-represented in this kind of multidisciplinary clinics and some very severe cases may have already died at a young age. Therefore, this survival curve only applies to patients who survived the neonatal period.

Table 2. Comparison of features of surviving and deceased patients with CHARGE syndrome

	Surviving patients ≥ 10 years		Patients who died < 10 years		Comparison
Features	**n=25**	**%**	**n=6**	**%**	**p-value ***
Male gender	12/25	48.0%	5/6	83.3%	NS
Bilateral choanal atresia	5/25	20.0%	1/6	16.7%	NS
Congenital heart defect	7/25	28.0%	5/6	83.3%	0.022
Tracheo-oesophageal anomaly	1/25	4.0%	2/6	33.3%	NS
Breathing difficulties[a]	8/24[c]	33.3%	4/6	66.7%	NS
Brain malformation	13/17[d]	76.5%	4/4[d]	100.0%	NS
GERD	6/24	25.0%	6/6	100.0%	0.002
Feeding difficulties[b]	14/24	58.3%	6/6	100.0%	NS
Epilepsy	3/24	12.5%	1/6	16.7%	NS
Breathing + feeding difficulties + GERD	4/24	16.7%	4/6	66.7%	0.029
Breathing + feeding difficulties	7/24	29.2%	4/6	66.7%	NS

[a] Breathing difficulties: patient had needed oxygen or tracheotomy. [b] Feeding difficulties: patient had required (short- or long-term) tube feeding. [c] Information on this specific feature was unknown in one patient, who was therefore not included in the analysis. [d] Brain scans were performed in only 17/25 surviving patients and in 4/6 deceased patients. * p-value (χ^2-test, univariate analysis); NS, not significant. GERD, gastro-oesophageal reflux disease.

In this study, we describe seven cases of post-neonatal death. In most of these, death occurred suddenly and unexpectedly. In five patients the cause of death was likely attributable to respiratory aspiration and/or circulatory arrest. One patient choked during eating and another patient died after post-operative complications. Our observations are in line with a previous study that reported the causes of death in five patients with CHARGE syndrome older than one year to be circulatory arrest (n=2), respiratory aspiration (n=2) and postoperative complications (n=1).[17] Two other studies[8,11] proposed respiratory aspiration as a major cause of mortality. Our study supports this theory, with respiratory aspiration likely contributing to early death in 5/7 patients (Table 1). However, due to the predominantly retrospective nature of our study and the lack of autopsy data, the cause of death remains speculative in five patients. It is possible that other causes may have contributed to

death, like congenital heart defects (present in 3/5 patients), brain abnormalities (present in the two patients who had a cerebral magnetic resonance imaging (MRI) scan of these five patients), epilepsy (1/5), or oesophageal atresia (1/5).

Unlike respiratory aspiration, choking has not been reported before as a cause of premature death in CHARGE syndrome. We presented the tragic history of patient A, who choked during eating when he was 8 years old. A major risk factor for choking is status post-repair of oesophageal atresia, but subglottic stenosis, swallowing difficulties and voracious eating behaviour could also have contributed to his death.[18-21]

Peri-operative complications are another important cause of death in post-neonatal CHARGE syndrome patients. Post-operative mortality rates are higher in patients with CHARGE syndrome than in the general population. Since patients with CHARGE syndrome generally have to undergo several surgical procedures, this risk should not be underestimated.[8] Patients with airway malformations, severe feeding difficulties and GERD have a high risk of post-operative complications. Cardiovascular surgery in particular seems to pose the highest risk. Post-operative airway events occur after 35% of anaesthetic procedures in CHARGE syndrome.[21] It has been hypothesized that CN defects might underlie post-operative airway problems, since these nerves play an important role in the proper handling of oral secretions (which are increased after anaesthesia).[21] Problems can also occur pre-operatively, since intubation problems are often seen in patients with CHARGE syndrome. This is mainly due to congenital airway malformations (choanal atresia, cleft lip/palate, micrognathia in combination with an anterior larynx, subglottic stenosis and laryngeal clefts).[22,23]

GERD, feeding/breathing difficulties and congenital heart defects were identified as risk factors for early death in our outpatient clinic population. On comparing the features of the surviving patients of ten years and older (n=25) to the patients who died before ten years of age (n=6), we saw an overrepresentation of congenital heart defects and GERD in the deceased patients (Table 2). In a previous study, these features were found in 4/5 and 2/5 deceased patients, respectively.[17] Congenital heart defects can lead to post-neonatal death due to rhythm disturbances and circulatory stand-still. However, in patients with no prior history of rhythm disturbances, this is not a common cause of death. The other risk factors, feeding difficulties and GERD, both predispose to respiratory aspiration, which can lead to premature death. Both are seen in the majority of CHARGE patients.[8,17,22-24] Respiratory aspiration is also common in CHARGE syndrome (prevalence 47-74%).[17,22,25] This explains why the combination of feeding/breathing problems and GERD have been determined as risk factors for premature death in our study group.

CN dysfunction is prevalent in CHARGE syndrome and is likely to be the underlying common risk factor, as it can cause both swallowing/breathing problems and GERD. CN V, VII, IX, X and XII play an important role in the swallowing process, as they coordinate muscle movements and supply sensory information from the oral, pharyngeal and laryngeal region (Table 3).[24,26] Blake *et al* reported dysfunction of at least one CN in 92% of patients with CHARGE syndrome and described multiple CN involvement in 72%.[5] The underlying cause of CN dysfunction in CHARGE syndrome is unknown,

but abnormalities of the brainstem or CN nuclei could play a role. Unfortunately, hardly any research has been performed by MRI brain imaging or by post-mortem brain examination, to unravel the anatomical substrates underlying the CN anomalies in CHARGE syndrome. Detailed clinical assessment of CN function in CHARGE syndrome patients may also shed light upon the precise role of CN dysfunction in swallowing, aspiration, GERD and other possibly fatal complications.

Table 3. Overview of cranial nerve involvement in phases of normal swallowing

Swallowing phase	Action	I/V[a]	Cranial nerves[b]	
			Motor	Sensory
Oral phase	Mastication of the food and mixing it with saliva for passage into the oropharynx	V	V3, VII, XII	V, VII, IX
Pharyngeal phase	Swallow reflex: soft palate moves upward, larynx and hyoid bone move forward and upward, epiglottis folds back, tongue pushes food bolus into the hypopharynx	I	V3, VII, IX, X, XII	IX, X
Oesophageal phase	Downward movement of food bolus by a peristaltic wave from the upper to the lower oesophageal sphincter, entrance of food bolus into the stomach	I	X	X

[a] I, involuntary; V, voluntary. [b] V3, mandibular branch of trigeminal nerve; VII, facial nerve; IX, glossopharyngeal nerve; X, vagus nerve; XII, hypoglossal nerve

In order to prevent premature death in patients with CHARGE syndrome, we recommend that every CHARGE patient with feeding difficulties (at present or in the past) should be thoroughly assessed by a multidisciplinary team (preferably consisting of an ear-nose-throat (ENT) surgeon, gastroenterologist, physiotherapist, occupational therapist, speech therapist and nutritionist). CN function should be investigated and should include analysis of chewing/biting (V), facial muscles (VII), velum function (IX), larynx mobility and closure of glottis (X), gag and cough reflexes (IX/X), tongue movement (XII), and the assessment of sensibility of the oropharynx (V, VII), pharynx (IX) and larynx (X). Swallowing studies, laryngopharyngoscopy, oesophageal pH studies, oesophageal manometry, and polysomnography may also be appropriate.[27] In order to prevent respiratory aspiration, swallowing problems and GERD should be treated without delay. Possible treatments include gastrostomy, anti-reflux medication and fundoplication. Respiratory problems can necessitate tracheotomy, but sometimes injection of botulinum toxin (botox) into the salivary glands can avert this.[5,28] In patients with a high risk of choking (as evaluated by the multidisciplinary team), we advise training the family or home carers in cardiopulmonary resuscitation and the Heimlich manoeuvre. For all patients with CHARGE syndrome, careful pre-operative assessment is also important and surgical procedures should be combined whenever possible. In addition,

a paediatric anaesthesiologist and/or laryngologist should be present in the operating room to anticipate possible intubation problems. Patients with CHARGE syndrome should be monitored for longer after surgery in order to identify and treat post-operative airway events. Finally, we strongly advise to perform autopsy in deceased CHARGE patients in order to gain more insight into causes of death.

In conclusion, post-neonatal death is not a rare event in patients with CHARGE syndrome and it often occurs unexpectedly. Gastro-oesophageal reflux and poor coordination of swallowing and breathing due to CN dysfunction seem to be the major risk factors.

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REFERENCES

1 Issekutz KA, Graham JM, Jr., Prasad C, Smith IM, Blake KD. An epidemiological analysis of CHARGE syndrome: preliminary results from a Canadian study. *Am J Med Genet A* 2005;133(3):309-317.

2 Sanlaville D, Verloes A. CHARGE syndrome: an update. *Eur J Hum Genet* 2007;15(4):389-399.

3 Pagon RA, Graham JM, Jr., Zonana J, Yong SL. Coloboma, congenital heart disease, and choanal atresia with multiple anomalies: CHARGE association. *J Pediatr* 1981;99(2):223-227.

4 Amiel J, Attiee-Bitach T, Marianowski R, Cormier-Daire V, Abadie V, Bonnet D, et al. Temporal bone anomaly proposed as a major criteria for diagnosis of CHARGE syndrome. *Am J Med Genet* 2001;99(2):124-127.

5 Blake KD, Hartshorne TS, Lawand C, Dailor AN, Thelin JW. Cranial nerve manifestations in CHARGE syndrome. *Am J Med Genet A* 2008;146(5):585-592.

6 Chalouhi C, Faulcon P, Le Bihan C, Hertz-Pannier L, Bonfils P, Abadie V. Olfactory evaluation in children: application to the CHARGE syndrome. *Pediatrics* 2005;116(1):e81-e88.

7 Vissers LE, van Ravenswaaij CM, Admiraal R, Hurst JA, De Vries BB, Janssen IM, et al. Mutations in a new member of the chromodomain gene family cause CHARGE syndrome. *Nat Genet* 2004;36(9):955-957.

8 Blake KD, Russell-Eggitt IM, Morgan DW, Ratcliffe JM, Wyse RK. Who's in CHARGE? Multidisciplinary management of patients with CHARGE association. *Arch Dis Child* 1990;65(2):217-223.

9 Tellier AL, Cormier-Daire V, Abadie V, Amiel J, Sigaudy S, Bonnet D, et al. CHARGE syndrome: report of 47 cases and review. *Am J Med Genet* 1998;76(5):402-409.

10 Writzl K, Cale CM, Pierce CM, Wilson LC, Hennekam RC. Immunological abnormalities in CHARGE syndrome. *Eur J Med Genet* 2007;50(5):338-345.

11 Wyse RK, al-Mahdawi S, Burn J, Blake K. Congenital heart disease in CHARGE association. *Pediatr Cardiol* 1993;14(2):75-81.

12 Jongmans MC, Admiraal RJ, van der Donk KP, Vissers LE, Baas AF, Kapusta L, et al. CHARGE syndrome: the phenotypic spectrum of mutations in the *CHD7* gene. *J Med Genet* 2006;43(4):306-314.

13 Stromland K, Sjogreen L, Johansson M, Ekman Joelsson BM, Miller M, Danielsson S, et al. CHARGE association in Sweden: malformations and functional deficits. *Am J Med Genet A* 2005;133(3):331-339.

14 Blake KD, Davenport SL, Hall BD, Hefner MA, Pagon RA, Williams MS, et al. CHARGE association: an update and review for the primary pediatrician. *Clin Pediatr (Phila)* 1998;37(3):159-173.

15 Verloes A. Updated diagnostic criteria for CHARGE syndrome: a proposal. *Am J Med Genet A* 2005;133(3):306-308.

16 Lin AE, Siebert JR, Graham JM,Jr. Central nervous system malformations in the CHARGE association. *Am J Med Genet* 1990;37(3):304-310.

17 Sporik R, Dinwiddie R, Wallis C. Lung involvement in the multisystem syndrome CHARGE association. *Eur Respir J* 1997;10(6):1354-1355.

18 Jacob B, Wiedbrauck C, Lamprecht J, Bonte W. Laryngologic aspects of bolus asphyxiation-bolus death. *Dysphagia* 1992;7(1):31-35.

19 Stevenson DA, Heinemann J, Angulo M, Butler MG, Loker J, Rupe N, et al. Deaths due to choking in Prader-Willi syndrome. *Am J Med Genet A* 2007;143(5):484-487.

20 Wick R, Gilbert JD, Byard RW. Cafe coronary syndrome-fatal choking on food: an autopsy approach. *J Clin Forensic Med* 2006;13(3):135-138.

21 Blake K, Maccuspie J, Hartshorne TS, Roy M, Davenport SL, Corsten G. Postoperative airway events of individuals with CHARGE syndrome. *Int J Pediatr Otorhinolaryngol* 2009;73(2):219-226.

22 Roger G, Morisseau-Durand MP, Van Den Abbeele T, Nicollas R, Triglia JM, Narcy P, et al. The CHARGE association: the role of tracheotomy. *Arch Otolaryngol Head Neck Surg* 1999;125(1):33-38.

23 StackCG,Wyse RK. Incidence and management of airway problems in the CHARGE Association. *Anaesthesia* 1991;46(7):582-585.

24 Dobbelsteyn C, Marche DM, Blake K, Rashid M. Early oral sensory experiences and feeding development in children with CHARGE syndrome: a report of five cases. *Dysphagia* 2005;20(2):89-100.

25 White DR, Giambra BK, Hopkin RJ, Daines CL, Rutter MJ. Aspiration in children with CHARGE syndrome. *Int J Pediatr Otorhinolaryngol* 2005;69(9):1205-1209.

26 Sanderson RJ, Tierney P, Adamson R. Neurological disease of the pharynx. In: Gleeson M, editor. Scott-Brown's otorhinolaryngoloy, head and neck surgery, London: Arnold, 2008:2074-2082.

27 Rogers B, Arvedson J. Assessment of infant oral sensorimotor and swallowing function. *Ment Retard Dev Disabil Res Rev* 2005;11(1):74-82.

28 Daniel SJ. Alternative to tracheotomy in a newborn with CHARGE association. *Arch Otolaryngol Head Neck Surg* 2008;134(3):322-323.

CHAPTER 4.1

CHD7 mutations in patients initially diagnosed with Kallmann syndrome - the clinical overlap with CHARGE syndrome

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ABSTRACT

Kallmann syndrome (KS) is the combination of hypogonadotropic hypogonadism and anosmia or hyposmia, two features that are also frequently present in CHARGE syndrome. CHARGE syndrome is caused by mutations in the *CHD7* gene. We performed analysis of *CHD7* in 36 patients with KS and 20 patients with normosmic idiopathic hypogonadotropic hypogonadism (nlHH) in whom mutations in *KAL1*, *FGFR1*, *PROK2* and *PROKR2* genes were excluded. Three of 56 KS/nlHH patients had *de novo* mutations in *CHD7*. In retrospect, these three *CHD7*-positive patients showed additional features that are seen in CHARGE syndrome. *CHD7* mutations can be present in KS patients who have additional features that are part of the CHARGE syndrome phenotype. We did not find mutations in patients with isolated KS. These findings imply that patients diagnosed with hypogonadotropic hypogonadism and anosmia should be screened for clinical features consistent with CHARGE syndrome. If such features are present, particularly deafness, dysmorphic ears and/or hypoplasia or aplasia of the semicircular canals, *CHD7* sequencing is recommended.

INTRODUCTION

Kallmann syndrome (KS) is a congenital disorder that combines hypogonadotropic hypogonadism and anosmia.[1] Three modes of inheritance have been described: X-linked recessive, autosomal dominant and more rarely autosomal recessive. To date, several genes have been identified to cause KS, either alone or in combination. Mutations in these genes together account for approximately 30% of all cases. *KAL1* encodes the protein anosmin and is involved in the X-linked form of KS (KAL1, OMIM #308700).[2,3] Loss-of-function mutations in the fibroblast growth factor receptor-1 gene (*FGFR1*) cause a form of KS (KAL2, OMIM #147950) that is generally inherited in an autosomal dominant way.[4,5] Dodé *et al* reported in a further 10% of patients mutations in the prokineticin receptor-2 (*PROKR2,* KAL3, OMIM #607123*)* and prokineticin-2 (*PROK2,* KAL4, OMIM #607002*)* genes, encoding a cell surface receptor and one of its ligands, respectively.[6] Mutations of the ligand, *PROK2,* can cause KS as well as normosmic idiopathic hypogonadotropic hypogonadism (nIHH) within the same family.[6,7] The same intrafamilial phenotypic variability is seen in patients with *FGFR1* mutations.[4] Thus, KS is a phenotypically and genotypically heterogeneous disorder. Not only the degree of hypogonadism and anosmia may vary significantly, but also other symptoms including bimanual synkinesia and dental agenesis (*KAL1* and *FGFR1*), renal anomalies (*KAL1*) and cleft lip/palate (*FGFR1*) occur with variable frequency.[8]

CHARGE syndrome (OMIM #214800) is an autosomal dominant condition characterized by a variety of congenital anomalies including coloboma, heart defects, choanal atresia, retarded growth and development, genital hypoplasia, ear anomalies and deafness. Other commonly observed congenital defects are semicircular canal hypoplasia, facial nerve palsy, cleft lip/palate and tracheo-oesophageal fistula.[9] Our group has discovered *CHD7* as the causative gene in CHARGE syndrome.[10] Since this discovery, several authors have reported on the phenotypic spectrum of *CHD7*-positive patients, including patients without typical CHARGE syndrome.[11-13] Therefore, we presume that the mild end of the phenotypic spectrum of *CHD7* mutations is not yet completely explored.

Recent studies revealed that anosmia and abnormal olfactory bulb development, as well as hypogonadotropic hypogonadism, are almost consistent findings in CHARGE syndrome, indicating that the key features of KS are also present in CHARGE syndrome.[14-16] For this reason, it has been suggested by others that *CHD7* may be considered a candidate locus in suspected KS cases without known mutations.[8] This hypothesis is worthwhile exploring, also because mutations in *CHD7* can result in a much milder phenotype than the classical CHARGE syndrome phenotype. Therefore, we sequenced *CHD7* in a large group of patients diagnosed as KS or nIHH but without mutations in *KAL1*, *FGFR1*, *PROK2* and *PROKR2*.

MATERIALS AND METHODS

Patients

A cohort of seven Japanese patients with a clinical diagnosis of KS, without mutations in *KAL1*, *FGFR1*, *PROK2* and *PROKR2*, was screened for *CHD7* mutations.[17] The diagnosis KS in this cohort was

based on an underdevelopment of secondary sexual characteristics in combination with anosmia or hyposmia. Subsequently, the cohort was enlarged by 49 *KAL1*, *FGFR1*, *PROK2* and *PROKR2* negative North American patients with KS or nIHH. GnRH deficiency in this cohort was defined by (a) absent/incomplete puberty by age 18 yr; (b) serum testosterone <100 ng/dL in men or estradiol <20 pg/mL in women in association with low or normal levels of serum gonadotropins; (c) otherwise normal pituitary function; (d) normal serum ferritin concentrations; and (e) normal magnetic resonance imaging (MRI) of the hypothalamic-pituitary region.[5]

The patients in whom *CHD7* mutations were identified were carefully evaluated for clinical features of CHARGE syndrome. The *CHD7* gene was analyzed in the parents. The patients or their legal representatives gave informed consent for the DNA studies and the collection of clinical data. The studies were approved by the institutional review boards.

Mutation screening

DNA was isolated according to standard procedures. The 37 coding exons of the *CHD7* gene (exon 2-38, accession number NM_017780, NCBI) and their flanking intron sequences were amplified by polymerase chain reaction (PCR). Subsequently, sequence analysis was performed using a 3730 automated sequencer (Applied Biosystems, Foster City, CA). Primer information and PCR conditions are given in a previous report of our group.[11]

The DNA samples of eleven mutation-negative patients were subsequently screened for exon deletions and/or duplications of the *CHD7* gene by multiplex ligation-dependent probe amplification (MLPA) analysis (Table 1). We used a commercially available set of probes, the SALSA P201 kit (MRC-Holland, Amsterdam, The Netherlands; http://www.mrc-holland.com). Further details are described in our recent report on MLPA analysis of the *CHD7* gene.[18]

RESULTS

The *CHD7* gene was first screened in a cohort of seven *KAL1*, *FGFR1*, *PROK2* and *PROKR2* negative patients of Japanese descent (five males, two females). All had hypogonadotropic hypogonadism and anosmia, whereas some had additional symptoms. Their clinical features are summarized in Table 1 and patient 2 is shown in Figure 1.

In two of the seven patients a heterozygous mutation in *CHD7* was identified: one nonsense mutation (c.8803G>T; p.Glu2935X) and one missense mutation (c.6347T>A; p.Ile2116Asn). The mutations were proven to be *de novo* in both patients and were not present in 600 alleles of healthy controls.

The study cohort was extended by 49 North American patients (28 males, 21 females), including 29 patients with KS and 20 with nIHH of whom three had a positive family history for KS. Some of these patients had additional phenotypic features (Table 1). In one of the patients (patient 8), a *de novo* pathogenic nonsense mutation in *CHD7* was found (c.6070C>T; p.Arg2935X).

As whole exon deletions or duplications will be missed by sequence analysis, we performed

Table 1. Clinical characteristics of all patients and results of *CHD7* analysis

No.	Sex	Diagnosis	Additional Features	Family	Mutation *CHD7*	Parents	MLPA performed
1	M	KS	Dental agenesis, high-arched palate, unilateral perceptive deafness and short stature	Sp	c.8803G>T; p.Glu2935X; exon 38	*De novo*	-
2	M	KS	Cleft palate, auricular dysplasia, nystagmus, bilateral perceptive deafness, hypoplasia of semi-circular canals	Sp	c.6347T>A; p.Ile2116Asn;exon 31	*De novo*	-
3	F	KS		Sp	-		-
4	F	KS		Sp	-		-
5	M	KS	High-arched palate	Sp	-		-
6	M	KS	Ptosis	Sp	-		-
7	M	KS		Sp	-		-
8	F	KS	Facial nerve palsy, bilateral colobomas, cleft lip/palate, deafness, short stature and developmental delay	Sp	c.6070C>T; p.Arg2935X; exon 30	*De novo*	-
9	F	KS		Fam	-		+
10	M	KS		Fam	-		-
11	F	KS	Crohn's disease and syndactyly	Fam	-		-
12	M	KS		Fam	-		-
13	M	KS		Sp	-		+
14	F	KS		Sp	-		-
15	F	KS,	Choanal atresia	Fam	-		+
16	M	KS		Fam	-		-
17	M	KS	Congenital deafness and Hirschsprung's disease	Sp	-		-
18	F	KS		Fam	-		-
19	M	KS		Fam	-		-
20	F	KS		Fam	-		+
21	F	KS	Hearing impairment	Fam	-		-
22	M	KS	Deafness	Sp	-		-
23	F	KS	Multiple cranial nerve abnormalities	Sp	-		+
24	F	KS		Fam	-		-
25	F	KS		Sp	-		+
26	M	KS		Fam	-		-
27	M	KS	Hearing impairment	Sp	-		-
28	M	KS		Fam	-		-
29	M	KS		Fam	-		-
30	M	KS	Cryptorchidism	Fam	-		-
31	M	KS		Fam	-		-

32	F	KS	Narrow palate	Fam	-	-
33	F	KS	High-arched palate and hyperlaxity of hand joints	Fam	-	-
34	M	KS	Macrocephaly, hypertelorism, high arched palate, ataxia, Dandy Walker malformation and developmental delay	U	-	-
35	M	KS		Fam	-	-
36	M	Partial KS	Spinal muscular atrophy	Sp	-	-
37	M	IHH, KS in family	Cardiac septum defect	Fam	-	+
38	M	IHH, KS in family	Hearing impairment	Fam	-	+
39	M	IHH, KS in family		Fam	-	-
40	F	IHH		Fam	-	+
41	M	IHH		Sp	-	+
42	F	IHH	Cardiac septum defect	Sp	-	+
43	M	IHH	Cryptorchidism	Fam	-	-
44	M	IHH	Growth hormone deficient	Fam	-	-
45	F	IHH		Fam	-	-
46	F	IHH		Fam	-	-
47	M	IHH	Cryptorchidism, blind, seizures, mental retardation and short stature	Sp	-	-
48	F	IHH		Fam	-	-
49	M	IHH	Ataxia	Sp	-	-
50	F	IHH		Fam	-	-
51	F	IHH		Fam	-	-
52	M	IHH		Fam	-	-
53	M	IHH		Fam	-	-
54	M	IHH	Developmental delay and high arched palate	Fam	-	-
55	M	IHH		Fam	-	-
56	F	IHH		Fam	-	-

[a]Patients 1-7 are of Japanese descent and patients 8-56 are from North America.
F, female; Fam, familial; IHH, idiopathic hypogonadotropic hypogonadism; KS, Kallmann syndrome (IHH + anosmia); M, male; MLPA, multiplex ligation-dependent probe amplification; partial KS, patient with IHH and anosmia, with some degree of spontaneous pubertal development; Sp, sporadic; U, unknown.

MLPA analysis. Due to a limited amount of available DNA, we were only able to finish this analysis in eleven patients. Two patients with a relatively high suspicion for CHARGE syndrome based on the features choanal atresia and multiple cranial nerve anomalies (respectively, patient 15 and 23; Table

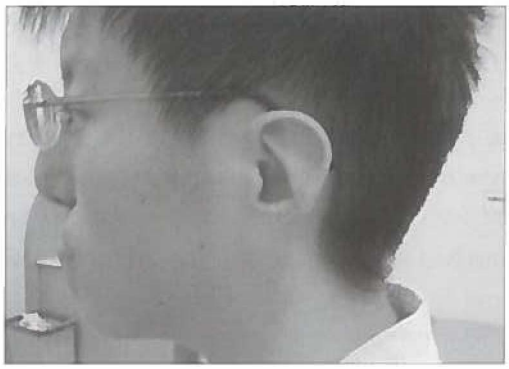

Figure 1. Lateral view of patient 2. Note the dysmorphic ears with absence of the earlobe and the lower helical fold, and a triangular concha. These dysmorphisms are typical for CHARGE syndrome.

1) were among those eleven patients. No exon copy number alterations were found.

The main features of the three patients carrying a mutation in *CHD7* are given in Table 1. All three patients were proven to be anosmic by formal smell tests. Audiometry revealed a left-sided hearing impairment of 70 dB in patient 1, a bilateral hearing impairment of 60-90 dB in patient 2, and left-sided complete sensorineural deafness and right-sided partial conductive hearing impairment in patient 8. Patient 1 had agenesis of four permanent teeth, the first upper and lower molars. No choanal atresia or heart defects were present in patients 1, 2 and 8. Colobomas were present in patient 8 but excluded by fundoscopy in patients 1 and 2. Patient 2 experienced feeding difficulties during infancy, but these were ascribed to the cleft palate. The dysmorphisms of the ears of patient 2 are very characteristic for CHARGE syndrome with absence of the earlobe and the lower helical fold, and a typical triangular concha (Figure 1). After identification of the *CHD7* mutation, a CT scan of the os petrosum showed bilateral hypoplasia of the semicircular canals. In patients 1 and 8, imaging studies of the temporal bones were not possible. Upon re-evaluation, patient 8 has not only deafness and bilateral colobomas but also left-sided facial nerve palsy, cleft lip and palate, short stature and developmental delay.

In retrospect, patients 2 and 8 have typical CHARGE syndrome according to the commonly used clinical criteria[9] while patient 1 has only some features of this syndrome.

DISCUSSION

Hypogonadotropic hypogonadism is a frequent feature in CHARGE syndrome. Chalouhi *et al* tested the olfactory function of 14 children with CHARGE syndrome and showed that all children had some degree of olfactory deficiency.[14] Pinto *et al* showed that olfactory deficiency and abnormal olfactory bulbs were present in all 18 CHARGE syndrome patients in their cohort.[15]

These observations prompted us to analyze the *CHD7* gene in 36 patients with KS and 20 patients with nlHH lacking mutations in *KAL1*, *FGFR1*, *PROK2* and *PROKR2*. *CHD7* mutations were identified by sequence analysis in two of seven Japanese KS patients and in one of 49 KS/nlHH North American

patients. By routine sequencing of the *CHD7* gene, we may have missed mutations located deep in introns or in the promoter region. Furthermore, MLPA analysis could not be performed in all patients.

Hypogonadism in KS is caused by GnRH deficiency. GnRH neurons of the forebrain are thought to originate from the nasal placode. During embryonic development, they migrate alongside the olfactory axons towards the hypothalamus. Mutations in *KAL1, FGFR1, PROK2* and *PROKR2* can result in hypogonadotropic hypogonadism and anosmia. Therefore, the protein products of these genes are thought to be involved in this combined migration process.[8,19] Since hypogonadotropic hypogonadism and anosmia are frequently present in CHARGE syndrome as well, it is possible that the same embryonic migration process is disturbed in CHARGE syndrome. *CHD7* encodes a protein of the chromodomain (chromatin organisation modifier) family. This family shares a unique combination of functional domains consisting of two N-terminal chromodomains, followed by a SWI2/SNF2-like ATPase/-helicase domain and a DNA-binding domain. It is assumed that CHD protein complexes affect chromatin structure and gene expression and thereby play important roles in regulating embryonic development.[20] Therefore, one might speculate that CHD7 has a possible influence on the expression or actions of *KAL1, FGFR1, PROK2* and/or *PROKR2* during development. However, because mutations in these genes account for only 30% of all KS cases, it is possible that CHD7 impacts on other, yet undiscovered, KS genes.

We identified a *de novo CHD7* mutation in three patients initially diagnosed with KS. The two nonsense mutations are known to be pathogenic. The missense mutation p.Ile2116Asn is not located in one of the known protein domains of the CHD7 protein, but it concerns a drastic amino acid change that has not been detected in over 600 control alleles. Furthermore, the p.Gly2108Arg mutation has been shown to be associated with CHARGE syndrome in two families with a variable phenotype, indicating that this part of the protein probably has an important function.[12] This indicates that the p.Ile2116Asn mutation is possibly pathogenic.

In retrospect two of the three *CHD7* positive patients (Patients 2 and 8) had typical CHARGE syndrome with the presence of at least three major features.[9] Patient 1 presented with only two additional CHARGE features (short stature and unilateral hearing impairment), although one should notice that vestibular function was not tested in this patient.

From this study, we conclude that it is important to evaluate patients with hypogonadotropic hypogonadism and anosmia for clinical features characteristic of CHARGE syndrome. All three patients were proven to be anosmic. Therefore, the chance to find a *CHD7* mutation seems higher in anosmic patients although the study group is too small to conclude that *CHD7* mutations cannot occur in patients with normosmic IHH. Indeed some patients with CHARGE syndrome are able to smell (personal observations). Because all three patients suffered from hearing impairment, it is tempting to regard this feature as discriminating. However, sensorineural hearing impairment is also an associated feature in males with *KAL1* mutations. Thus, hearing abnormalities may be a sensitive but not very specific symptom of *CHD7* mutations. Hypoplasia or aplasia of the semicircular canals is a much more consistent feature in CHARGE syndrome, even in mildly affected patients.[9,12] Therefore,

history taking regarding balance disturbances and gross motor development might reveal indicative information for the presence of a *CHD7* mutation. Abadie *et al* have described a specific pattern of postural behaviour related to vestibular anomalies in CHARGE syndrome. They noticed a frequent inability to crawl on all fours without resting the head on the floor (5-point crawl), a prolonged duration of standing with support stage and an inability to ride a bike without stabilizers.[21] After the first years of life, balance disturbances may not be unequivocally present as a result of visual compensation. In these patients, disequilibrium in the dark is a helpful indication of vestibular deficit. If there is doubt about the vestibular function, screening for vestibular areflexia or imaging of the semicircular canals will be helpful. In the newborn, agenesis of the semicircular canals can be visualized on plain profile X-ray of the skull.[9] In older patients, computerized tomography or MRI is necessary.

Finally, dilated fundus examination can be performed to reveal an optic disc coloboma. A less invasive, but of course also less accurate method, would be to ask for the presence of an optic field defect.

CHD7 screening in the large North-American cohort revealed only one mutation. In general, these patients underwent a more extensive clinical work-up.[5] From this cohort, we learned that it is not useful to screen the *CHD7* gene in each patient diagnosed with KS or nIHH; additional CHARGE features should be present. Such additional features do not imply that a *CHD7* mutation will be present as has been demonstrated by patient 15 who has choanal atresia but no *CHD7* mutation.

The patients carrying a mutation in *CHD7* in this cohort and the mild *CHD7*-positive patients reported by us in a previous study[12] show that the current diagnostic criteria can not always discriminate between patients with and without a mutation in *CHD7*.[9,12]

We conclude that it is useful to screen patients with hypogonadotropic hypogonadism and anosmia for clinical features consistent with CHARGE syndrome, particularly hearing impairment, vestibular dysfunction and dysmorphisms of the ears. If additional features of CHARGE syndrome are present, *CHD7* sequencing is recommended.

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REFERENCES

1 Kallmann FJ, Schoenfeld WA, Barrera SE. The genetic aspects of primary eunuchoidism. *Am J Ment Defic* 1944;48:203-236.

2 Franco B, Guioli S, Pragliola A, Incerti B, Bardoni B, Tonlorenzi R, et al. A gene deleted in Kallmann's syndrome shares homology with neural cell adhesion and axonal path-finding molecules. *Nature* 1991;353(6344):529-536.

3 Legouis R, Hardelin JP, Levilliers J, Claverie JM, Compain S, Wunderle V, et al. The candidate gene for the X-linked Kallmann syndrome encodes a protein related to adhesion molecules. *Cell* 1991;67(2):423-435.

4 Dode C, Levilliers J, Dupont JM, De Paepe A, Le Du N, Soussi-Yanicostas N, et al. Loss-of-function mutations in *FGFR1* cause autosomal dominant Kallmann syndrome. *Nat Genet* 2003;33(4):463-465.

5 Pitteloud N, Meysing A, Quinton R, Acierno JS,Jr., Dwyer AA, Plummer L, et al. Mutations in fibroblast growth factor receptor 1 cause Kallmann syndrome with a wide spectrum of reproductive phenotypes. *Mol Cell Endocrinol* 2006;254-255:60-69.

6 Dode C, Teixeira L, Levilliers J, Fouveaut C, Bouchard P, Kottler ML, et al. Kallmann syndrome: mutations in the genes encoding prokineticin-2 and prokineticin receptor-2. *PLoS Genet* 2006;2(10):1648-1652.

7 Pitteloud N, Zhang C, Pignatelli D, Li JD, Raivio T, Cole LW, et al. Loss-of-function mutation in the prokineticin 2 gene causes Kallmann syndrome and normosmic idiopathic hypogonadotropic hypogonadism. *Proc Natl Acad Sci USA* 2007;104(44):17447-17452.

8 Kim SH, Hu Y, Cadman S, Bouloux P. Diversity in fibroblast growth factor receptor 1 regulation: learning from the investigation of Kallmann syndrome. *J Neuroendocrinol* 2008;20(2):141-163.

9 Sanlaville D, Verloes A. CHARGE syndrome: an update. *Eur J Hum Genet* 2007;15(4):389-399.

10 Vissers LE, van Ravenswaaij CM, Admiraal R, Hurst JA, De Vries BB, Janssen IM, et al. Mutations in a new member of the chromodomain gene family cause CHARGE syndrome. *Nat Genet* 2004;36(9):955-957.

11 Jongmans MC, Admiraal RJ, van der Donk KP, Vissers LE, Baas AF, Kapusta L, et al. CHARGE syndrome: the phenotypic spectrum of mutations in the *CHD7* gene. *J Med Genet* 2006;43(4):306-314.

12 Jongmans MC, Hoefsloot LH, van der Donk KP, Admiraal RJ, Magee A, van de Laar I, et al. Familial CHARGE syndrome and the *CHD7* gene: a recurrent missense mutation, intrafamilial recurrence and variability. *Am J Med Genet A* 2008;146(1):43-50.

13 Lalani SR, Safiullah AM, Fernbach SD, Harutyunyan KG, Thaller C, Peterson LE, et al. Spectrum of *CHD7* mutations in 110 individuals with CHARGE syndrome and genotype-phenotype correlation. *Am J Hum Genet* 2006;78(2):303-314.

14 Chalouhi C, Faulcon P, Le Bihan C, Hertz-Pannier L, Bonfils P, Abadie V. Olfactory evaluation in children: application to the CHARGE syndrome. *Pediatrics* 2005;116(1):e81-e88.

15 Pinto G, Abadie V, Mesnage R, Blustajn J, Cabrol S, Amiel J, et al. CHARGE syndrome includes hypogonadotropic hypogonadism and abnormal olfactory bulb development. *J Clin Endocrinol Metab* 2005;90(10):5621-5626.

16 Ogata T, Fujiwara I, Ogawa E, Sato N, Udaka T, Kosaki K. Kallmann syndrome phenotype in a female patient with CHARGE syndrome and *CHD7* mutation. *Endocr J* 2006;53(6):741-743.

17 Sato N, Katsumata N, Kagami M, Hasegawa T, Hori N, Kawakita S, et al. Clinical assessment and mutation analysis of Kallmann syndrome 1 (*KAL1*) and fibroblast growth factor receptor 1 (*FGFR1*, or *KAL2*) in five families and 18 sporadic patients. *J Clin Endocrinol Metab* 2004;89(3):1079-1088.

18 Bergman JE, de Wijs I, Jongmans MC, Admiraal RJ, Hoefsloot LH, Ravenswaaij-Arts CM. Exon copy number alterations of the *CHD7* gene are not a major cause of CHARGE and CHARGE-like syndrome. *Eur J Med Genet* 2008;51(5):417-425.

19 Oliveira LM, Seminara SB, Beranova M, Hayes FJ, Valkenburgh SB, Schipani E, et al. The importance of autosomal genes in Kallmann syndrome: genotype-phenotype correlations and neuroendocrine characteristics. *J Clin Endocrinol Metab* 2001;86(4):1532-1538.

20 Higgs DR, Vernimmen D, Hughes J, Gibbons R. Using genomics to study how chromatin influences gene expression. *Annu Rev Genomics Hum Genet* 2007;8:299-325.

21 Abadie V, Wiener-Vacher S, Morisseau-Durand MP, Poree C, Amiel J, Amanou L, et al. Vestibular anomalies in CHARGE syndrome: investigations on and consequences for postural development. *Eur J Pediatr* 2000;159(8):569-574.

CHAPTER 4.2

The results of *CHD7* analysis in clinically well-characterised patients with Kallmann syndrome

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Submitted

ABSTRACT

Context Kallmann syndrome (KS) and CHARGE syndrome are rare heritable disorders in which anosmia and hypogonadotropic hypogonadism co-occur. KS is genetically heterogeneous and there are at least eight genes involved in its pathogenesis, whereas CHARGE syndrome is caused by autosomal dominant mutations in only one gene, the *CHD7* gene. Two independent studies showed that *CHD7* mutations can also be found in a small minority of KS patients.

Objective We aimed to investigate whether *CHD7* mutations can give rise to isolated KS or whether additional features of CHARGE syndrome always occur.

Design We performed *CHD7* analysis in a cohort of 36 clinically well-characterised Dutch patients with KS but without mutations in *KAL1*. The KS genes with known incomplete penetrance, *FGFR1*, *PROK2*, *PROKR2* and *FGF8*, were also studied.

Results We identified six heterozygous *PROKR2* mutations, one heterozygous *FGF8* mutation and three heterozygous *CHD7* mutations. The *CHD7*-positive patients were carefully re-examined and were all found to have additional features of CHARGE syndrome.

Conclusions The yield of *CHD7* analysis in patients with isolated KS seems very low, but increases when additional CHARGE features are present. Therefore, we recommend performing *CHD7* analysis in KS patients who have at least two additional CHARGE features or semicircular canal anomalies. Identifying a *CHD7* mutation has important clinical implications for the surveillance and genetic counselling of patients.

INTRODUCTION

Kallmann syndrome (KS) is characterised by the combination of anosmia and hypogonadotropic hypogonadism (HH).[1] Anosmia, or the inability to smell, is the result of olfactory bulb defects,[2,3] while HH presents as absent or impaired pubertal maturation and is caused by gonadotropin-releasing hormone (GnRH) deficiency.[4] Besides anosmia and HH, other features are occasionally present in KS, e.g. bimanual synkinesia, unilateral renal agenesis, cleft lip/palate, hypodontia, hearing loss and, rarely, hand or feet anomalies.[5,6] KS is very heterogeneous, both clinically and genetically. At present, eight genes are known to be involved in KS, but mutations in these genes are found in only 25-35% of cases.[7-12] Inheritance patterns are variable in KS: X-linked recessive (*KAL1*[13,14]), autosomal dominant (*FGFR1*,[15] *FGF8*,[16] *WDR11*[11] and *CHD7*[9,10]), autosomal recessive (*PROK2*, *PROKR2*[17] and *NELF*[12]) and digenic inheritance have been described.[16-21] Mutations in *KAL1* are always associated with HH and a smell deficit in males, whereas mutations in *FGFR1*, *PROK2*, *PROKR2* and *FGF8* show variable expression and incomplete penetrance, indicating that patients can be asymptomatic, can have solely anosmia, solely HH, or KS.[22] Heterozygous loss-of-function mutations in the *CHD7* gene were identified in patients with normosmic idiopathic HH (nlHH), KS and CHARGE syndrome.[9,10]

CHARGE syndrome is a highly variable disorder in which congenital anomalies, multi-sensory impairment and variable mental retardation can occur.[23] CHARGE is an acronym for ocular coloboma, heart defects, atresia of choanae, retardation of growth and/or development, genital hypoplasia and ear anomalies combined with deafness.[24] The phenotypic spectrum of CHARGE syndrome is very broad, with some patients having few or mild features and other patients being severely affected.[25] HH and anosmia are present in the majority of patients with CHARGE syndrome.[26] Recently, we showed that HH and anosmia always co-occur in CHARGE syndrome,[27] which means that KS is part of the phenotypic spectrum of CHARGE syndrome. The *CHD7* gene is the major gene involved in CHARGE syndrome, with a *CHD7* mutation being present in more than 90% of the patients who fulfil the clinical criteria for CHARGE syndrome.[26,28-30]

Conversely, *CHD7* mutations are not a major cause of KS, as only 3-5% of patients with nlHH or KS were found to have a *CHD7* mutation in two independent studies.[9,10] The first study identified seven *CHD7* mutations in a cohort of 197 patients with nlHH or KS (7/197 = 3.6%).[10] Four of the *CHD7*-positive patients were reported to have nlHH, while three patients had anosmia and thus KS. In the *CHD7*-positive patients, no other features of CHARGE syndrome were present, except for cleft lip/palate and hearing loss, which can occur in both CHARGE and Kallmann syndromes. The authors concluded that *CHD7* mutations can give rise to isolated nlHH and KS. Unfortunately, it is not clear whether formal smell tests were used in this study or whether all the patients with a *CHD7* mutation were carefully re-evaluated for CHARGE features, e.g. fundoscopy to screen for retinal coloboma. The second study was performed by our group and found three *CHD7* mutations in a cohort of 56 nlHH/KS patients from Japan and North America in whom mutations in *KAL1*, *FGFR1*, *PROK2* and *PROKR2* had been excluded (3/56 = 5.4%).[9] The three *CHD7*-positive patients were all diagnosed with KS, but on extensive clinical re-evaluation were found to have several other features

of CHARGE syndrome. It was concluded that *CHD7* mutations in KS patients represent the mild end of the CHARGE spectrum and that it is only useful to perform *CHD7* analysis in KS patients who have additional features of CHARGE syndrome.

Because of the conflicting data from these two studies, we decided to investigate whether *CHD7* mutations can give rise to isolated KS in an independent cohort. We therefore analysed the *CHD7* gene in 36 clinically well-characterised Dutch patients with KS but without mutations in *KAL1*. The KS genes with known incomplete penetrance, *FGFR1*, *PROK2*, *PROKR2*, and *FGF8*, were also studied. The patients found to have a *CHD7* mutation were carefully re-evaluated for features of CHARGE syndrome.

PATIENTS AND METHODS

Patients

A cohort of 36 Dutch KS patients (7 women, 29 men), without a hemizygous mutation in *KAL1* (*KAL1* analysis was only performed in male patients), was informed about this study via their paediatric endocrinologist, endocrinologist, gynaecologist or clinical geneticist. The patients gave their informed consent for sequence analysis of *FGFR1*, *PROK2*, *PROKR2*, *FGF8* and *CHD7* and for collection of their medical data via a questionnaire and/or retrospective chart review. The KS diagnosis was based on the presence of HH in combination with a smell deficit. HH was defined as no pubertal maturation in combination with low serum gonadotropins and sex steroids. The smell deficit was identified from the patient's history and/or formal smell testing. The *CHD7*-positive patients who were identified in this study, were carefully re-evaluated for features of CHARGE syndrome and underwent formal smell testing (University of Pennsylvania Smell Identification test, UPSIT, Sensonics Inc, Haddon Heights, New Jersey, USA; www.sensonics.com[31]). This study was approved by the ethical review board of the University Medical Centre Groningen (UMCG).

DNA analysis

DNA was extracted from peripheral blood lymphocytes using standard procedures. All individual exons of the respective genes were amplified by polymerase chain reaction (PCR, primers and PCR conditions are available upon request) and direct sequencing was performed on an ABI 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The following GenBank accession numbers were used as reference sequences for the *KAL1*, *FGFR1*, *PROK2*, *PROKR2*, *FGF8* and *CHD7* genes, respectively: NM_000216.2, NM_023110.2, NM_021935.3, NM_144773.2, NM_033163.3 and NM_017780.2. In all cases, the A of ATG was designated number 1. The intron sequences of the *KAL1*, *FGFR1*, *PROK2*, *PROKR2*, *FGF8* and *CHD7* genes can be found in NG_007088.1, NG_007729.1, NG_008275.1, NG_008132.1, NG_007151.1 and NG_007009.1, respectively.

RESULTS

The clinical features of the 36 Dutch KS patients and the results of DNA analysis are summarised in Table 1. A heterozygous *CHD7* mutation was identified in three patients: two patients had a nonsense mutation (c.4015C>T; p.Arg1339X and c.5316G>A; p.Trp1772X) and one patient had a missense mutation (c.6322G>A; p.Gly2108Arg). The missense mutation had occurred *de novo* and was previously identified in three other index patients with CHARGE syndrome (Janssen *et al*, submitted, see also the locus-specific *CHD7* mutation database available at www.CHD7.org).

The three *CHD7*-positive patients were carefully re-examined for additional features of CHARGE syndrome. Patient 1 had been diagnosed with KS at the age of 16 and was then started on hormone replacement therapy (HRT). She had a bilateral cleft lip and palate and bilateral mixed hearing loss. She was 20 years old when she was re-evaluated at the UMCG's multidisciplinary CHARGE outpatient clinic. Her history revealed delayed motor development (independent walking at age 2.5 years) and feeding difficulties that had been attributed to the cleft lip and palate. She also had balance disturbance (but a CT scan of the temporal bone had not been performed), mild scoliosis, and impaired vision. We referred her for ophthalmological examination, which revealed bilateral retinal colobomas. Her external ears were normal and ultrasound of the heart and kidneys showed no abnormalities. Anosmia was confirmed by formal smell testing (UPSIT score 12/40) and re-evaluation of an earlier MRI brain scan showed olfactory bulb aplasia. In retrospect, she has typical CHARGE syndrome.

Patient 2 had been diagnosed with KS when he was 16 years old and was started on HRT. He also had severe bilateral hearing loss. He was re-examined at the age of 31 at the Department of Human Genetics, Radboud University Nijmegen Medical Centre. His case history revealed mildly delayed motor development and surgery for a cleft palate. Physical examination showed that he had short stature (length -2.5 SD), synkinesia and abnormal external ears with small earlobes. An ultrasound of the heart and kidneys was performed after identification of the *CHD7* mutation and revealed a bicuspid aortic valve and normal kidneys. Formal smell testing showed that he had anosmia (UPSIT score 7/40).

Patient 3 had been diagnosed with KS at the age of 14 years and started on HRT. He also had severe bilateral sensorineural hearing loss. His clinical data were recently published.[25] He was re-evaluated at the age of 17 years at the UMCG's CHARGE outpatient clinic. His case history revealed delayed motor development (independent walking at 22 months) and balance disturbance. He had a normal intelligence and normal external ears, eyes, kidneys and heart. Anosmia was confirmed with the UPSIT (score 9/40). A CT scan of the temporal bone showed hypoplasia of the cochlea and semicircular canals. This patient did not fulfil the clinical criteria for CHARGE syndrome.[28,30]

On analysis of *FGFR1*, *PROK2*, *PROKR2* and *FGF8*, seven additional mutations were found in our Dutch KS cohort. Six patients harboured a heterozygous missense variant in the *PROKR2* gene (c.254G>A; p.Arg85His (4x), c.254G>T; p.Arg85Leu and c.791G>A; p.Arg264His) and in one patient a heterozygous *FGF8* variant (c.86_103dup; p.Gly29_Arg34dup) was identified. The *FGF8* variant is

Table 1. Dutch patients with Kallmann syndrome who did not have a mutation in the *KAL1* gene: results of *FGFR1*, *PROK2*, *PROKR2*, *FGF8* and *CHD7* analysis and an overview of the clinical characteristics and family history

Patient no. (n=36)	Sex[a]	FGFR1, PROK2, PROKR2 and FGF8 results[b]	CHD7 results[c]	Clinical characteristics besides HH and anosmia[d]	Family history[e]
1	F	-	c.4015C>T; p.Arg1339X	Cleft lip and palate, mixed hearing loss, retinal coloboma, balance disturbance, mild scoliosis, olfactory bulb aplasia	-
2	M	-	c.5316G>A; p.Trp1772X	Hearing loss, cleft palate, short stature, bicuspid aortic valve, abnormal external ears, synkinesia	-
3	M	-	c.6322G>A; p.Gly2108Arg *de novo*	Sensorineural hearing loss, hypoplasia of cochlea and semicircular canals	-
4	M	*PROKR2*: c.254G>A; p.Arg85His	-	Obesity (BMI 35 kg/m^2)	?
5	M	*PROKR2*: c.254G>A; p.Arg85His	-	-	?
6	M	*PROKR2*: c.254G>A; p.Arg85His	-	Hypospadia	-
7	F	*PROKR2*: c.254G>A; p.Arg85His	-	Myopia (-10 dioptres)	Grandmother anosmia, uncle cleft lip/palate
8	M	*PROKR2*: c.254G>T; p.Arg85Leu	-	Myopia (-5 dioptres)	-
9	F	*PROKR2*: c.791G>A; p.Arg264His	-	Unilateral renal agenesis, synkinesia	Father unilateral renal agenesis
10	F	*FGF8*: c.86_103dup; p.Gly29_Arg34dup	-	-	-
11	F	-	-	-	Mother and sister anosmia
12	F	-	-	Myopia (-10 dioptres)	?
13	F	-	-	-	?
14	M	-	-	Obesity	-
15	M	-	-	-	Mother primary amenorrhea and anosmia
16	M	-	-	-	Two sisters smell deficit

17	M	-	-	Obesity	?
18	M	-	-	-	?
19	M	-	-	-	Mother Kallmann syndrome
20	M	-	-	-	-
21	M	-	-	Myopia (-9.5 dioptres)	Sister and aunt decreased fertility
22	M	-	-	Epilepsy and brachycephaly	?
23	M	-	-	Cleft lip/palate	?
24	M	-, *FGF8* pending	-	-	?
25	M	-	-	Obesity	-
26	M	-	-	-	-
27	M	-	-	Epilepsy, obesity	-
28	M	-	-	-	-
29	M	-	-	-	-
30	M	-	-	Obesity	-
31	M	-	-	Neonatal hypotonia, bifid scrotum	-
32	M	-	-	Deafness	-
33	M	-	-	-	7 family members with HH, anosmia and/ or cleft lip/palate
34	M	-	-	-	-
35	M	-	-	-	-
36	M	-	-	-	-

[a] F, female; M, male
[b] -, normal results of *FGFR1*, *PROK2*, *PROKR2* and *FGF8* analysis
[c] -, normal result of *CHD7* analysis
[d] -, no additional features of CHARGE and/or Kallmann syndromes; BMI, body mass index; HH, hypogonadotropic hypogonadism
[e] -, negative family history; ?, unknown family history

located in the differentially spliced exon 1C of the *FGF8* gene and only affects FGF8e and FGF8f isoforms (and not FGF8a and FGF8b isoforms).

DISCUSSION

We have identified three *CHD7* mutations in 36 Dutch KS patients who had no mutation in the *KAL1* gene (3/36 = 8.3%). Here, we only studied KS patients and did not include patients with nIHH, because we have previously shown that HH is always associated with anosmia in patients with a *CHD7* mutation.[27] The three *CHD7*-positive patients that we identified all proved to have additional features of CHARGE syndrome on careful re-examination, which is in agreement with our previous study.[9] Two of the *CHD7*-positive patients had typical CHARGE syndrome (patients 1 and 2), but the third patient had only a few features of CHARGE syndrome. However, he did have semicircular canal anomalies, which are typical for CHARGE syndrome.[30,32,33] Hearing loss was the most frequent feature seen in the *CHD7*-positive KS patients in our study (Table 1) and in other published studies[9,10]; it was found in 7/13 *CHD7*-positive KS patients. However, hearing loss is not specific for the presence of a *CHD7* mutation, because it can also occur in patients with a mutation in the *KAL1*, *FGFR1* or *FGF8* gene.[6,16] Other features that were repeatedly found in *CHD7*-positive KS patients were a cleft lip and/or palate (5/13), short stature (3/13) and balance disturbance (3/13) (Table 1, [9,10]).

We did not identify a *CHD7* mutation in 30 KS patients without additional CHARGE features (Table 1), which suggests that *CHD7* mutations are not a frequent cause of isolated KS. Recently, *CHD7* analysis was also performed in a cohort of 30 Finnish KS patients.[34] Although three KS patients displayed additional CHARGE features, no *CHD7* mutations were identified in this cohort. Further studies in large cohorts of clinically well-characterised KS patients are needed to estimate the frequency of *CHD7* mutations in KS patients more reliably. In addition, it would be useful to know whether the *CHD7*-positive patients in the study by Kim *et al*[10] underwent formal smell testing and were carefully re-evaluated after the *CHD7* mutation was identified. Without extensive clinical examination, it is possible that subtle features of CHARGE syndrome were missed in their *CHD7*-positive patients, thereby undermining their claim that *CHD7* mutations can give rise to isolated nIHH and KS. This is well illustrated by our patients 1 and 3 in whom retinal colobomas and semicircular canal hypoplasia were only detected by fundoscopy and CT imaging, respectively, after the *CHD7* mutation was identified. Another limitation of the study by Kim *et al*[10] is that it is unclear whether *CHD7* analysis was performed in the parents of the patients with a *CHD7* missense variant. Segregation analysis is helpful to predict whether *CHD7* missense variants are pathogenic or benign (Bergman *et al*, submitted). Only one of the five missense mutations that Kim *et al*[10] identified, p.Ser834Phe, has been previously reported and it is probably pathogenic. The other four missense mutations were not identified in 180 healthy controls, but they may still be rare benign variants. The *CHD7* missense mutation (p.Gly2108Arg) identified in our patient 3 is probably pathogenic, because it occurred *de novo* and had previously been identified in three other index patients with CHARGE syndrome (Jansen *et al*, submitted and www.CHD7.org). In addition, the glycine at position 2108 is

highly conserved and the amino acid substitution is predicted to be pathogenic by SIFT (Sorting Intolerant From Tolerant[35,36]), PolyPhen-2 (Polymorphism Phenotyping program-2[37,38]) and Align-GVGD.[39,40]

Based on the results of this study and the literature, our advice is to evaluate KS patients carefully for features of CHARGE syndrome by taking a detailed case history and physical examination. Balance disturbance is almost always present in patients with CHARGE syndrome, but it often goes unnoticed because balance problems decrease after the first few years of life due to visual compensation. However, if the case history reveals that walking without support was delayed, that the patient was unable to crawl without resting the head on the floor (5-point crawling) and that the patient was unable to ride a bicycle without side-stabilisers, the patient probably suffered from balance disturbance.[41] Imaging of the semicircular canals (preferably by a CT scan of the temporal bones) is indicated in all KS patients who are suspected of balance disturbance. Our advice is to perform *CHD7* analysis in KS patients who have at least two of the following features of CHARGE syndrome: ocular coloboma, choanal atresia or stenosis, characteristic external ear anomaly, cranial nerve dysfunction (facial palsy, sensorineural hearing loss or hypoplasia of cranial nerves on imaging) or balance disturbance. In addition, *CHD7* analysis is recommended in KS patients with semicircular canal anomalies, but who do not show other evident CHARGE features. These recommendations are in line with our 2011 guideline for *CHD7* analysis in patients suspected of CHARGE syndrome.[25]

Identifying a *CHD7* mutation has important clinical implications. Firstly, the *CHD7*-positive patient should be screened for additional CHARGE features, because subtle features can remain undetected but can have therapeutic consequences, e.g. unilateral renal agenesis. For recommendations on screening, we refer to the clinical surveillance schedule in Bergman *et al.*[25] Secondly, genetic counselling is indicated, because the patient has a 50% chance of transmitting the *CHD7* mutation to his or her offspring. The offspring may develop a more severe manifestation of CHARGE syndrome, because the syndrome is highly variable, even within families.[25,42-45] The possibility of prenatal diagnosis and pre-implantation genetic diagnosis should therefore be discussed with the patient and his or her partner.

Six patients in our Dutch KS cohort harboured a heterozygous missense mutation in the *PROKR2* gene, which alone is thought to be insufficient for the development of HH or KS. One of the *PROKR2* mutations that we identified, p.Arg85His, was previously found in a heterozygous state in patients with HH, KS, functional amenorrhea, or isolated anosmia, but also in unaffected family members.[17,20,46] It was also identified in a homozygous state in a KS patient.[17] Functional assays showed that the p.Arg85His mutation results in a mild loss-of-receptor function.[47] The other *PROKR2* missense mutations that we identified, p.Arg85Leu and p.Arg264His, have not been reported previously. However, since both mutations affect an amino acid that is conserved in bovine, murine and rat orthologous sequences,[17] they are thought to have a deleterious effect.

The heterozygous *FGF8* mutation that we identified in patient 10 is also expected to be insufficient alone for the development of KS. Evidence comes from a previous study in which

heterozygous *FGF8* missense variants (p.Pro26Leu and p.Arg209Leu) were identified in unaffected controls.[21] The *FGF8* variant that we identified in patient 10 (p.Gly29_Arg34dup) has not been reported previously, but two other variants in exon 1C of the *FGF8* gene have been described.[16] One *FGF8* variant was found in a heterozygous state (p.Pro26Ile) in a father with anosmia and in his son with Kallmann syndrome. The other *FGF8* variant was found in a homozygous state (p.Phe40Leu) in combination with a double heterozygous *FGFR1* mutation (p.Gln764His/p.Asp768Tyr) in a sporadic male patient with nIHH. Functional assays showed that these two *FGF8* mutations reduce the FGFR1c activity levels and both were therefore classified as loss-of-function mutations.[16]

KS is both genetically and clinically very heterogeneous. New genes implicated in KS will probably be identified in the near future, which will further increase the complexity of the inheritance patterns. KS is expected to follow oligogenic inheritance in most patients, with mutations in the *KAL1* gene as a possible exception (X-linked recessive inheritance, fully penetrant in males). Modifier genes and environmental factors will probably further increase the phenotypic variability of KS.[48,49] This hypothesis is supported by the identification of heterozygous mutations in several KS genes in unaffected persons, implying that heterozygous KS mutations are insufficient for the development of GnRH deficiency and anosmia.[21] In our cohort, oligogenic inheritance was not identified. However, we expect most of our KS patients will carry other mutations in genes yet to be associated with KS. In our *CHD7*-positive patients, however, we do not expect oligogenic inheritance to be responsible for their observed phenotypes. In the literature, mutations in *CHD7* have never been described in combination with mutations in other KS genes. In theory, digenic inheritance may occur by coincidence, but *CHD7* mutations are, on their own, sufficient for the development of HH and anosmia. Moreover, we question whether *CHD7* should be labelled as a KS gene because most, if not all, *CHD7*-positive KS patients have been shown to have additional features of CHARGE syndrome and therefore the diagnosis of KS could be revised to CHARGE syndrome, which is known to follow pure autosomal dominant inheritance.

There is still much to learn about the underlying pathogenesis of HH and anosmia, but at present it is thought these features are the result of a failed embryonic migration process in patients with KS and CHARGE syndrome.[48,50] During normal embryogenesis, the GnRH neurons migrate alongside the olfactory axons from the nasal placode into the brain.[48] Pathological examination of two male foetuses with KS due to a *KAL1* mutation revealed that the migration of both GnRH and olfactory neurons was disturbed, as evidenced by the presence of entangled nerve fibres on the dorsal side of the cribriform plate and the absence of GnRH neurons in the hypothalamus.[50,51] Comparable migration defects were also present in a foetus with CHARGE syndrome due to a frameshift mutation in the *CHD7* gene.[50] Studies in *Chd7*$^{Gt/+}$ mice, however, suggested that GnRH deficiency in mice with *Chd7* haploinsufficiency is not caused by GnRH migration defects, but rather by reduced GnRH cellular proliferation in the embryonic olfactory placode.[52] The olfactory bulb defects of these mice were possibly caused by reduced basal cell proliferation in the adult olfactory epithelium.[53]

CHD7 is a member of the chromodomain helicase DNA-binding protein family. This family

consists of adenosine triphosphate (ATP)-dependent chromatin remodelers. Recent studies have indicated that CHD7 regulates the transcription of multiple genes during embryonic development in a tissue- and time-dependent manner.[54-56] It is therefore hypothesized that CHD7 reduces the expression of one or more KS genes, which would explain the co-occurrence of anosmia and HH in CHARGE and Kallmann syndromes. In *Chd7*$^{Gt/+}$ mice, *Chd7* haploinsufficiency was found to be associated with reduced expression of *Fgfr1*, *Bmp4* and *Otx2*, but not *Fgf8* in the embryonic olfactory placode and reduced expression of *Otx2* and *GnRH1* in the adult hypothalamus.[52] These data suggest that Chd7 regulates GnRH neurogenesis and signalling through transcription regulation of key target genes in mice. Future research in human foetal tissues is needed to investigate whether CHD7 also regulates the transcription of KS genes in humans.

In conclusion, this study shows that *CHD7* mutations can be identified in a proportion of KS patients. The yield of *CHD7* analysis in patients with isolated KS seems very low, but increases when additional CHARGE features are present. We recommend evaluating all KS patients for additional CHARGE features by taking a detailed case history and physical examination. *CHD7* analysis is indicated when at least two additional CHARGE features or semicircular canal anomalies are present. Identifying a *CHD7* mutation has important clinical implications for the surveillance and genetic counselling of the patient.

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REFERENCES

1 Kallmann FJ, Schoenfeld WA, Barrera SE. The genetic aspects of primary eunuchoidism. *Am J Ment Def* 1944;48:203-236.

2 Qu Q, Liu J, Ni D, Zhang Q, Yang D, Wang N, et al. Diagnosis and clinical characteristics of congenital anosmia: case series report. *J Otolaryngol Head Neck Surg* 2010;39(6):723-731.

3 Yousem DM, Turner WJ, Li C, Snyder PJ, Doty RL. Kallmann syndrome: MR evaluation of olfactory system. *Am J Neuroradiol* 1993;14(4):839-843.

4 Pitteloud N, Meysing A, Quinton R, Acierno JS,Jr., Dwyer AA, Plummer L, et al. Mutations in fibroblast growth factor receptor 1 cause Kallmann syndrome with a wide spectrum of reproductive phenotypes. *Mol Cell Endocrinol* 2006;254-255:60-69.

5 Kaplan JD, Bernstein JA, Kwan A, Hudgins L. Clues to an early diagnosis of Kallmann syndrome. *Am J Med*

Genet A 2010;152A(11):2796-2801.

6 Tsai PS, Gill JC. Mechanisms of disease: Insights into X-linked and autosomal-dominant Kallmann syndrome. *Nat Clin Pract Endocrinol Metab* 2006;2(3):160-171.

7 Dode C, Hardelin JP. Clinical genetics of Kallmann syndrome. *Ann Endocrinol (Paris)* 2010;71(3):149-157.

8 Hardelin JP, Dode C. The complex genetics of Kallmann syndrome: *KAL1*, *FGFR1*, *FGF8*, *PROKR2*, *PROK2*, et al. *Sex Dev* 2008;2(4-5):181-193.

9 Jongmans MC, Ravenswaaij-Arts CM, Pitteloud N, Ogata T, Sato N, Claahsen-van der Grinten HL, et al. *CHD7* mutations in patients initially diagnosed with Kallmann syndrome--the clinical overlap with CHARGE syndrome. *Clin Genet* 2009;75(1):65-71.

10 Kim HG, Kurth I, Lan F, Meliciani I, Wenzel W, Eom SH, et al. Mutations in *CHD7*, encoding a chromatin-remodeling protein, cause idiopathic hypogonadotropic hypogonadism and Kallmann syndrome. *Am J Hum Genet* 2008;83(4):511-519.

11 Kim HG, Ahn JW, Kurth I, Ullmann R, Kim HT, Kulharya A, et al. WDR11, a WD protein that interacts with transcription factor EMX1, is mutated in idiopathic hypogonadotropic hypogonadism and Kallmann syndrome. *Am J Hum Genet* 2010;87(4):465-479.

12 Xu N, Kim HG, Bhagavath B, Cho SG, Lee JH, Ha K, et al. Nasal embryonic LHRH factor (*NELF*) mutations in patients with normosmic hypogonadotropic hypogonadism and Kallmann syndrome. *Fertil Steril* 2011;95(5):1613-1620.

13 Franco B, Guioli S, Pragliola A, Incerti B, Bardoni B, Tonlorenzi R, et al. A gene deleted in Kallmann's syndrome shares homology with neural cell adhesion and axonal path-finding molecules. *Nature* 1991;353(6344):529-536.

14 Legouis R, Hardelin JP, Levilliers J, Claverie JM, Compain S, Wunderle V, et al. The candidate gene for the X-linked Kallmann syndrome encodes a protein related to adhesion molecules. *Cell* 1991;67(2):423-435.

15 Dode C, Levilliers J, Dupont JM, De Paepe A, Le Du N, Soussi-Yanicostas N, et al. Loss-of-function mutations in *FGFR1* cause autosomal dominant Kallmann syndrome. *Nat Genet* 2003;33(4):463-465.

16 Falardeau J, Chung WC, Beenken A, Raivio T, Plummer L, Sidis Y, et al. Decreased FGF8 signaling causes deficiency of gonadotropin-releasing hormone in humans and mice. *J Clin Invest* 2008;118(8):2822-2831.

17 Dode C, Teixeira L, Levilliers J, Fouveaut C, Bouchard P, Kottler ML, et al. Kallmann syndrome: mutations in the genes encoding prokineticin-2 and prokineticin receptor-2. *PLoS Genet* 2006;2(10):1648-1652.

18 Cole LW, Sidis Y, Zhang C, Quinton R, Plummer L, Pignatelli D, et al. Mutations in prokineticin 2 and prokineticin receptor 2 genes in human gonadotrophin-releasing hormone deficiency: molecular genetics and clinical spectrum. *J Clin Endocrinol Metab* 2008;93(9):3551-3559.

19 Pitteloud N, Quinton R, Pearce S, Raivio T, Acierno J, Dwyer A, et al. Digenic mutations account for variable phenotypes in idiopathic hypogonadotropic hypogonadism. *J Clin Invest* 2007;117(2):457-463.

20 Sarfati J, Guiochon-Mantel A, Rondard P, Arnulf I, Garcia-Pinero A, Wolczynski S, et al. A comparative phenotypic study of Kallmann syndrome patients carrying monoallelic and biallelic mutations in the prokineticin 2 or prokineticin receptor 2 genes. *J Clin Endocrinol Metab* 2010;95(2):659-669.

21 Sykiotis GP, Plummer L, Hughes VA, Au M, Durrani S, Nayak-Young S, et al. Oligogenic basis of isolated gonadotropin-releasing hormone deficiency. *Proc Natl Acad Sci U S A* 2010;107(34):15140-15144.

22 Pallais JC, Au M, Pitteloud N, Seminara S, Crowley WF. Kallmann syndrome. GeneReviews 2011. http://www.ncbi.nlm.nih.gov/books/NBK1334/ (accessed 2011).

23 Sanlaville D, Verloes A. CHARGE syndrome: an update. *Eur J Hum Genet* 2007;15(4):389-399.

24 Pagon RA, Graham JM, Jr., Zonana J, Yong SL. Coloboma, congenital heart disease, and choanal atresia with multiple anomalies: CHARGE association. *J Pediatr* 1981;99(2):223-227.

25 Bergman JE, Janssen N, Hoefsloot LH, Jongmans MC, Hofstra RM, van Ravenswaaij-Arts CM. *CHD7* mutations and CHARGE syndrome: the clinical implications of an expanding phenotype. *J Med Genet* 2011;48(5):334-

342.

26 Jongmans MC, Admiraal RJ, van der Donk KP, Vissers LE, Baas AF, Kapusta L, et al. CHARGE syndrome: the phenotypic spectrum of mutations in the *CHD7* gene. *J Med Genet* 2006;43(4):306-314.

27 Bergman JE, Bocca G, Hoefsloot LH, Meiners LC, Ravenswaaij-Arts CM. Anosmia predicts hypogonadotropic hypogonadism in CHARGE syndrome. *J Pediatr* 2011;158(3):474-479.

28 Blake KD, Davenport SL, Hall BD, Hefner MA, Pagon RA, Williams MS, et al. CHARGE association: an update and review for the primary pediatrician. *Clin Pediatr (Phila)* 1998;37(3):159-173.

29 Blake K, Ravenswaaij-Arts CM, Hoefsloot L, Verloes A. Clinical utility gene card for: CHARGE syndrome. *Eur J Hum Genet* 2011;Epub ahead of print, doi: 10.1038/ejhg.2011.45.

30 Verloes A. Updated diagnostic criteria for CHARGE syndrome: a proposal. *Am J Med Genet A* 2005;133(3):306-308.

31 Doty RL, Shaman P, Dann M. Development of the University of Pennsylvania Smell Identification Test: a standardized microencapsulated test of olfactory function. *Physiol Behav* 1984;32(3):489-502.

32 Admiraal RJ, Joosten FB, Huygen PL. Temporal bone CT findings in the CHARGE association. *Int J Pediatr Otorhinolaryngol* 1998;45(2):151-162.

33 Amiel J, Attiee-Bitach T, Marianowski R, Cormier-Daire V, Abadie V, Bonnet D, et al. Temporal bone anomaly proposed as a major criteria for diagnosis of CHARGE syndrome. *Am J Med Genet* 2001;99(2):124-127.

34 Laitinen EM, Vaaralahti K, Tommiska J, Eklund E, Tervaniemi M, Valanne L, et al. Incidence, phenotypic features and molecular genetics of Kallmann syndrome in Finland. *Orphanet J Rare Dis* 2011;6:41.

35 Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc* 2009;4(7):1073-1081.

36 Ng PC, Henikoff S. Predicting the effects of amino acid substitutions on protein function. *Annu Rev Genomics Hum Genet* 2006;7:61-80.

37 Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. *Nat Methods* 2010;7(4):248-249.

38 Ramensky V, Bork P, Sunyaev S. Human non-synonymous SNPs: server and survey. *Nucleic Acids Res* 2002;30(17):3894-3900.

39 Mathe E, Olivier M, Kato S, Ishioka C, Hainaut P, Tavtigian SV. Computational approaches for predicting the biological effect of *p53* missense mutations: a comparison of three sequence analysis based methods. *Nucleic Acids Res* 2006;34(5):1317-1325.

40 Tavtigian SV, Deffenbaugh AM, Yin L, Judkins T, Scholl T, Samollow PB, et al. Comprehensive statistical study of 452 *BRCA1* missense substitutions with classification of eight recurrent substitutions as neutral. *J Med Genet* 2006;43(4):295-305.

41 Abadie V, Wiener-Vacher S, Morisseau-Durand MP, Poree C, Amiel J, Amanou L, et al. Vestibular anomalies in CHARGE syndrome: investigations on and consequences for postural development. *Eur J Pediatr* 2000;159(8):569-574.

42 Delahaye A, Sznajer Y, Lyonnet S, Elmaleh-Berges M, Delpierre I, Audollent S, et al. Familial CHARGE syndrome because of *CHD7* mutation: clinical intra- and interfamilial variability. *Clin Genet* 2007;72(2):112-121.

43 Feret H, McDonald-McGinn DM, Santani A, Zackai EH. Expanding the phenotypic overlap between CHARGE and Kallmann syndromes due to *CHD7* mutations. 2010;Abstract of poster presented at the 60th Annual ASHG Meeting, Washington DC, 2-6 Nov. 2010. No.1671:www.ashg.org/cgi-bin/2010/ashg10s.

44 Jongmans MC, Hoefsloot LH, van der Donk KP, Admiraal RJ, Magee A, van de Laar I, et al. Familial CHARGE syndrome and the *CHD7* gene: a recurrent missense mutation, intrafamilial recurrence and variability. *Am J Med Genet A* 2008;146(1):43-50.

45 Lalani SR, Safiullah AM, Fernbach SD, Harutyunyan KG, Thaller C, Peterson LE, et al. Spectrum of *CHD7*

mutations in 110 individuals with CHARGE syndrome and genotype-phenotype correlation. *Am J Hum Genet* 2006;78(2):303-314.

46 Caronia LM, Martin C, Welt CK, Sykiotis GP, Quinton R, Thambundit A, et al. A genetic basis for functional hypothalamic amenorrhea. *N Engl J Med* 2011;364(3):215-225.

47 Monnier C, Dode C, Fabre L, Teixeira L, Labesse G, Pin JP, et al. *PROKR2* missense mutations associated with Kallmann syndrome impair receptor signalling activity. *Hum Mol Genet* 2009;18(1):75-81.

48 Balasubramanian R, Dwyer A, Seminara SB, Pitteloud N, Kaiser UB, Crowley WF,Jr. Human GnRH deficiency: a unique disease model to unravel the ontogeny of GnRH neurons. *Neuroendocrinology* 2010;92(2):81-99.

49 Mitchell AL, Dwyer A, Pitteloud N, Quinton R. Genetic basis and variable phenotypic expression of Kallmann syndrome: towards a unifying theory. *Trends Endocrinol Metab* 2011;22(7):249-258.

50 Teixeira L, Guimiot F, Dode C, Fallet-Bianco C, Millar RP, Delezoide AL, et al. Defective migration of neuroendocrine GnRH cells in human arrhinencephalic conditions. *J Clin Invest* 2010;120(10):3668-3672.

51 Schwanzel-Fukuda M, Bick D, Pfaff DW. Luteinizing hormone-releasing hormone (LHRH)-expressing cells do not migrate normally in an inherited hypogonadal (Kallmann) syndrome. *Brain Res Mol Brain Res* 1989;6(4):311-326.

52 Layman WS, Hurd EA, Martin DM. Reproductive dysfunction and decreased GnRH neurogenesis in a mouse model of CHARGE syndrome. *Hum Mol Genet* 2011;20(16):3138-1350.

53 Layman WS, McEwen DP, Beyer LA, Lalani SR, Fernbach SD, Oh E, et al. Defects in neural stem cell proliferation and olfaction in *Chd7* deficient mice indicate a mechanism for hyposmia in human CHARGE syndrome. *Hum Mol Genet* 2009;18(11):1909-1923.

54 Bajpai R, Chen DA, Rada-Iglesias A, Zhang J, Xiong Y, Helms J, et al. CHD7 cooperates with PBAF to control multipotent neural crest formation. *Nature* 2010;463(7283):958-962.

55 Ho L, Crabtree GR. Chromatin remodelling during development. *Nature* 2010;463(7280):474-484.

56 Schnetz MP, Bartels CF, Shastri K, Balasubramanian D, Zentner GE, Balaji R, et al. Genomic distribution of CHD7 on chromatin tracks H3K4 methylation patterns. *Genome Res* 2009;19(4):590-601.

CHAPTER 5.1

CHD7 mutations and CHARGE syndrome: the clinical implications of an expanding phenotype

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ABSTRACT

Background CHARGE syndrome is a highly variable, multiple congenital anomaly syndrome, of which the complete phenotypic spectrum was only revealed after identification of the causative gene in 2004. CHARGE is an acronym for ocular coloboma, congenital heart defects, choanal atresia, retardation of growth and development, genital hypoplasia and ear anomalies associated with deafness. This typical combination of clinical features is caused by autosomal dominant mutations in the *CHD7* gene.

Objective To explore the emerging phenotypic spectrum of *CHD7* mutations, with a special focus on the mild end of the spectrum.

Methods We evaluated the clinical characteristics in our own cohort of 280 *CHD7*-positive patients and in previously reported patients with *CHD7* mutations and compared these with previously reported patients with CHARGE syndrome but an unknown *CHD7* status. We then further explored the mild end of the phenotypic spectrum of *CHD7* mutations.

Results We discuss that CHARGE syndrome is primarily a clinical diagnosis. In addition, we propose guidelines for *CHD7* analysis and indicate when evaluation of the semicircular canals is helpful in the diagnostic process. Finally, we give updated recommendations for clinical surveillance of patients with a *CHD7* mutation, based on our exploration of the phenotypic spectrum and on our experience in a multidisciplinary outpatient clinic for CHARGE syndrome.

Conclusion CHARGE syndrome is an extremely variable clinical syndrome. *CHD7* analysis can be helpful in the diagnostic process, but the phenotype cannot be predicted from the genotype.

INTRODUCTION

The first patients with what later became known as CHARGE syndrome (OMIM *214800) were described in 1961.[1,2] In 1979, two independent clinicians recognised that coloboma, choanal atresia and congenital heart defects clustered together in several patients.[3,4] The acronym CHARGE dates from 1981 and summarises some of the cardinal features: ocular coloboma, congenital heart defects, choanal atresia, retardation of growth and/or development, genital anomalies, and ear anomalies associated with deafness.[5] In 2004, mutations in the *CHD7* gene were identified as the major cause and 'CHARGE association' was changed to 'CHARGE syndrome'.[6] CHARGE syndrome occurs in approximately 1 in 10,000 newborns.[7] The inheritance pattern is autosomal dominant with variable expressivity. Almost all mutations occur *de novo*, but parent-to-child transmission has occasionally been reported.[8] In this review, we explore the phenotypic spectrum of *CHD7* mutations with special focus on the mild end of the spectrum. In the light of this expanding phenotype, we discuss whether CHARGE syndrome is a clinical or a molecular diagnosis, we propose guidelines for *CHD7* analysis, and give updated recommendations for the clinical surveillance of *CHD7*-positive patients.

BACKGROUND

Clinical diagnosis

Before discovery of the causative gene, CHARGE syndrome was a clinical diagnosis (clinical features summarised in Figure 1). Pagon was the first to introduce diagnostic criteria for CHARGE syndrome in 1981,[5] but these criteria are no longer in use. At present, the clinical criteria by Blake *et al* and Verloes are used in conjunction (Table 1).[9,10]

The Blake criteria[9] were slightly adjusted by a consortium and last updated in 2009.[11] These criteria encompass four major and seven minor criteria. The four major criteria are coloboma, choanal atresia, cranial nerve dysfunction, and abnormalities of the inner, middle, or external ear. At least four major, or three major and three minor, criteria must be present in order to diagnose CHARGE syndrome. In 2005, Verloes proposed renewed criteria.[10] He included semicircular canal defects as a major criterion, as these defects were shown to be a very specific and consistent feature in CHARGE syndrome.[12] Verloes also anticipated broadening of the phenotypic spectrum and reduced the number of features necessary for a diagnosis of CHARGE (to only three major, or two major and two minor, criteria) and he made his criteria less age- and sex-dependent. A common feature of both sets of criteria is that either coloboma or choanal atresia (which can sometimes be replaced by cleft palate, Table 1[13]) must be present in order to diagnose CHARGE syndrome.

Molecular diagnosis

Nowadays, CHARGE syndrome can also be diagnosed by a molecular genetic test. The *CHD7* gene, mutated in the majority of patients with CHARGE syndrome, consists of 37 coding exons and one non-coding exon.[6] The gene encodes for a 2997 amino acid long protein that belongs to the

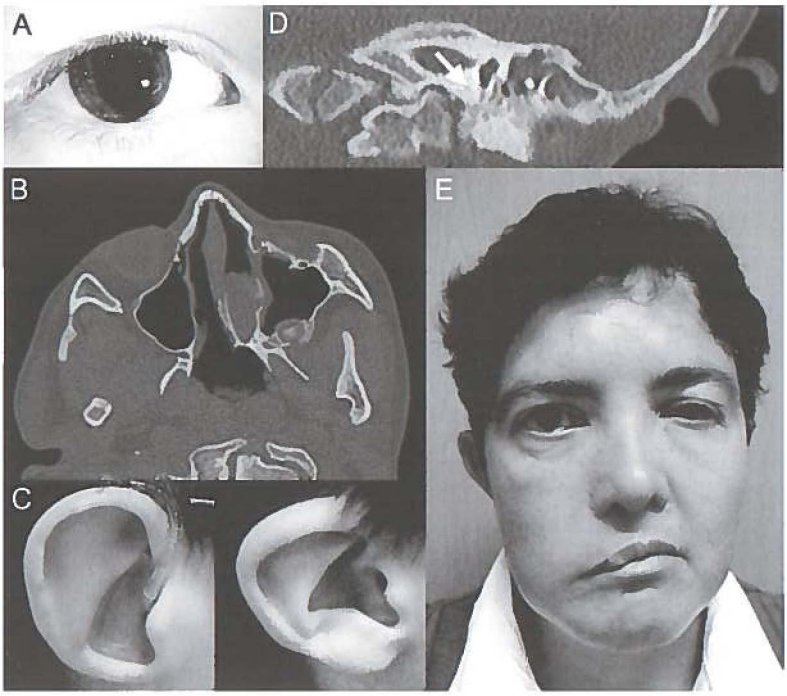


Figure 1. Overview of features occurring in CHARGE syndrome (frequencies are shown in Table 2).
Major features Coloboma the of iris (A) and/or retina, with or without microphthalmia, often only visible by fundoscopy. Choanal atresia (B, unilateral) or stenosis. Characteristic ear anomaly (C): cup shaped ear with triangular conchae and small/absent ear lobes. Middle or inner ear malformations may be present as well. Semicircular canal hypoplasia or aplasia (D arrow, semicircular canal aplasia of the left ear on a coronal CT scan). Cranial nerve dysfunction: oculomotor dysfunction (III/VI), less powerful chewing (V), facial palsy (VII) (E, right-sided), hearing loss/vestibular problems (VIII), swallowing and feeding problems (IX/X).
Minor features/occasional findings Hypothalamo-hypophyseal dysfunction: gonadotropin deficiency (hypogonadotropic hypogonadism), growth hormone deficiency. Other congenital anomalies: cleft lip/palate, congenital heart defects, tracheo-oesophageal anomalies, kidney anomalies, brain anomalies (including olfactory bulb hypoplasia), lacrimal duct atresia. Developmental delay: delayed motor development and/or cognitive delay. Characteristic face: broad forehead, square face, facial asymmetry. Other features: behavioural problems, sleep disturbance, scoliosis, respiratory aspiration, gastro-oesophageal reflux, postoperative complications, sudden death, obstructive sleep apnoea, enuresis nocturna, hockey stick palmar crease, webbed neck/sloping shoulders.
Rare features Immune deficiency, limb anomalies, epilepsy, oligodontia, anal atresia.
Informed consent was obtained for publication of the photographs.

Chromodomain Helicase DNA binding (CHD) family.[14] CHD7 can form complexes with different proteins, thereby ensuring specific binding to different enhancer regions leading to time and tissue specific regulation of gene expression.[15] One example is the association of CHD7 with PBAF (polybromo- and BRG1-associated factor containing complex) that is essential for neural crest gene expression and cell migration.[16] This is in line with previous assumptions that many of the congenital defects seen in CHARGE syndrome may be neural crest related.[17] CHD7 was also shown to associate with rDNA and was therefore suggested to play a role as positive regulator of rRNA synthesis.[18]

Haploinsufficiency of the *CHD7* gene leads to CHARGE syndrome and, as expected, most patients are found to have truncating *CHD7* mutations.[19-24] Missense mutations occur in a minority of patients and partial or full deletions of the *CHD7* gene are rare events.[6,19,23,25-31] Most *CHD7* mutations occur *de novo*. There are no mutational hotspots and recurrent mutations are rare.[20] No clear genotype-phenotype correlation exists, although it seems that missense mutations in general are associated with a milder phenotype.[20]

Table 1. Clinical criteria for CHARGE syndrome

	Major criteria	Minor criteria	Inclusion rule
Blake[#9]	1. Coloboma, microphthalmia 2. Choanal atresia or stenosis* 3. Characteristic external ear anomaly, middle/inner ear malformations, mixed deafness 4. Cranial nerve dysfunction	1. Cardiovascular malformations 2. Tracheo-oesophageal defects 3. Genital hypoplasia or delayed pubertal development 4. Cleft lip and/or palate 5. Developmental delay 6. Growth retardation 7. Characteristic face	Typical CHARGE: 4 major *or* 3 major + 3 minor
Verloes[10]	1. Ocular coloboma 2. Choanal atresia 3. Hypoplastic semicircular canals	1. Heart or oesophagus malformation 2. Malformation of the middle or external ear 3. Rhombencephalic dysfunction including sensorineural deafness 4. Hypothalamo-hypophyseal dysfunction (gonadotropin or growth hormone deficiency) 5. Mental retardation	Typical CHARGE: 3 major *or* 2 major + 2 minor Partial CHARGE: 2 major + 1 minor Atypical CHARGE: 2 major + 0 minor *or* 1 major + 3 minor

[#] Updated by a consortium in 2006 and 2009.[11]

* Cleft palate can be substituted for choanal atresia, since these anomalies rarely occur together.[13]

CHD7 analysis detects mutations in more than 90% of patients fulfilling the clinical criteria for CHARGE syndrome. The lack of mutation detection in the remaining 5–10% of patients suggests genetic heterogeneity. The *SEMA3E* gene was proposed as a candidate gene, but it seems to play a minor role as only two *SEMA3E* alterations have been described in patients with CHARGE syndrome.[32] Besides genetic heterogeneity, it is also possible that mutations in intronic regions, 5' or 3' untranslated regions, or in regulatory elements of *CHD7* underlie the *CHD7*-negative cases. Phenocopies of CHARGE or CHARGE-like syndrome can be due to teratogen exposure (e.g. thalidomide, retinoic acid, maternal diabetes) or chromosomal aberrations.[8]

PHENOTYPIC SPECTRUM OF PATIENTS WITH A MUTATION IN THE *CHD7* GENE

Phenotypic spectrum in our *CHD7*-positive cohort compared to two other cohorts

Our *CHD7*-positive cohort consists of patients who had *CHD7* analysis done in Nijmegen, the

Netherlands. In Nijmegen, *CHD7* analysis was performed in 863 patients suspected of CHARGE syndrome and 360 *CHD7* mutations were found (360/863 = 42%). The mutations were scattered throughout the entire coding region and splice sites of the *CHD7* gene. One third of the mutations were found in exons 2, 3, 30, and 31 (34% of mutations, 33% of genomic size). However, exons 8, 12, 26, 30, and 36 showed a remarkably high number of mutations relative to their genomic size (19% of mutations, 9% of genomic size). No mutations were found in exons 6, 7, 20, and 28, but these comprise only 3% of the coding genome of *CHD7*. Apart from the high number of mutations in exon 2 (the largest exon), our results do not agree with a previous report (n=91).[33] Most mutations were nonsense (38%) or frameshift mutations (32%). Missense mutations and splice site mutations occurred in 13% and 17%, respectively, and deletions were rarely present (<1%). The phenotypic spectrum of the missense mutations was more variable and on average milder when compared to the truncating mutations.

In Table 2 we present an overview of the clinical features of 280 of our *CHD7*-positive patients, the *CHD7*-positive cohort reported in the literature (reviewed by Zentner *et al*, n=254[24]) and a cohort of patients clinically diagnosed with CHARGE syndrome, but of whom the *CHD7* status is unknown (n=124[7,34]). We only included 280 of our 360 *CHD7*-positive patients, because clinical data were lacking in the other 80 patients. The phenotypes of 64 of the 280 patients have been published previously (Table 2).[20,26,35-40]

The clinical features of the *CHD7*-positive patients, previously reported or presented here, are rarely completely known. When calculating the percentage of patients who exhibit a certain feature, the incompleteness of the clinical data will have a major effect on the accuracy of the percentage. In order to compensate for this inaccuracy, we also calculated the frequency range. The minimum frequency is defined as the number of patients with a particular feature divided by the total number of patients in the cohort. The maximum frequency is defined as the number of patients with a particular feature plus patients for whom it is unknown whether they have the feature, divided by the total number of patients in the cohort.

Four features are almost always present in patients with a *CHD7* mutation: external ear anomalies, cranial nerve dysfunction, semicircular canal hypoplasia, and delayed attainment of motor milestones (Table 2). The characteristic external ear anomaly consists of triangular conchae or cup-shaped ears (Figure 1) and occurs in more than 90% of patients with a *CHD7* mutation. The second feature, cranial nerve dysfunction, is present in more than 95% of patients. The seventh and eighth cranial nerves are most often affected, leading to facial palsy and sensorineural hearing loss, respectively. Dysfunction of other cranial nerves can also occur. The third feature, semicircular canal hypoplasia, is not always assessed, but when investigated it is found to be present in over 90% of patients. The high frequency of semicircular canal hypoplasia is reflected in the delayed attainment of motor milestones (often scored as developmental delay in previous papers), that is almost universally present in patients with CHARGE syndrome. A delay in speech development is also common in these patients who suffer from multiple sensory impairment (e.g. blindness and/or

Table 2. Clinical features of patients with a *CHD7* mutation compared to clinically diagnosed patients with CHARGE syndrome

Feature	Our *CHD7*-positive cohort (n=280)	*CHD7*-positive cohort from the literature (n=254)#	CHARGE patients before *CHD7* discovery (n=124)*
External ear anomaly	224/231^ **97%** (80-98%)†	214/235 **91%**	74/77 **96%**
Cranial nerve dysfunction (VII, VIII and others)	173/174 **99%** (62-100%)	?	107/124 **86%**
Semicircular canal anomaly	110/117 **94%** (39-98%)	94/96 **98%**	12/12 **100%**
Coloboma	189/234 **81%** (68-84%)	190/253 **75%**	96/124 **77%**
Choanal atresia	99/179 **55%** (35-71%)	95/247 **38%**	76/124 **61%**
Cleft lip and/or palate	79/163 **48%** (28-70%)	79/242 **33%**	22/124 **18%***
Feeding difficulties necessitating tube feeding	90/110 **82%** (32-93%)	?	40/47 **85%**
Facial palsy	80/121 **66%** (29-85%)	72/187 **39%**	17/47 **36%**
Anosmia on formal smell testing	24/30 **80%**	?	?
Genital hypoplasia	118/145 **81%** (42-90%)	116/187 **62%**	45/124 **36%***
Congenital heart defect	191/252 **76%** (68-78%)	193/250 **77%**	105/124 **85%***
Tracheo-oesophageal anomaly	42/146 **29%** (15-63%)	35/185 **19%**	22/124 **18%**
Developmental delay	Delayed motor milestones 147/149 **99%** (53-99%) Intellectual disability 108/134 **74%** (39-91%)	Developmental delay 107/141 **76%**	Developmental delay 47/47 **100%**
Growth retardation	35/94 **37%** (13-79%)	101/141 **72%**	80/124 **65%**

CHD7-positive cohort from the literature as reviewed by Zentner *et al* in 2010.[24] This cohort partially overlaps with our *CHD7*-positive cohort because the phenotypes of 64 of our patients were published previously.[20,26,35-40]

* Cohort of patients with clinically diagnosed CHARGE syndrome reported by Tellier *et al* in 1998 and Issekutz *et al* in 2005, before *CHD7* analysis was possible.[7,34]

^ Frequencies are represented as the number of patients with a particular feature/the total number of patients that were tested for that particular feature

† The range of percentages presented between brackets was calculated as: (positive/total)x100% - (positive +unknown/total)x100% (for further explanation see text)

* Outside the frequency range of patients with a *CHD7* mutation

deafness).[41,42] In our cohort, approximately 75% of patients had intellectual disability, indicating that one quarter had a normal intelligence.

Two features seem to occur more frequently since *CHD7* analysis has become available as a diagnostic tool in CHARGE syndrome (Table 2). These are cleft lip and/or palate and genital hypoplasia; in the study by Tellier *et al*,[34] the percentages of these two features were below our frequency range. The most likely explanation is that in the past, patients with cleft palate, and thus often without choanal atresia, were not recognised as having CHARGE syndrome. Mutation analysis enables a diagnosis in these clinically less typical patients. The higher prevalence of genital hypoplasia in patients with a *CHD7* mutation can be explained by a higher mean age in the patients for whom molecular studies have been performed, but it may also be due to an increased awareness that genital hypoplasia is a frequent feature in patients with a *CHD7* mutation.

One feature seems to occur less frequently since *CHD7* analysis became available: congenital heart defects were present in 76% of *CHD7*-positive patients and in 85% of patients with a clinical diagnosis of CHARGE syndrome. The most likely explanation is that the clinical diagnosis was more readily made in hospitalised children with a heart defect and that, like children with cleft palate, children without a heart defect were more likely to remain unrecognised as having CHARGE syndrome before *CHD7* analysis.

Exploration of the mild end of the phenotypic spectrum of *CHD7* mutations

Patients with a typical presentation of CHARGE syndrome are easily clinically recognised, but those who are mildly affected can be missed, as the mild end of the CHARGE spectrum is only recently starting to emerge. Several studies have shown that an increasing number of patients with a *CHD7* mutation do not fulfil the clinical criteria, as they do not have coloboma or choanal atresia or cleft palate.[20] Exploration of the mild end of the CHARGE spectrum can be undertaken in four ways: by studying familial CHARGE syndrome, by evaluating very mildly affected patients who are picked up with *CHD7* analysis, by performing *CHD7* analysis in cohorts of patients with only one CHARGE feature, and finally by studying syndromes that show clinical overlap with CHARGE syndrome (e.g. 22q11 deletion syndrome and Kallmann syndrome).

Familial CHARGE syndrome

Very mildly affected patients with CHARGE syndrome can be identified by studying familial CHARGE syndrome. In the literature, only 16 families have been described with recurrence of molecularly confirmed CHARGE syndrome.[20,21,23,37,43-45] These families include seven sib-pairs, three monozygotic twin-pairs, and six two-generation families. In this review, we describe another two-generation family from our *CHD7*-positive cohort, making a total of 17 families (Table 3).

Of the 39 *CHD7*-positive individuals, only 24 (62%) fulfilled the clinical criteria for CHARGE syndrome as defined by either Blake *et al*[9] or Verloes.[10] Atypical CHARGE patients are most frequently seen in the two-generation families. Often, the mildly affected individuals were recognised only

Table 3. Familial CHARGE syndrome

Reference	*CHD7* mutation	Fulfilling clinical criteria			Segregation
Sib-pairs		**Sib 1**	**Sib 2**		
1. Wincent 2008[23]	c.4015C>T; p.R1339X	+ (case 11a)	+ (case 11b)		Father no mutation
2. Pauli 2009[44]	c.7302dupA	+ (girl)	+ (boy)		Germline mosaicism in father
3. Lalani 2006[21]	p.W2332X	+ (died)	- (case CHA76)		Parents no mutation
4. Jongmans 2008[37]	c.2442+5G>C	- (case 1)	+ (case 2)		Mother no mutation
5. Jongmans 2008[37]	c.2520G>A; p.W840X	+ (case 3)	+ (case 4)		Somatic mosaicism in father
6. Jongmans 2008[37]	c.1610G>A; p.W537X	+ (case 5)	+ (case 6)		Parents no mutation
7. Jongmans 2006[20]	c.5982G>A; p.W1994X	+ (case 29)	+ (case 30)		Somatic mosaicism in mother
Monozygotic twins		**Twin 1**	**Twin 2**		
1. Wincent 2008[23]	c.5428C>T; p.R1810X	+ (case 13a)	+ (case 13b)		*De novo*
2. Lalani 2006[21]	p.E1271X	+ (case A)	+ (case B)		Unknown
3. Jongmans 2006[20]	c.5752_5753 dupA; p.T1918fs	+ (case 26)	- (case 27)		Parents were not tested
Parent - child		**Child 1**	**Child 2**	**Parent**	
1. Vuorela 2008[45]	c.4795C>T; p.Q1599X	+ (case 1)	+ (case 2)	- (case 3)	*De novo* in father*
2. Delahaye 2007[43]	c.2501C>T; p.S834F	+ (case A III-2)	+ (case A III-3)	- (case A II-2)	*De novo* in mother
3. Delahaye 2007[43]	c.469>T; p.R157X	+ (B III-1)	+ (B III-3)	- (B II-2)	*De novo* in father
4. Lalani 2006[21]	p.R2319S	- (case CHA166)		-	Unknown
5. Jongmans 2008[37]	c.6322G>A; p.G2108R	- (case 7)		- (case 8)	*De novo* in mother*
6. Jongmans 2008[37]	c.6322G>A; p.G2108R	- (case 9)	+ (case 10)	- (case 11)	*De novo* in mother
7. This study	c.7769del	-	-	-	Unknown
Total clinical criteria positive		**Children 24/32**		**Parents 0/7**	

* Somatic mosaicism was excluded (the *CHD7* mutation was present in both peripheral blood lymphocytes and buccal cells). +, fulfilling the criteria, -, not fulfilling the clinical criteria of Blake and/or Verloes.[9,10]

after a *CHD7* mutation was found in a more severely affected family member. The most mildly affected patients described in the literature had dysmorphic ears and balance disturbance as the only manifestations of CHARGE syndrome. Somatic mosaicism was considered unlikely in two of the very mildly affected parents, because the *CHD7* mutation was found in different tissues.[37,45]

The monozygotic twin-pairs showed strikingly discordant features and underscore the great intra-familial variability seen in CHARGE syndrome.[20,21,23] This variability might be explained by differential epigenetic regulation or fluctuating embryonic CHD7 levels in relation to a time and tissue dependent critical threshold during embryonic development.

Mildly affected patients from our *CHD7*-positive cohort

The most widely used criteria are those of Blake *et al*[9] and Lalani *et al*[11] Interestingly, 18 out of the 131 (14%) *CHD7*-positive patients that could be scored for these criteria had only one or two major Blake features and thus could not be clinically diagnosed as having CHARGE syndrome. Based on the presence of none, or only one major Verloes feature, as many as 17% (22/124 patients) could not be clinically diagnosed with CHARGE syndrome using the Verloes criteria. The phenotypes of the three most mildly affected (previously unpublished) patients are presented below.

The first patient had abnormal external ears and a congenital heart defect, but no other features of CHARGE syndrome. She had normal semicircular canals, no cranial nerve dysfunction, and a normal pubertal development. She had a *de novo* pathogenic missense mutation in the *CHD7* gene that had not been described before (c.4406A>G; p.Tyr1469Cys in exon 19).

The second patient had mild semicircular canal anomalies and a mild hearing loss. His external ears were normal. He was only recognised as having CHARGE syndrome after a *CHD7* splice site mutation was found in his more severely affected children (Table 3, two-generation family from this study).

The third patient was diagnosed with Kallmann syndrome and had sensorineural hearing loss. After a *de novo* pathogenic missense mutation in the *CHD7* gene (c.6322G>A; p.Gly2108Arg in exon 31) was identified, a CT scan of his temporal bone was re-evaluated and semicircular canal hypoplasia was seen. He had normal external ears.

***CHD7* analysis in cohorts of patients with only one CHARGE feature**

Some authors have undertaken *CHD7* screening in patients with only one CHARGE syndrome feature - e.g. cleft lip and/or palate,[46] congenital heart disease,[47] or scoliosis.[48] These studies did not identify pathogenic *CHD7* mutations. The general impression is that in the absence of other CHARGE features, the chance of finding a *CHD7* mutation is very low.

Studies in syndromes that overlap with CHARGE syndrome

Thus far, two clinically overlapping syndromes have been studied in relation to *CHD7* mutations: velocardiofacial syndrome (VCFS) and Kallmann syndrome.

Velocardiofacial or 22q11 deletion syndrome, shares many features with CHARGE syndrome, including congenital heart defects, cleft palate, developmental delay, renal anomalies, growth retardation, ear anomalies, hearing loss, hypoglycaemia and lymphopenia.[49] In particular, thymus aplasia and hypoparathyroidism are increasingly recognised in CHARGE syndrome and mark the

clinical overlap with the DiGeorge phenotype of 22q11 deletions.[50,51] In approximately 85% of VCFS patients, a common 3 Mb heterozygous deletion of 22q11.2 is present, resulting in *TBX1* haploinsufficiency. Mutations in the *TBX1* gene are present in a minority of VCFS patients. Array comparative genomic hybridisation (CGH) in a cohort of VCFS patients without 22q11 deletion or *TBX1* mutation revealed one heterozygous deletion encompassing the *CHD7* gene in a patient with features typical of VCFS.[52] This patient had a learning difficulty with speech delay, severe feeding difficulties, a congenital heart defect (interruption of the aortic arch, coarctation of the aorta, bicuspid aortic valve, ventricular and atrial septal defect), long slender fingers and low set, over-folded ear helices. The patient did not have coloboma, choanal atresia or cleft palate, but did have typical CHARGE ears with triangular conchae. To our knowledge, *CHD7* sequence analysis has not yet been performed in a cohort of VCFS patients without deletion or mutation of *TBX1*. In Figure 2 we illustrate how difficult it can be to distinguish between CHARGE syndrome and 22q11 deletion syndrome. The phenotypic similarity between VCFS and CHARGE syndrome is also apparent in mice with haploinsufficiency of *Tbx1* and *Chd7*.[52] Both genes are required in pharyngeal ectoderm for fourth pharyngeal artery development. In addition, both genes are important in the development of the thymus and semicircular canals. The *Tbx1* and *Chd7* genes were shown to interact in mice, but a direct regulatory effect of Chd7 on *Tbx1* expression could not been demonstrated.[52]

Kallmann syndrome usually presents as the combination of hypogonadotropic hypogonadism (HH) and anosmia. Both features also occur in the majority of patients with CHARGE syndrome.[53-56]

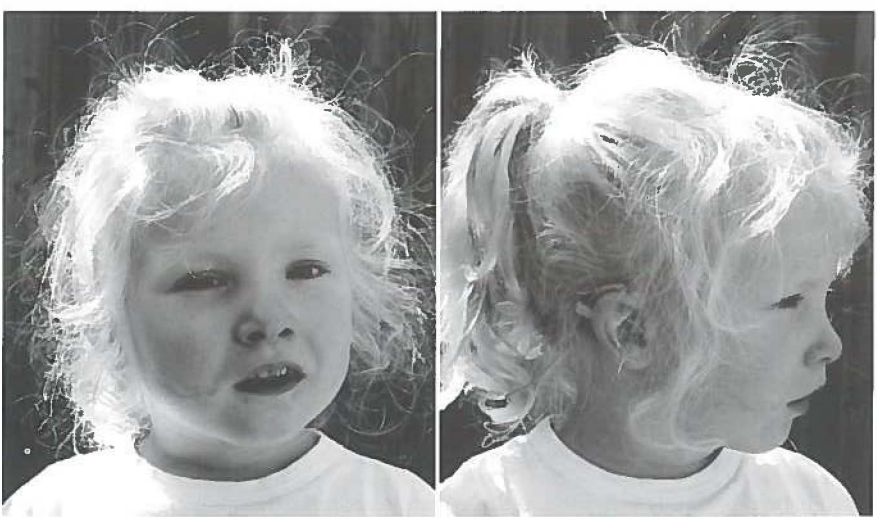


Figure 2. Patient with typical CHARGE syndrome and a 22q11 deletion.
This 3½-year-old girl presented with retinal and iris coloboma, unilateral choanal stenosis, abnormal semicircular canals, mixed hearing loss, pulmonary valve stenosis and simple ears. Clinically she has typical CHARGE syndrome, but neither a *CHD7* mutation nor a deletion could be detected by sequence analysis and multiplex ligation-dependent probe amplification (MLPA).[26] Subsequently, array comparative genomic hybridisation (CGH) was performed (Agilent 180K custom HD-DGH microarray) and revealed a *de novo* 3 Mb 22q11.2 loss, suggestive for the typical DiGeorge/velocardiofacial syndrome deletion.
Informed consent was obtained for publication of the photographs.

Other features that can be present in both syndromes are hearing loss, cleft lip/palate and renal malformations. Two studies have been performed in which patients with normosmic HH or Kallmann syndrome were screened for *CHD7* mutations. *CHD7* mutations were reported in seven out of 197 patients with normosmic HH or Kallmann syndrome,[57] in three out of 36 patients with Kallmann syndrome (confirmed by a smell test), but in none of 20 patients with normosmic HH.[58] The second study showed that after thorough clinical examination of the *CHD7*-positive Kallmann patients, other CHARGE features were universally present. The authors concluded that these patients represent the mild end of the CHARGE phenotypic spectrum, as we also demonstrated in our patient who was referred with Kallmann syndrome (see the section "Mildly affected patients from our *CHD7*-positive cohort").

CHD7 AND CHARGE SYNDROME: THE CLINICAL IMPLICATIONS

Based on the studies conducted after the identification of *CHD7* and summarised above, we discuss whether CHARGE syndrome is a clinical or molecular diagnosis, propose a new guideline for *CHD7* analysis and give recommendations for clinical surveillance of *CHD7*-positive patients.

CHARGE syndrome, a clinical or molecular diagnosis?

In our opinion, CHARGE syndrome is primarily a clinical diagnosis. If patients fulfil the clinical criteria of Blake or Verloes and chromosomal aberrations and teratogenic exposure effects fully explaining the clinical features have been ruled out, then they have CHARGE syndrome, irrespective of the results of *CHD7* analysis. On the other hand, patients who do not completely fulfil the clinical criteria should not be excluded from *CHD7* analysis. If a mutation is found in these patients, clinical follow-up and genetic counselling should be performed as in clinically diagnosed patients with CHARGE syndrome.

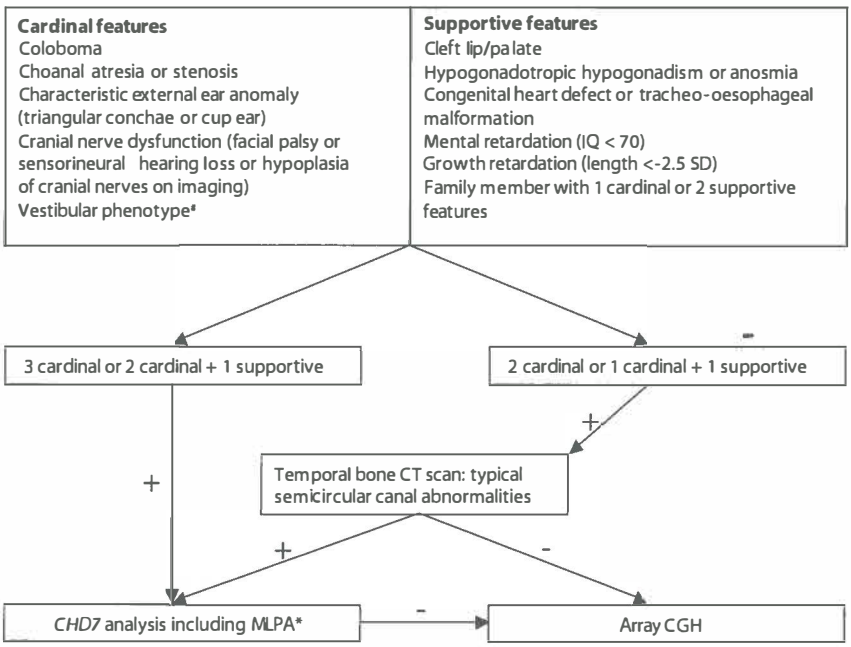
Guideline for *CHD7* analysis

Considering the broad phenotypic spectrum, it is evident that *CHD7* analysis should not be restricted to patients fulfilling the clinical criteria for CHARGE syndrome. Coloboma and choanal atresia (or cleft palate) are not always present in CHARGE syndrome. Therefore, patients with other CHARGE features, but without those cardinal features, should not be excluded from *CHD7* analysis. When a patient is suspected of CHARGE syndrome, the external ears, cranial nerve function and semicircular canals should be thoroughly examined, as these features occur in the great majority of patients with a *CHD7* mutation (Table 2).

We propose a guideline for *CHD7* analysis in Figure 3. In our experience, imaging of the semicircular canals is not an easy routine in daily clinical practice, especially in children in whom sedation can be complicated (see "Clinical surveillance" and Table 4). Therefore, in our guideline we have indicated when imaging of the semicircular canals is needed to support the decision for *CHD7* analysis. We based our guideline on the clinical features that were present in our *CHD7*-positive

patients (n=280). When applying our guideline, *CHD7* analysis would not have been recommended in one of our patients. This patient is the first one described in the section "Mildly affected patients from our *CHD7*-positive cohort" and is extremely mildly affected. A prospective study is needed to evaluate the usefulness of this guideline in clinical practice.

Figure 3. Guideline for *CHD7* analysis in patients suspected of CHARGE syndrome.



CGH, comparative genomic hybridisation; MLPA, multiplex ligation-dependent probe amplification.
A convincing history of vestibular problems (e.g. five-point crawl) or abnormal vestibular test or semicircular canal hypoplasia. * If clinical presentation is very atypical, it is recommended to perform array CGH first. Patients with velocardiofacial syndrome, but without a mutation or deletion of the *TBX1* gene, are also good candidates for *CHD7* analysis

Clinical surveillance of patients with a *CHD7* mutation or typical CHARGE syndrome

Ideally, follow-up of patients with a *CHD7* mutation or typical CHARGE syndrome should be done by an expert multidisciplinary team, because this approach will ensure optimal treatment of this very complex patient group. In the Netherlands, several specialities are involved in the CHARGE outpatient clinic of the University Medical Centre Groningen: clinical genetics, paediatric endocrinology, ear nose throat (ENT), speech and occupational therapy, ophthalmology, child and youth psychiatry, social paediatrics, gynaecology, endocrinology, paediatric cardiology, neuroradiology and dentistry. In Table 4, we show updated recommendations for clinical surveillance of patients with a *CHD7* mutation based on the experiences of our CHARGE outpatient clinic, on the clinical features in our *CHD7*-positive cohort (Table 2), and on a literature review.

Table 4. Clinical surveillance of patients with a *CHD7* mutation

Evaluation	Tests	Treatment/advice	Be aware of
Ophthalmology	Full ophthalmological examination including fundoscopy	Tinted spectacles for photophobia (iris coloboma) Artificial tears in case of facial palsy Correction of refraction errors	Retinal detachment (in case of retinal coloboma)
ENT, audiology, occupational/ speech therapy, gastroenterology	Multidisciplinary evaluation: Assess patency of choanae (CT scan or nasal endoscopy) Evaluation for cleft palate and tracheo-oesophageal anomalies Audiometry (BAER), tympanometry Temporal bone CT scan (pathology of middle ear, inner ear, cranial nerves, semicircular canals, aberrant course of blood vessels or cranial nerves) Cranial nerve function tests Swallowing studies, pH monitoring, reflux scan in case of feeding/swallowing difficulties University of Pennsylvania Smell Identification Test	Surgical correction of choanal atresia Hearing aids, ventilation tubes Sign language and speech training GERD: Nissen fundoplication, antispasmodics Gastrostomy/tracheotomy in case of severe swallowing problems Surgery of tracheo-oesophageal abnormalities Advice concerning anosmia	Respiratory aspiration (recurrent pneumonias) Aberrant course of blood vessels or cranial nerves when surgery for cochlear implants Obstructive sleep apnoea
Paediatrics/ endocrinology	Renal ultrasound, voiding cysto-urethrogram in case of urinary infections Immunological studies in case of recurrent infections or suspected hypocalcaemia Follow-up of growth and development (growth hormone stimulation test if indicated) Monitor cryptorchidism Gonadotropin levels (age 6-8 weeks) and follow-up of pubertal development DEXA scan (when suspected for osteoporosis) Monitor for scoliosis	Early treatment of bladder infections (especially in case of unilateral renal agenesis or vesico-urethral reflux) Growth hormone treatment if growth hormone deficiency is present Orchidopexy when indicated Gonadotropin treatment in case of hypogonadotropic hypogonadism Corset or surgery when severe progressive scoliosis is present	
Cardiology	Cardiac evaluation including ultrasound	Cardiac surgery and/or antibiotic prophylaxis	
Anaesthesiology	Extensive preoperative assessment	Combine surgical procedures whenever possible Longer surveillance after surgery	Postoperative complications (due to aspiration/cranial nerve dysfunction) Problems with intubation

Neurology	Cerebral MRI scan (including visualisation of olfactory bulbs, and inner ear if no temporal bone CT scan has been performed) EEG (only when clinically seizures are observed)	Anticonvulsants if overt epilepsy seen	
Behaviour, developmental and educational services	Extensive multidisciplinary evaluation of developmental and sensory impairments and behavioural problems Use formal tests in order to screen for autism spectrum, obsessive compulsive disorders and ADHD Perform IQ tests regularly	Integrated individualised therapy with special attention for optimising communication	
Physiotherapy	Assessment of balance problems, motor delay, visiospatial coordination and hypotonia	Therapy for hypotonia and devices to overcome balance impairment	
Genetics	*CHD7* analysis (when no *CHD7* mutation or deletion is found, perform array CGH)	Genetic counselling, options for prenatal diagnosis	Intra-familial variability in CHARGE syndrome

ADHD, attention deficit hyperactivity disorder; BAER, brain stem auditory evoked response; array CGH, array comparative genomic hybridisation; DEXA, dual energy x-ray absorptiometry; EEG, electroencephalogram; ENT, ear nose throat; GERD, gastro-oesophageal reflux disease.

An ultrasound of the heart and kidneys should be done in all patients, because mild congenital anomalies can remain undetected until adulthood, but may have therapeutic consequences (e.g. early treatment of urinary tract infections in case of renal anomalies).

Cranial nerve investigation is important. Dysfunction of the seventh, ninth and tenth cranial nerve can lead to severe feeding and swallowing problems, can result in respiratory aspiration and postoperative complications and might be involved in sudden death.[59-62]

HH should be diagnosed at an early stage, because patients are at risk for osteoporosis if hormone replacement therapy is not started in time. We recently demonstrated that anosmia and HH are 100% correlated in CHARGE syndrome and we proposed smell testing as a predictive test for HH.[63]

Last, but not least, an individualised educational program is needed in order to stimulate fully the intellectual potential of a child with CHARGE syndrome and to manage behavioural problems.[64-68] Clinicians should be aware that semicircular canal hypoplasia, a very frequent feature in CHARGE syndrome, causes balance problems and therefore a delay in motor development. This motor retardation may erroneously lead to the suspicion of intellectual disability, although approximately 25% of patients have a normal intelligence.

In addition, identifying a *CHD7* mutation gives further tools for genetic counselling of both the parents and the patients themselves. When the *CHD7* mutation has occurred *de novo* in the index patient, the recurrence risk for the parents is 2–3% because both germline and somatic mosaicism have been described in CHARGE syndrome.[20,37,44] Patients themselves, when fertile with or without appropriate hormone replacement therapy, have a 50% chance of transmitting the *CHD7* mutation to their offspring. The severity of CHARGE syndrome in offspring cannot be predicted, because intra-familial variability is large. Prenatal diagnosis, either by molecular analysis or ultrasound, and pre-implantation genetic diagnosis, when appropriate, should be discussed with parents and patients.

CONCLUSIONS

CHARGE syndrome is extremely variable, an observation that has been strongly underscored since the discovery of the *CHD7* gene. The phenotype cannot be predicted from the genotype, as exemplified by intra-familial variability. CHARGE syndrome remains primarily a clinical diagnosis, but molecular testing can confirm the diagnosis in mildly affected patients. Guidelines for *CHD7* analysis in individuals suspected of having CHARGE syndrome are proposed in Figure 3. In addition, updated guidelines for the surveillance of patients with a *CHD7* mutation or typical CHARGE syndrome are presented in Table 4.

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REFERENCES

1 Angelman H. Syndrome of coloboma with multiple congenital abnormalities in infancy. *Br Med J* 1961;1(5234):1212-1214.

2 Edwards JH, Finlay HVL, Young RB. Coloboma with Multiple Congenital Anomalies. *Br Med J* 1961;2(525):586-587.

3 Hall BD. Choanal atresia and associated multiple anomalies. *J Pediatr* 1979;95(3):395-398.

4 Hittner HM, Hirsch NJ, Kreh GM, Rudolph AJ. Colobomatous microphthalmia, heart disease, hearing loss, and mental retardation--a syndrome. *J Pediatr Ophthalmol Strabismus* 1979;16(2):122-128.

5 Pagon RA, Graham JM, Jr., Zonana J, Yong SL. Coloboma, congenital heart disease, and choanal atresia with multiple anomalies: CHARGE association. *J Pediatr* 1981;99(2):223-227.

6 Vissers LE, van Ravenswaaij CM, Admiraal R, Hurst JA, De Vries BB, Janssen IM, et al. Mutations in a new member of the chromodomain gene family cause CHARGE syndrome. *Nat Genet* 2004;36(9):955-957.

7 Issekutz KA, Graham JM, Jr., Prasad C, Smith IM, Blake KD. An epidemiological analysis of CHARGE syndrome: preliminary results from a Canadian study. *Am J Med Genet A* 2005;133(3):309-317.

8 Sanlaville D, Verloes A. CHARGE syndrome: an update. *Eur J Hum Genet* 2007;15(4):389-399.

9 Blake KD, Davenport SL, Hall BD, Hefner MA, Pagon RA, Williams MS, et al. CHARGE association: an update and review for the primary pediatrician. *Clin Pediatr (Phila)* 1998;37(3):159-173.

10 Verloes A. Updated diagnostic criteria for CHARGE syndrome: a proposal. *Am J Med Genet A* 2005;133(3):306-308.

11 Lalani SR, Hefner MA, Belmont JW, Davenport SLH. CHARGE Syndrome. GeneReviews 2009. http://www.ncbi.nlm.nih.gov/books/NBK1117/ (accessed 2011).

12 Admiraal RJ, Joosten FB, Huygen PL. Temporal bone CT findings in the CHARGE association. *Int J Pediatr Otorhinolaryngol* 1998;45(2):151-162.

13 Blake KD, Prasad C. CHARGE syndrome. *Orphanet J Rare Dis* 2006;1(1):34.

14 Woodage T, Basrai MA, Baxevanis AD, Hieter P, Collins FS. Characterization of the CHD family of proteins. *Proc Natl Acad Sci USA* 1997;94(21):11472-11477.

15 Layman WS, Hurd EA, Martin DM. Chromodomain proteins in development: lessons from CHARGE syndrome. *Clin Genet* 2010;78(1):11-20.

16 Bajpai R, Chen DA, Rada-Iglesias A, Zhang J, Xiong Y, Helms J, et al. CHD7 cooperates with PBAF to control multipotent neural crest formation. *Nature* 2010;463(7283):958-962.

17 Siebert JR, Graham JM, Jr., MacDonald C. Pathologic features of the CHARGE association: support for involvement of the neural crest. *Teratology* 1985;31(3):331-336.

18 Zentner GE, Hurd EA, Schnetz MP, Handoko L, Wang C, Wang Z, et al. CHD7 functions in the nucleolus as a positive regulator of ribosomal RNA biogenesis. *Hum Mol Genet* 2010;19(18):3491-3501.

19 Aramaki M, Udaka T, Kosaki R, Makita Y, Okamoto N, Yoshihashi H, et al. Phenotypic spectrum of CHARGE syndrome with *CHD7* mutations. *J Pediatr* 2006;148(3):410-414.

20 Jongmans MC, Admiraal RJ, van der Donk KP, Vissers LE, Baas AF, Kapusta L, et al. CHARGE syndrome: the phenotypic spectrum of mutations in the *CHD7* gene. *J Med Genet* 2006;43(4):306-314.

21 Lalani SR, Safiullah AM, Fernbach SD, Harutyunyan KG, Thaller C, Peterson LE, et al. Spectrum of *CHD7* mutations in 110 individuals with CHARGE syndrome and genotype-phenotype correlation. *Am J Hum Genet* 2006;78(2):303-314.

22 Lee YW, Kim SC, Shin YL, Kim JW, Hong HS, Lee YK, et al. Clinical and genetic analysis of the *CHD7* gene in Korean patients with CHARGE syndrome. *Clin Genet* 2009;75(3):290-293.

23 Wincent J, Holmberg E, Stromland K, Soller M, Mirzaei L, Djureinovic T, et al. *CHD7* mutation spectrum in 28 Swedish patients diagnosed with CHARGE syndrome. *Clin Genet* 2008;74(1):31-38.

24 Zentner GE, Layman WS, Martin DM, Scacheri PC. Molecular and phenotypic aspects of *CHD7* mutation in CHARGE syndrome. *Am J Med Genet A* 2010;152A(3):674-686.

25 Arrington CB, Cowley BC, Nightingale DR, Zhou H, Brothman AR, Viskochil DH. Interstitial deletion 8q11.2-q13 with congenital anomalies of CHARGE association. *Am J Med Genet A* 2005;133(3):326-330.

26 Bergman JE, de Wijs I, Jongmans MC, Admiraal RJ, Hoefsloot LH, Ravenswaaij-Arts CM. Exon copy number alterations of the *CHD7* gene are not a major cause of CHARGE and CHARGE-like syndrome. *Eur J Med Genet* 2008;51(5):417-425.

27 Hurst JA, Meinecke P, Baraitser M. Balanced t(6;8)(6p8p;6q8q) and the CHARGE association. *J Med Genet* 1991;28(1):54-55.

28 Johnson D, Morrison N, Grant L, Turner T, Fantes J, Connor JM, et al. Confirmation of *CHD7* as a cause of CHARGE association identified by mapping a balanced chromosome translocation in affected monozygotic twins. *J Med Genet* 2006;43(3):280-284.

29 Udaka T, Okamoto N, Aramaki M, Torii C, Kosaki R, Hosokai N, et al. An Alu retrotransposition-mediated deletion of *CHD7* in a patient with CHARGE syndrome. *Am J Med Genet A* 2007;143(7):721-726.

30 Vuorela P, la-Mello S, Saloranta C, Penttinen M, Poyhonen M, Huoponen K, et al. Molecular analysis of the *CHD7* gene in CHARGE syndrome: identification of 22 novel mutations and evidence for a low contribution of large *CHD7* deletions. *Genet Med* 2007;9(10):690-694.

31 Wincent J, Schulze A, Schoumans J. Detection of *CHD7* deletions by MLPA in CHARGE syndrome patients with a less typical phenotype. *Eur J Med Genet* 2009;52(4):271-272.

32 Lalani SR, Safiullah AM, Molinari LM, Fernbach SD, Martin DM, Belmont JW. *SEMA3E* mutation in a patient with CHARGE syndrome. *J Med Genet* 2004;41(7):e94.

33 Sanlaville D, Audollent S, Goudefroye G, Amiel J, Abadie V, Baumann C, et al. CHARGE syndrome: genotype-phenotype correlations and mutational hot-spots at the *CHD7* locus. 2006;Abstract of poster presented at the 56th annual ASHG meeting, New Orleans, Louisiana, USA, 9-13 Oct. 2006. No.1943: www.ashg.org/meetings/2006.

34 Tellier AL, Cormier-Daire V, Abadie V, Amiel J, Sigaudy S, Bonnet D, et al. CHARGE syndrome: report of 47 cases and review. *Am J Med Genet* 1998;76(5):402-409.

35 Bech AP, op den Akker JO, Matthijsse PR. Isolation of the left subclavian artery from the pulmonary artery in a patient with CHARGE association. *Congenit Anom (Kyoto)* 2010;50(3):200-202.

36 Douglas AG, Lam W. Extending the phenotypic spectrum of CHARGE syndrome: a case with preaxial polydactyly. *Clin Dysmorphol* 2009;19(1):33-34.

37 Jongmans MC, Hoefsloot LH, van der Donk KP, Admiraal RJ, Magee A, van de Laar I, et al. Familial CHARGE syndrome and the *CHD7* gene: a recurrent missense mutation, intrafamilial recurrence and variability. *Am J Med Genet A* 2008;146(1):43-50.

38 Van de Laar I, Dooijes D, Hoefsloot L, Simon M, Hoogeboom J, Devriendt K. Limb anomalies in patients with CHARGE syndrome: an expansion of the phenotype. *Am J Med Genet A* 2007;143(22):2712-2715.

39 Pedersen AM, Skovby F. [Molecular diagnosis of CHARGE syndrome]. *Ugeskr Laeger* 2007;169(5):402-406.

40 Writzl K, Cale CM, Pierce CM, Wilson LC, Hennekam RC. Immunological abnormalities in CHARGE syndrome. *Eur J Med Genet* 2007;50(5):338-345.

41 Goldson E, Smith AC, Stewart JM. The CHARGE association. How well can they do? *Am J Dis Child* 1986;140(9):918-921.

42 Money J, Norman BF. Pedagogical handicap associated with micropenis and other CHARGE syndrome anomalies of embryogenesis: four 46, XY cases reared as girls. *Am J Psychother* 1988;42(3):354-379.

43 Delahaye A, Sznajer Y, Lyonnet S, Elmaleh-Berges M, Delpierre I, Audollent S, et al. Familial CHARGE syndrome because of *CHD7* mutation: clinical intra- and interfamilial variability. *Clin Genet* 2007;72(2):112-121.

44 Pauli S, Pieper L, Haeberle J, Grzmil P, Burfeind P, Steckel M, et al. Proven germline mosaicism in a father of two children with CHARGE syndrome. *Clin Genet* 2009;75(5):473-479.

45 Vuorela PE, Penttinen MT, Hietala MH, Laine JO, Huoponen KA, Kaariainen HA. A familial CHARGE syndrome with a *CHD7* nonsense mutation and new clinical features. *Clin Dysmorphol* 2008;17(4):249-253.

46 Felix TM, Hanshaw BC, Mueller R, Bitoun P, Murray JC. *CHD7* gene and non-syndromic cleft lip and palate. *Am J Med Genet A* 2006;140(19):2110-2114.

47 Qi Q, Yi L, Yang C, Chen H, Shen L, Mo X, et al. [Mutation analysis of the *CHD7* gene in patients with congenital heart disease]. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi* 2008;25(6):637-641.

48 Gao X, Gordon D, Zhang D, Browne R, Helms C, Gillum J, et al. *CHD7* Gene polymorphisms are associated with susceptibility to idiopathic scoliosis. *Am J Hum Genet* 2007;80(5):957-965.

49 Jyonouchi S, McDonald-Mcginn DM, Bale S, Zackai EH, Sullivan KE. CHARGE (coloboma, heart defect, atresia choanae, retarded growth and development, genital hypoplasia, ear anomalies/deafness) syndrome and chromosome 22q11.2 deletion syndrome: a comparison of immunologic and nonimmunologic phenotypic features. *Pediatrics* 2009;123(5):e871-e877.

50 Inoue H, Takada H, Kusuda T, Goto T, Ochiai M, Kinjo T, et al. Successful cord blood transplantation for a CHARGE syndrome with *CHD7* mutation showing DiGeorge sequence including hypoparathyroidism. *Eur J Pediatr* 2010;169(7):839-844.

51 Sanlaville D, Etchevers HC, Gonzales M, Martinovic J, Clement-Ziza M, Delezoide AL, et al. Phenotypic spectrum of CHARGE syndrome in fetuses with *CHD7* truncating mutations correlates with expression during human development. *J Med Genet* 2006;43(3):211-217.

52 Randall V, McCue K, Roberts C, Kyriakopoulou V, Beddow S, Barrett AN, et al. Great vessel development requires biallelic expression of *Chd7* and *Tbx1* in pharyngeal ectoderm in mice. *J Clin Invest* 2009;119(11):3301-3310.

53 Asakura Y, Toyota Y, Muroya K, Kurosawa K, Fujita K, Aida N, et al. Endocrine and radiological studies in patients with molecularly confirmed CHARGE syndrome. *J Clin Endocrinol Metab* 2008;93(3):920-924.

54 Blustajn J, Kirsch CF, Panigrahy A, Netchine I. Olfactory anomalies in CHARGE syndrome: imaging findings of a potential major diagnostic criterion. *Am J Neuroradiol* 2008;29(7):1266-1269.

55 Chalouhi C, Faulcon P, Le Bihan C, Hertz-Pannier L, Bonfils P, Abadie V. Olfactory evaluation in children: application to the CHARGE syndrome. *Pediatrics* 2005;116(1):e81-e88.

56 Pinto G, Abadie V, Mesnage R, Blustajn J, Cabrol S, Amiel J, et al. CHARGE syndrome includes hypogonadotropic hypogonadism and abnormal olfactory bulb development. *J Clin Endocrinol Metab* 2005;90(10):5621-5626.

57 Kim HG, Kurth I, Lan F, Meliciani I, Wenzel W, Eom SH, et al. Mutations in *CHD7*, encoding a chromatin-remodeling protein, cause idiopathic hypogonadotropic hypogonadism and Kallmann syndrome. *Am J Hum Genet* 2008;83(4):511-519.

58 Jongmans MC, Ravenswaaij-Arts CM, Pitteloud N, Ogata T, Sato N, Claahsen-van der Grinten HL, et al. *CHD7* mutations in patients initially diagnosed with Kallmann syndrome–the clinical overlap with CHARGE syndrome. *Clin Genet* 2009;75(1):65-71.

59 Bergman JE, Blake KD, Bakker MK, du Marchie Sarvaas GJ, Free RH, Ravenswaaij-Arts CM. Death in CHARGE syndrome after the neonatal period. *Clin Genet* 2010;77(3):232-240.

60 Dobbelsteyn C, Marche DM, Blake K, Rashid M. Early oral sensory experiences and feeding development in children with CHARGE syndrome: a report of five cases. *Dysphagia* 2005;20(2):89-100.

61 Dobbelsteyn C, Peacocke SD, Blake K, Crist W, Rashid M. Feeding difficulties in children with CHARGE syndrome: prevalence, risk factors, and prognosis. *Dysphagia* 2008;23(2):127-135.

62 Sporik R, Dinwiddie R, Wallis C. Lung involvement in the multisystem syndrome CHARGE association. *Eur Respir J* 1997;10(6):1354-1355.

63 Bergman JE, Bocca G, Hoefsloot LH, Meiners LC, Ravenswaaij-Arts CM. Anosmia predicts hypogonadotropic

hypogonadism in CHARGE syndrome. *J Pediatr* 2011;158(3):474-479.

64 Brown D. CHARGE syndrome "behaviors": challenges or adaptations? *Am J Med Genet A* 2005;133(3):268-272.

65 Hartshorne TS, Hefner MA, Davenport SL. Behavior in CHARGE syndrome: introduction to the special topic. *Am J Med Genet A* 2005;133(3):228-231.

66 Hartshorne TS, Grialou TL, Parker KR. Autistic-like behavior in CHARGE syndrome. *Am J Med Genet A* 2005;133(3):257-261.

67 Johansson M, Gillberg C, Rastam M. Autism spectrum conditions in individuals with Mobius sequence, CHARGE syndrome and oculo-auriculo-vertebral spectrum: Diagnostic aspects. *Res Dev Disabil* 2010;31(1):9-24.

68 Souriau J, Gimenes M, Blouin C, Benbrik I, Benbrik E, Churakowskyi A, et al. CHARGE syndrome: developmental and behavioral data. *Am J Med Genet A* 2005;133(3):278-281.

CHAPTER 5.2

Discussion and future perspectives

THE *CHD7* GENE IN CHARGE SYNDROME

CHD7 mutation database

The *CHD7* gene is the major gene involved in CHARGE syndrome.[1,2] A *CHD7* mutation can be identified in more than 90% of patients who fulfil the clinical diagnostic criteria.[3-5] In **chapter 2.1**, a comprehensive *CHD7* mutation database was presented which contained 531 different pathogenic *CHD7* mutations (June 15th 2011). The database contains all the *CHD7* mutations reported in the literature, in addition to mutations identified by the molecular genetic laboratories of the Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands and the Department of Cellular and Molecular Medicine, the Panum Institute, University of Copenhagen, Copenhagen, Denmark. Nonsense and frameshift mutations were identified in the majority of patients (77%), while splice site mutations were found in 11%, missense mutations in 9% and deletions/duplications and genomic rearrangements occurred only rarely (3%). Several recurrent mutations (n=96) were found, but most mutations were unique. The database will be helpful for clinicians as well as scientists, as it gives a complete and readily available overview of all *CHD7* mutations in combination with any relevant clinical information on them. We will continue to add new *CHD7* mutations to the database and we hope that other laboratories will submit their *CHD7* mutations to the database, because any additional information, including new patients with a recurrent mutation, can be valuable. For example, it might become clear that a particular mutation infers a high risk for a specific congenital anomaly. At present, however, no clear genotype-phenotype correlations have been identified and the database cannot therefore be used for individual predictions. The current lack of genotype-phenotype correlations is underscored by observations in familial CHARGE syndrome, where family members with the same *CHD7* mutation can be affected in a very variable manner, and where monozygotic twins with a *CHD7* mutation have been shown to have different congenital anomalies (see also **chapter 5.1**).

Pathogenicity of *CHD7* missense variants

Assessing the pathogenicity of missense variants is difficult, especially when the variant has not been published in the literature or recorded in our database. Computational algorithms (e.g. SIFT,[6,7] PolyPhen[8,9] and Align GVGD[10,11]) can give an indication of pathogenicity, but often show conflicting results. Since correct interpretation of the pathogenicity of missense variants is essential for genetic counselling, we developed a novel classification system for *CHD7* missense variants (described in **chapter 2.2**). The classification is based on the output of SIFT, PolyPhen and Align GVGD combined with data from segregation analysis and it assigns a score to each missense variant that reflects the likelihood that the variant is pathogenic. Structural modelling of the CHD7 protein was used for further confirmation of our prediction. With our system, we classified 30% (43/145) as 'probably pathogenic', 23% (33/145) as 'probably benign', and 48% (69/145) as 'unclassified variant', due to lacking segregation or phenotypic data. Our classification was frequently in agreement with previous predictions from the literature. Unfortunately, we cannot test the validity of our classifications,

because there is no a gold standard for the classification of *CHD7* missense variants. We had to classify many variants as 'unknown', mainly because of a lack of segregation and phenotypic data. Segregation analysis can be helpful in classifying *CHD7* variants, but should be done with care. If a parent is found to carry the missense variant of interest, a detailed clinical examination of this parent should be performed. If the carrier parent has subtle features of CHARGE syndrome, the chance that the missense variant is pathogenic will increase. However, it is still possible that the variant is not pathogenic, but that it is in linkage disequilibrium with an unidentified pathogenic mutation. It would also be worthwhile to determine how many *CHD7* variants are present in the healthy population, because such data can help to calculate the prior chance that a certain *CHD7* variant is pathogenic. The 1000 Genomes Project and the Genome of the Netherlands could supply useful data. The ongoing submission of our own data and that from other laboratories to the *CHD7* mutation database will contribute to a better classification of *CHD7* missense variants. This will improve the molecular diagnosis of CHARGE syndrome and facilitate genotype-phenotype studies. Both aspects are important for the genetic counselling and surveillance of patients with CHARGE syndrome. The classification system that we developed for *CHD7* missense variants may also be applicable to other predominantly sporadic, autosomal dominant disorders. Future studies could explore this.

In addition to the classification system described in **chapter 2.2**, it would be helpful to develop a functional assay to assess the effects of missense variants on CHD7 function. The first requirement for developing such functional assay is to determine the precise function of the protein of interest. For CHD7, it is generally accepted that the protein has an important role in controlling the expression of multiple genes in a cell type- and embryonic stage-dependent manner.[12,13] Many binding partners of CHD7 have been identified, but the underlying molecular pathways have not yet been elucidated.[12-18] One study identified CHD8 as a binding partner of CHD7 through a yeast two-hybrid library screen and also investigated the effect of four different missense variants of the *CHD7* gene on the binding affinity to CHD8.[14] Three of the four missense variants showed disrupted binding of CHD7 to CHD8 in the yeast two-hybrid system, but not in the co-immunoprecipitation studies, indicating that CHD7 and CHD8 might be components of a larger protein complex. If the binding of CHD7 to CHD8 is an essential step in the development of CHARGE syndrome, then three of the four missense mutations would be considered pathogenic. This prediction agrees with the results of our classification system, which predicted that the three missense variants that disrupted binding to CHD8 were pathogenic, whereas the fourth variant was predicted to be benign. Other potential means of investigating functional effects of *CHD7* missense variants are the *in vivo* migration assay of cephalic neural crest cells, phenotypic analysis of mutant mRNA injected tadpoles, and investigating the binding affinity of CHD7 to other partners (e.g. PBAF components, NLK, SETDB1, PPAR-gamma and specific DNA regions).[12] At present, however, too little is known about the interacting partners of CHD7 that are functionally important, or about the pathways that CHD7 is involved in, for developing a functional assay that is fast and reliable enough for use in a

diagnostic setting.

Phenotypic spectrum of *CHD7* missense mutations

In **chapter 2.2**, we compared the clinical features of patients with missense mutations with those of patients with truncating mutations (nonsense and frameshift mutations and deletions). The patients with missense mutations showed a highly variable phenotype and were, on average, less severely affected than those with a truncating mutation in the *CHD7* gene. It is therefore not possible to predict the disease severity in an individual patient with a *CHD7* missense mutation, but there is a chance that the patient will only be mildly affected. Our observations are in line with data from studies of familial CHARGE syndrome, in which missense and splice site mutations are often present.[19-21] However, the same mutation can lead to an extremely variable phenotype among family members (see also **chapter 5.1**). Based on our clinical observations, I hypothesise that complete haploinsufficiency (due to truncating mutations) has more severe effects on CHD7 function than missense mutations that lead to an altered, but possibly partly functional protein.

Cause of CHARGE syndrome in patients without a *CHD7* mutation

In some patients highly suspected of having CHARGE syndrome, no *CHD7* mutation can be found. The initial discovery of the gene was based on a microdeletion in 8q12, including the *CHD7* gene,[1] and thus the *CHD7* haploinsufficiency may be due to a deletion in a subset of patients. However, partial or complete deletions of the gene will not be detected by routine Sanger sequencing. In **chapter 2.3**, I described the results of multiplex ligation-dependent probe amplification (MLPA) in a cohort of 54 patients with features of CHARGE syndrome but who did not have a *CHD7* mutation on sequence analysis. Eighteen of these 54 patients fulfilled the clinical criteria of Blake and/or Verloes[3,5] and therefore presented with typical CHARGE syndrome. The other 36 patients had features of CHARGE syndrome, but did not fulfil the clinical criteria and therefore had atypical CHARGE syndrome. We identified a deletion of exons 13 to 38 in one patient with typical CHARGE syndrome (1/54 = 1.9%). Because *CHD7* deletions were not found in the atypical patients of our cohort, the yield of MLPA in atypical patients was assumed to be very low. In a subsequent Swedish study, however, the yield of MLPA was much higher. Four *CHD7* deletions were identified in eighteen Swedish patients with features of CHARGE syndrome, but without a *CHD7* mutation upon sequencing.[22,23] In two patients a deletion of the entire *CHD7* gene was found, one patient had a *de novo* deletion of exon 1, and the other patient had a *de novo* deletion of exon 4. Three of the four patients did not fulfil the clinical criteria and it was therefore advised to perform MLPA in all patients who are suspected of CHARGE syndrome.[23] I agree with Wincent *et al*'s advice, but want to warn about the possibility of obtaining false-positive results in MLPA. If a single nucleotide polymorphism (SNP) is located underneath an MLPA probe, the MLPA will detect a deletion that is not actually present. If a single exon deletion is detected by MLPA, it is advisable to confirm the deletion with another molecular technique. Another possibility is to sequence the region of the MLPA probe that is found to be deleted, in order

to exclude SNPs that prohibit binding of the probe.

CHD7 sequence analysis and MLPA should be performed in all patients who are suspected of CHARGE syndrome. However, after sequence analysis and MLPA, 5–10% of the patients with typical CHARGE syndrome and a substantial number of patients with atypical CHARGE syndrome still have no molecular diagnosis. The cause of CHARGE syndrome might differ in the typical and atypical cases. Particularly in patients with typical CHARGE syndrome, it is possible that they do have a *CHD7* mutation, but that the mutation cannot be identified with current molecular techniques. For example, mutations in the deep intronic regions, 5' or 3' untranslated regions, or critical regulatory elements of the *CHD7* gene will currently not be identified. To investigate this possibility, RNA analysis could be performed. However, it is difficult to find a cell type that expresses a gene like *CHD7*, which is mainly active during embryogenesis. One report showed that *CHD7* is expressed in adult retina, cornea, brain, skeletal muscle, heart, kidney and lung,[1] but these cells are difficult to ascertain for RNA analysis in patients. We therefore examined whether *CHD7* was expressed in a control EBV cell line, but unfortunately found no *CHD7* expression. Further studies are needed to identify a suitable cell type for *CHD7* RNA analysis.

A second possibility is that the *CHD7*-negative patients have a mutation in another, as yet unidentified, gene for CHARGE syndrome. Several candidate genes, e.g. *PAX2*, *PITX2* and *CHD8*, have been analysed in patients, but no mutations were identified.[14,24,25] In 2004, alterations of the *SEMA3E* gene were found in two patients with CHARGE syndrome,[26] but no additional *SEMA3E* mutations have been reported since then. In searching for a new gene for CHARGE or CHARGE-like syndromes, our group will perform exome sequencing in a selected group of *CHD7*-negative patients with features of CHARGE syndrome.

CHARGE SYNDROME: STUDIES IN MICE AND MEN

Studies in *Chd7*$^{Whi/+}$ and *Chd7*$^{Gt/+}$ mice

Mouse models can be helpful in gaining insight into the pathogenesis of human disease. Two mouse models are commonly used to study CHARGE syndrome; the *Whirligig* mouse (*Chd7*$^{Whi/+}$), which carries a heterozygous nonsense mutation in the *Chd7* gene,[27] and a mouse that is heterozygous for a gene-trapped *lacZ* allele (*Chd7*$^{Gt/+}$).[28] The *Chd7*$^{Whi/+}$ mice have been most extensively studied and have many features in common with human CHARGE syndrome, e.g. fully penetrant semicircular canal anomalies and occasional congenital heart defects, cleft palate, choanal atresia and reduced body weight.[27] However, sense of smell and reproductive organ anatomy had not been studied in detail in *Chd7*$^{Whi/+}$ mice. Since we were interested in anosmia and hypogonadotropic hypogonadism (HH), which are the main features of Kallmann syndrome and which also occur in the majority of patients with CHARGE syndrome, we studied olfaction and aspects of the reproductive system in *Chd7*$^{Whi/+}$ mice (in **chapter 3.1**). We found that Chd7 is expressed in brain regions involved in olfaction and reproduction during embryonic development, which is in concordance with previous studies.[27-30] We showed that *Chd7*$^{Whi/+}$ mice maintained on a C3HeB/FeJ background performed

less well on a smell test than wild-type mice. Unfortunately, our study design did not allow us to discriminate between an olfactory deficit and severe balance disturbance, and we were therefore uncertain whether hyposmia was present in *Chd7*$^{Whi/+}$ mice. A concurrent study, however, confirmed the presence of hyposmia in adult *Chd7*$^{Gt/+}$ mice maintained on a 129SI/SvImJ background, by using odour-evoked electro-olfactograms.[31] In our study, we observed olfactory bulb hypoplasia and reproductive organ anomalies (uterine abnormalities in females and testes hypoplasia in males) in some of the *Chd7*$^{Whi/+}$ mice. The reproductive performance of both male and female *Chd7*$^{Whi/+}$ mice was slightly impaired compared to wild-type mice. In addition, the gonadotropin-releasing hormone (GnRH) neuron density appeared to be lower in adult female *Chd7*$^{Whi/+}$ mice, suggesting that GnRH deficiency could underlie their reproductive defects. A subsequent study also identified reproductive defects in *Chd7*$^{Gt/+}$ mice maintained on a 129SI/SvImJ background. These mice had delayed puberty, erratic oestrus cycles, decreased levels of circulating LH and FSH, and a reduced GnRH neuron count in the hypothalamus.[32] It was suggested that the reduced GnRH neuron count was caused by reduced GnRH cellular proliferation in the embryonic olfactory placode rather than by GnRH migration defects or increased cell death of GnRH neurons. In addition, Layman *et al* found that *Chd7* haploinsufficiency was associated with a decreased expression of *Fgfr1*, *Bmp4* and *Otx2*, but not *Fgf8*, in the embryonic olfactory placode and *Otx2* and *GnRH1* in the adult hypothalamus.[32] Combined with the results from their previous study,[31] the authors suggested that the reproductive and olfactory defects in *Chd7*-deficient mice were caused by a decreased proliferation of GnRH neurons in the embryonic olfactory placode, together with a decreased proliferation of basal cells in the adult olfactory epithelium. Chd7 could possibly affect GnRH neurogenesis and signalling by influencing transcriptional regulation of target genes involved in the BMP and FGF pathway.[32]

In our study, we crossed *Chd7*$^{Whi/+}$ mice with *Fgfr1*$^{Hspy/+}$ mice, and showed that a mutation in both *Chd7* and *Fgfr1* was associated with perinatal or early postnatal death. This is in contrast to the normal viability of *Fgfr1*$^{Hspy/+}$ mice and the 50% lethality of *Chd7*$^{Whi/+}$ mice and suggests that mutations in *Fgfr1* and *Chd7* interact synergistically. Both Chd7 and Fgfr1 are expressed in multiple tissues throughout embryogenesis and will probably interact in an unidentified pathway that is essential for survival. Olfactory bulbs and GnRH neuron distribution were, however, normal in the double heterozygous embryos, arguing against a genetic interaction between *Chd7* and *Fgfr1* in the development of the olfactory bulb and hypothalamus in mice.

Extrapolating the results from mouse studies to human CHARGE syndrome could prove difficult because of species-specific differences. The *Chd7*-deficient mice did not have complete aplasia of olfactory bulbs, nor were they infertile. Neither did the *Fgfr1*$^{Hspy/+}$ mice have overt features of Kallmann syndrome.[33] The phenotypic discrepancies between humans and mice might be due to species-specific differences in the developmental requirements for CHD7 and FGFR1. It is interesting that the *KAL1* gene (which is implicated in X-linked Kallmann syndrome in humans) has not been identified in mice, but it is hypothesised to have a pseudo-autosomal location.[34,35] The *KAL1* gene product, anosmin-1, is expected to interact with FGFR1 and FGF8. Theoretically, a higher *Kal1*

expression level in mice could make *Fgfr1*$^{Hspy/+}$ mice less susceptible for HH. The genetic background of the mice could also play a major role, with modifier genes having an effect on the penetrance and expressivity of different phenotypic features. This was already shown for the olfactory and ear phenotype, with *Chd7*$^{Gt/+}$ mice maintained on a 129SI/SvlmJ background having a more severe olfactory phenotype, and mice on a C57BL/6J background having a more severe ear phenotype compared to mice maintained on a mixed background.[2] The difference in genetic background of the *Chd7*$^{Whi/+}$ mice (C3HeB/FeJ) and the *Chd7*$^{Gt/+}$ mice (129S1/Sv1 mJ) could also have an effect on the severity of the olfactory and reproductive phenotype.

In our study, we were surprised to find that even the genetically identical *Chd7*$^{Whi/+}$ mice maintained on a homogeneous C3HeB/FeJ background showed incomplete penetrance of congenital anomalies. This observation supports the theory that the extreme variability of the CHARGE phenotype in both humans and mice is due to variants in the foetal micro-environment, leading to small fluctuations of the local Chd7 level or of the downstream gene products. Congenital anomalies will only occur if the Chd7 level is below a critical threshold that is tissue- and embryonic-stage-dependent. Some tissues, for example the inner ear, appear to be highly sensitive to Chd7 dosage, and are frequently affected in CHARGE syndrome. Other tissues, for example choanae, palate, eye and kidney, seem less sensitive to Chd7 dosage and are less frequently affected in CHARGE syndrome.[2,36]

In the future our group would like to study the pathogenesis of congenital heart defects in *Chd7*$^{Gt/+}$ mice. We hypothesise that *Chd7* haploinsufficiency in neural-crest-derived cardiac cells will alter the transcription of cardiogenic genes and that this will result in cardiac outflow tract defects. Candidate genes for outflow tract defects can possibly be identified by comparing the gene expression patterns of *Chd7*$^{Gt/+}$ mice with those of their wild-type litter mates during different embryonic stages in neural-crest-derived cardiac cells. Such a study could provide insight into the pathogenetic mechanisms underlying *CHD7*-related heart defects. If novel genes for cardiac defects are discovered, more aetiological diagnoses can be made in patients with congenital heart defects and this would lead to a more accurate determination of the recurrence risk and would improve genetic counselling.

Study of anosmia and HH in patients with CHARGE syndrome

After studying the occurrence of anosmia and HH in *Chd7*$^{Whi/+}$ mice, we investigated the prevalence of these two features in human CHARGE syndrome. We hypothesised that HH and anosmia might co-occur in CHARGE syndrome, as is the case in Kallmann syndrome. The results, presented in **chapter 3.2**, showed that anosmia and HH were indeed 100% correlated in our cohort of 35 adolescent patients with CHARGE syndrome. The co-occurrence of anosmia and HH had previously been reported for six female patients with CHARGE syndrome[37,38] In addition, a recent study reported arrhinencephaly and defective migration of GnRH neurons in a foetus with CHARGE syndrome, offering further support of the link between anosmia and HH.[39] In the foetus with CHARGE syndrome,

there were very few GnRH neurons in the hypothalamic region and entangled nerve fibres were found on the dorsal side of the cribriform plate, as was also the case in a foetus with Kallmann syndrome. These results are in contrast to data from mouse studies, where normal migration of GnRH neurons was observed in *Chd7*-deficient mice.[32] These observations shows that extrapolating results from mouse studies to the human situation is not always possible. It would therefore be interesting to investigate the effect of *CHD7* haploinsufficiency on the expression of several genes (e.g. *KAL1*, *FGFR1*, *PROK2*, *PROKR2*, *FGF8*, *NELF*, *WDR11*, *OTX2*, *BMP4* and *GnRH1*) in human embryonic olfactory placode and hypothalamus. In this way, we could gain more insight into the underlying pathogenesis of HH and anosmia in human CHARGE syndrome.

Our findings and the literature provide convincing evidence that anosmia and HH always co-occur in CHARGE syndrome, which means that a smell test can be used to predict the occurrence of HH in patients with CHARGE syndrome. This gives the opportunity to start with hormone replacement therapy (HRT) at an age-appropriate time in anosmic children with CHARGE syndrome, thereby reducing socio-emotional problems and lowering the risk of osteoporosis and cardiovascular disease, which are related to low sex hormone levels. Although there are many advantages to early HRT, there are also some drawbacks. Testosterone treatment increases muscle mass in boys and may aggravate behavioural problems. In girls, menstruation can be a burden, but can be minimized to three menstrual bleeds a year. The advantages and disadvantages of HRT should be discussed with the patient and parents in order to provide optimal patient care. At our CHARGE outpatient clinic, we are following patients who have received HRT and will investigate whether these patients or their parents report negative side effects. At present, it is not known how many patients with CHARGE syndrome experience problems due to HRT or how severe these problems are. The results from this study will be used to counsel patients more accurately about the negative side-effects of HRT in CHARGE syndrome.

Study of sudden death in patients with CHARGE syndrome

CHARGE syndrome is a rare disease, which makes it difficult to ascertain a large enough cohort for clinical studies. Our group is fortunate to have approximately 70 patients in the CHARGE outpatient clinic to follow, which greatly facilitates our research. The phenotypic variability of CHARGE syndrome is impressive; some patients have no health issues and a normal intelligence, whereas others die shortly after birth due to bilateral choanal atresia or complex congenital heart defects. We had gained the impression that the life expectancy of patients with CHARGE syndrome who survived the neonatal period was not significantly decreased. We were therefore surprised to hear that three patients had died unexpectedly after the neonatal period. We collected their clinical data and that for four other patients who had died after the neonatal period to study the risk factors for premature demise. The results, presented in **chapter 3.3**, showed that gastro-oesophageal reflux disease (GERD) and poor coordination of swallowing and breathing, leading to respiratory aspiration and post-operative airway events, were risk factors for post-neonatal death in CHARGE

syndrome. These observations are in line with previous studies.[40-42] Cranial nerve dysfunction was proposed as the underlying mechanism. We advised multidisciplinary follow-up of patients with CHARGE syndrome who have feeding difficulties, in combination with assessing their swallowing function and cranial nerve function. Treatment of swallowing problems and GERD should not be delayed and surgical procedures should be combined whenever possible in such patients. Further research is needed to elucidate the underlying cause of cranial nerve dysfunction in CHARGE syndrome. Detailed MRI brain imaging or post-mortem brain examination might shed light on the anatomical substrates of cranial nerve dysfunction in CHARGE syndrome. In addition, it is worth studying cranial nerve function in relation to swallowing problems, respiratory aspiration, GERD, and post-operative complications. Autopsy data from patients with CHARGE syndrome who died unexpectedly could lead to more knowledge on causes of death. This could help to identify more accurately which patients are at high risk for sudden death and, ideally, preventive measures could be taken to reduce the risk. Another factor that might contribute to sudden death in CHARGE syndrome is central adrenal insufficiency during infection-related stress (as was postulated for patients with Prader Willi syndrome by De Lind van Wijngaarden *et al*).[43] Adrenal insufficiency has not been reported in CHARGE syndrome, but it is known that patients with CHARGE syndrome can have other hypothalamic insufficiencies, for example growth hormone deficiency (present in a small minority of patients) or GnRH deficiency (leading to HH).[44]

Study of other aspects of CHARGE syndrome

We have studied only three aspects of CHARGE syndrome (olfaction, pubertal development and sudden death), but many other aspects of this complex syndrome deserve further study. At our outpatient clinic we gain insight into the questions that are relevant for the patients and their parents. One question that is frequently voiced concerns nocturnal enuresis. Many parents report that the young patient is still wetting the bed long after the age that children normally stay dry at night (6 to 7 years of age). This aspect of CHARGE syndrome has, to our knowledge, never been studied. In the general population, at least three pathogenic mechanisms are known to contribute to nocturnal enuresis: nocturnal polyuria, nocturnal detrusor overactivity, and high arousal thresholds.[45] Polyuria is often caused by nocturnal lack of vasopressin, an antidiuretic hormone that is produced in the pituitary gland. Brainstem disturbance is thought to be the underlying cause for all three mechanisms.[46-50] It is tempting to speculate that brainstem pathology could also be the cause of nocturnal enuresis in CHARGE syndrome. Future research is needed to gain insight into the pathophysiological mechanisms of this problem. This could contribute to better treatment options for children with CHARGE syndrome with nocturnal enuresis.

Another problem that is frequently reported by parents is disturbed sleep. A previous study investigated sleep disturbance in 87 children with CHARGE syndrome and found that the majority had a clinically significant disturbance as measured on the sleep disturbance scale for children.[51] Disorders of initiating and maintaining sleep, sleep breathing, and sleep-wake transition were most

commonly found, were associated with behavioural problems, and had negative effects on the parents' mental health. The cause of sleep disturbance in CHARGE syndrome is unknown, but could be related to pain (e.g. due to ear infections) or breathing problems caused by congenital anomalies (e.g. choanal atresia). Another possibility is that patients have a disturbed circadian rhythm. Support for this last possibility comes from mouse studies. The *Wheels* mouse, an ENU-generated mouse with a *Chd7* mutation, was reported to have a disturbed circadian rhythm.[27,52] The *Wheels* mouse has a lengthened circadian period and is more active in light conditions, which is aberrant behaviour for a nocturnal rodent. An altered melatonin secretion pattern is known to underlie sleep disturbance in other syndromes, for example in Smith Magenis syndrome,[53] Angelman syndrome,[54] and fragile X syndrome,[55] but it has not been studied in CHARGE syndrome. Our group would therefore like to study melatonin secretion patterns in patients with CHARGE syndrome with severe sleep disturbance. If altered patterns are found in such patients, we will investigate whether melatonin supplementation can be used to improve sleeping patterns.

Other aspects of CHARGE syndrome that need studying are hearing loss (and the benefit of cochlear implants), speech development, dental anomalies, adrenal gland function, and health problems in adult and elderly patients with CHARGE syndrome. Some of these studies are already being carried out in collaboration with the dentists and ear-nose-throat (ENT) specialists from the CHARGE outpatient clinic at the UMCG and the ENT department of the Radboud University Nijmegen Medical Centre.

Eventually, we hope an evidence-based guideline can be developed for all aspects of CHARGE syndrome. Ideally, this guideline will be used by all doctors and support workers who are caring for patients with CHARGE syndrome. The implementation of a best clinical practice guideline will improve the quality of life of patients and may contribute to an increased life expectancy.

THE *CHD7* GENE IN KALLMANN SYNDROME

CHD7 analysis in patients with Kallmann syndrome

Because the key features of Kallmann syndrome, anosmia and hypogonadotropic hypogonadism (HH), are also frequently encountered in CHARGE syndrome, we hypothesised that the *CHD7* gene could be a candidate gene for Kallmann syndrome. In a pilot study, we therefore analysed the *CHD7* gene in 36 patients with Kallmann syndrome and in 20 patients with HH but a normal sense of smell from Japan and North America who did not have a mutation in *KAL1*, *FGFR1*, *PROK2* and *PROKR2*. We presented the results of this study in **chapter 4.1**. Three *de novo CHD7* mutations were found in the patients with Kallmann syndrome (3/36 = 8.3%), whereas none of the patients with only HH had a *CHD7* mutation. Thus, a close relationship between anosmia, HH, and *CHD7* mutations was again observed. Upon re-evaluation, it became evident that the *CHD7*-positive patients all had additional features of CHARGE syndrome. We concluded that Kallmann patients should be evaluated for any features consistent with CHARGE syndrome and advised performing *CHD7* analysis if additional features are found.

A concurrent study performed *CHD7* analysis in 50 patients with Kallmann syndrome and in 51 patients with normosmic idiopathic HH (nlHH).[29] They also screened exons 6-10 of the *CHD7* gene in 96 additional patients with Kallmann syndrome or nlHH. In total, they found seven *CHD7* mutations: two splice site mutations and five missense mutations. Three of the *CHD7*-positive patients had Kallmann syndrome, whereas four of the *CHD7*-positive patients had nlHH. Besides a cleft lip (present in two *CHD7*-positive patients) and hearing loss (in one *CHD7*-positive patient), no additional features of CHARGE syndrome were reported. Although this study confirms that *CHD7* mutations can be found in patients with Kallmann syndrome, *CHD7* mutations were also identified in patients with nlHH but no other features of CHARGE syndrome. Unfortunately, the authors do not report whether their *CHD7*-positive patients underwent formal smell testing or if they were later re-evaluated for other features of CHARGE syndrome (e.g. fundoscopy). It is therefore possible that subtle features of CHARGE syndrome could have been missed. Another limitation of this study is that *CHD7* analysis was not performed in the parents of the *CHD7*-positive patients. Segregation analysis can help to predict whether missense variants are pathogenic or benign (see **chapter 2.2**). Only one of the five missense mutations that the authors identified had been previously reported and was known to be pathogenic. The other four missense mutations are of uncertain pathogenicity according to our classification system (with summed scores ranging between 0 and 1.5). We cannot therefore exclude the possibility that four of the seven *CHD7* mutations identified by Kim *et al*[29] are actually benign polymorphisms.

Because of the conflicting results of the studies described above, we decided to perform *CHD7* analysis in a well-characterised cohort of Dutch patients with Kallmann syndrome without mutations in the *KAL1* gene. All the patients were screened for mutations in *FGFR1*, *PROK2*, *PROKR2*, *FGF8* and *CHD7*. We have included 36 patients with Kallmann syndrome in this study and have identified six heterozygous variants in *PROKR2*, one heterozygous variant in *FGF8*, and three pathogenic *CHD7* mutations (see **chapter 4.2**). Both the *FGF8* and *PROKR2* variants are thought to contribute to the development of Kallmann syndrome in combination with one or more other mutations, but on their own they seem to be insufficient for the development of GnRH deficiency. Re-evaluation of the three *CHD7*-positive patients revealed that other CHARGE features were present (e.g. hearing loss, cleft lip and palate, balance disturbance, coloboma, short stature and scoliosis). Our results are in agreement with those from our pilot study (presented in **chapter 4.1**).

We and others have shown that Kallmann syndrome can be part of the CHARGE spectrum. CHARGE syndrome can have a very mild presentation, but we believe that every *CHD7*-positive patient will display at least one other CHARGE feature upon detailed examination. We therefore recommend evaluating all KS patients for additional CHARGE features by history taking and physical examination. *CHD7* analysis is indicated when at least two additional major CHARGE features or semicircular canal anomalies are present. The major features are ocular coloboma, choanal atresia or stenosis, external ear anomalies, cranial nerve dysfunction (including sensorineural hearing loss) and balance disturbance. Identifying a *CHD7* mutation in a patient with Kallmann syndrome has

important implications for their clinical treatment. A *CHD7*-positive patient should be screened for other CHARGE features (e.g. heart defects and renal anomalies; see our recommendations for surveillance of *CHD7*-positive patients in **chapter 5.1**). In addition, genetic counselling is indicated, because the patient has a 50% chance of transmitting the *CHD7* mutation to his/her offspring. Because of the great intrafamilial variability of CHARGE syndrome, the offspring could suffer from severe CHARGE syndrome.[19-21,56] Prenatal diagnosis and pre-implantation genetic diagnosis should be discussed.

***CHD7* analysis in other cohorts**

Considering the important implications of identifying a *CHD7* mutation, it is worth performing *CHD7* analysis in other cohorts of patients with one or a few features of CHARGE syndrome. At present, our group is collecting a cohort of patients with a congenital heart defect and one other CHARGE feature for analysis of the *CHD7* gene. It would also be interesting to perform *CHD7* analysis in a cohort of patients with semicircular canal anomalies and in a cohort of patients with velocardiofacial syndrome who do not have a 22q11 deletion or *TBX1* mutation. I would expect to find *CHD7* mutations in these cohorts and likely some other features of CHARGE syndrome upon detailed examination of *CHD7*-positive patients. In addition, it would be interesting to perform *CHD7* analysis in patients with Goldenhar syndrome (oculo-auriculo-vertebral spectrum) and patients with the VACTERL association (vertebral anomalies, anal atresia, cardiac defects, tracheo-oesophageal fistula, oesophageal atresia, renal anomalies and limb defects). These two conditions show some clinical overlap with CHARGE syndrome, but the underlying genetic defect is largely unknown (mutations in the *ZIC3* gene are known to underlie some cases of X-linked VACTERL-H[57]). By performing *CHD7* analysis in patients with other syndromes, we can further explore the full phenotypic spectrum of *CHD7* mutations and may shed light on the underlying pathogenesis.

CONCLUSION

Our research has provided new insights into the clinical effects of mutations in the *CHD7* gene in the context of CHARGE and Kallmann syndromes. We have shown that the phenotypic spectrum of *CHD7* mutations is even broader than was originally anticipated. We have also constructed a comprehensive *CHD7* mutation database and presented a classification system for missense variants in the *CHD7* gene. We investigated olfaction and aspects of the reproductive system in patients and mice with heterozygous *CHD7* mutations. We found that anosmia and hypogonadotropic hypogonadism (HH) always co-occurred in patients with CHARGE syndrome and therefore proposed that a smell test could be used to predict the occurrence of HH in patients with CHARGE syndrome. We also showed that *CHD7* mutations are occasionally present in patients initially diagnosed with Kallmann syndrome. Our studies have further expanded the knowledge of CHARGE and Kallmann syndromes and our results have raised many new questions for further research in this field.

REFERENCES

1 Vissers LE, van Ravenswaaij CM, Admiraal R, Hurst JA, De Vries BB, Janssen IM, et al. Mutations in a new member of the chromodomain gene family cause CHARGE syndrome. *Nat Genet* 2004;36(9):955-957.

2 Zentner GE, Layman WS, Martin DM, Scacheri PC. Molecular and phenotypic aspects of *CHD7* mutation in CHARGE syndrome. *Am J Med Genet A* 2010;152A(3):674-686.

3 Blake KD, Davenport SL, Hall BD, Hefner MA, Pagon RA, Williams MS, et al. CHARGE association: an update and review for the primary pediatrician. *Clin Pediatr (Phila)* 1998;37(3):159-173.

4 Jongmans MC, Admiraal RJ, van der Donk KP, Vissers LE, Baas AF, Kapusta L, et al. CHARGE syndrome: the phenotypic spectrum of mutations in the *CHD7* gene. *J Med Genet* 2006;43(4):306-314.

5 Verloes A. Updated diagnostic criteria for CHARGE syndrome: a proposal. *Am J Med Genet A* 2005;133(3):306-308.

6 Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc* 2009;4(7):1073-1081.

7 Ng PC, Henikoff S. Predicting the effects of amino acid substitutions on protein function. *Annu Rev Genomics Hum Genet* 2006;7:61-80.

8 Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. *Nat Methods* 2010;7(4):248-249.

9 Ramensky V, Bork P, Sunyaev S. Human non-synonymous SNPs: server and survey. *Nucleic Acids Res* 2002;30(17):3894-3900.

10 Mathe E, Olivier M, Kato S, Ishioka C, Hainaut P, Tavtigian SV. Computational approaches for predicting the biological effect of *p53* missense mutations: a comparison of three sequence analysis based methods. *Nucleic Acids Res* 2006;34(5):1317-1325.

11 Tavtigian SV, Deffenbaugh AM, Yin L, Judkins T, Scholl T, Samollow PB, et al. Comprehensive statistical study of 452 *BRCA1* missense substitutions with classification of eight recurrent substitutions as neutral. *J Med Genet* 2006;43(4):295-305.

12 Bajpai R, Chen DA, Rada-Iglesias A, Zhang J, Xiong Y, Helms J, et al. CHD7 cooperates with PBAF to control multipotent neural crest formation. *Nature* 2010;463(7283):958-962.

13 Schnetz MP, Bartels CF, Shastri K, Balasubramanian D, Zentner GE, Balaji R, et al. Genomic distribution of CHD7 on chromatin tracks H3K4 methylation patterns. *Genome Res* 2009;19:590-601.

14 Batsukh T, Pieper L, Koszucka AM, von Velsen N, Hoyer-Fender S, Elbracht M, et al. CHD8 interacts with CHD7, a protein which is mutated in CHARGE syndrome. *Hum Mol Genet* 2010;19(14):2858-2866.

15 Engelen E, Akinci U, Bryne JC, Hou J, Gontan C, Moen M, et al. Sox2 cooperates with Chd7 to regulate genes that are mutated in human syndromes. *Nat Genet* 2011;43(6):607-611.

16 Schnetz MP, Handoko L, Akhtar-Zaidi B, Bartels CF, Pereira CF, Fisher AG, et al. CHD7 targets active gene enhancer elements to modulate ES cell-specific gene expression. *PLoS Genet* 2010;6(7):e1001023.

17 Takada I, Mihara M, Suzawa M, Ohtake F, Kobayashi S, Igarashi M, et al. A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR-gamma transactivation. *Nat Cell Biol* 2007;9(11):1273-1285.

18 Zentner GE, Hurd EA, Schnetz M P, Handoko L, Wang C, Wang Z, et al. CHD7 functions in the nucleolus as a positive regulator of ribosomal RNA biogenesis. *Hum Mol Genet* 2010;19(18):3491-3501.

19 Delahaye A, Sznajer Y, Lyonnet S, Elmaleh-Berges M, Delpierre I, Audollent S, et al. Familial CHARGE syndrome because of *CHD7* mutation: clinical intra- and interfamilial variability. *Clin Genet* 2007;72(2):112-121.

20 Jongmans MC, Hoefsloot LH, van der Donk KP, Admiraal RJ, Magee A, van de Laar I, et al. Familial CHARGE syndrome and the *CHD7* gene: a recurrent missense mutation, intrafamilial recurrence and variability. *Am J Med Genet A* 2008;146(1):43-50.

21 Lalani SR, Safiullah AM, Fernbach SD, Harutyunyan KG, Thaller C, Peterson LE, et al. Spectrum of *CHD7* mutations in 110 individuals with CHARGE syndrome and genotype-phenotype correlation. *Am J Hum Genet* 2006;78(2):303-314.

22 Wincent J, Holmberg E, Stromland K, Soller M, Mirzaei L, Djureinovic T, et al. *CHD7* mutation spectrum in 28 Swedish patients diagnosed with CHARGE syndrome. *Clin Genet* 2008;74(1):31-38.

23 Wincent J, Schulze A, Schoumans J. Detection of *CHD7* deletions by MLPA in CHARGE syndrome patients with a less typical phenotype. *Eur J Med Genet* 2009;52(4):271-272.

24 Martin DM, Probst FJ, Fox SE, Schimmenti LA, Semina EV, Hefner MA, et al. Exclusion of *PITX2* mutations as a major cause of CHARGE association. *Am J Med Genet* 2002;111(1):27-30.

25 Tellier AL, Amiel J, Delezoide AL, Audollent S, Auge J, Esnault D, et al. Expression of the *PAX2* gene in human embryos and exclusion in the CHARGE syndrome. *Am J Med Genet* 2000;93(2):85-88.

26 Lalani SR, Safiullah AM, Molinari LM, Fernbach SD, Martin DM, Belmont JW. *SEMA3E* mutation in a patient with CHARGE syndrome. *J Med Genet* 2004;41(7):e94.

27 Bosman EA, Penn AC, Ambrose JC, Kettleborough R, Stemple DL, Steel KP. Multiple mutations in mouse *Chd7* provide models for CHARGE syndrome. *Hum Mol Genet* 2005;14(22):3463-3476.

28 Hurd EA, Capers PL, Blauwkamp MN, Adams ME, Raphael Y, Poucher HK, et al. Loss of Chd7 function in gene-trapped reporter mice is embryonic lethal and associated with severe defects in multiple developing tissues. *Mamm Genome* 2007;18(2):94-104.

29 Kim HG, Kurth I, Lan F, Meliciani I, Wenzel W, Eom SH, et al. Mutations in *CHD7*, encoding a chromatin-remodeling protein, cause idiopathic hypogonadotropic hypogonadism and Kallmann syndrome. *Am J Hum Genet* 2008;83(4):511-519.

30 Sanlaville D, Etchevers HC, Gonzales M, Martinovic J, Clement-Ziza M, Delezoide AL, et al. Phenotypic spectrum of CHARGE syndrome in fetuses with *CHD7* truncating mutations correlates with expression during human development. *J Med Genet* 2006;43(3):211-217.

31 Layman WS, McEwen DP, Beyer LA, Lalani SR, Fernbach SD, Oh E, et al. Defects in neural stem cell proliferation and olfaction in Chd7 deficient mice indicate a mechanism for hyposmia in human CHARGE syndrome. *Hum Mol Genet* 2009;18(11):1909-1923.

32 Layman WS, Hurd EA, Martin DM. Reproductive dysfunction and decreased GnRH neurogenesis in a mouse model of CHARGE syndrome. *Hum Mol Genet* 2011;20(16):3138-3150.

33 Pau H, Fuchs H, de Angelis MH, Steel KP. Hush puppy: a new mouse mutant with pinna, ossicle, and inner ear defects. *Laryngoscope* 2005;115(1):116-124.

34 Hardelin JP. Kallmann syndrome: towards molecular pathogenesis. *Mol Cell Endocrinol* 2001;179(1-2):75-81.

35 MacColl GS, Quinton R, Bulow HE. Biology of KAL1 and its orthologs: implications for X-linked Kallmann syndrome and the search for novel candidate genes. *Front Horm Res* 2010;39:62-77.

36 Layman WS, Hurd EA, Martin DM. Chromodomain proteins in development: lessons from CHARGE syndrome. *Clin Genet* 2010;78(1):11-20.

37 Ogata T, Fujiwara I, Ogawa E, Sato N, Udaka T, Kosaki K. Kallmann syndrome phenotype in a female patient with CHARGE syndrome and *CHD7* mutation. *Endocr J* 2006;53(6):741-743.

38 Pinto G, Abadie V, Mesnage R, Blustajn J, Cabrol S, Amiel J, et al. CHARGE syndrome includes hypogonadotropic hypogonadism and abnormal olfactory bulb development. *J Clin Endocrinol Metab* 2005;90(10):5621-5626.

39 Teixeira L, Guimiot F, Dode C, Fallet-Bianco C, Millar RP, Delezoide AL, et al. Defective migration of neuroendocrine GnRH cells in human arrhinencephalic conditions. *J Clin Invest* 2010;120(10):3668-3672.

40 Blake KD, Russell-Eggitt IM, Morgan DW, Ratcliffe JM, Wyse RK. Who's in CHARGE? Multidisciplinary management of patients with CHARGE association. *Arch Dis Child* 1990;65(2):217-223.

41 Sporik R, Dinwiddie R, Wallis C. Lung involvement in the multisystem syndrome CHARGE association. *Eur Respir J* 1997;10(6):1354-1355.

42 Wyse RK, al-Mahdawi S, Burn J, Blake K. Congenital heart disease in CHARGE association. *Pediatr Cardiol* 1993;14(2):75-81.

43 de Lind van Wijngaarden RF, Otten BJ, Festen DA, Joosten KF, de Jong FH, Sweep FC, et al. High prevalence of central adrenal insufficiency in patients with Prader-Willi syndrome. *J Clin Endocrinol Metab* 2008;93(5):1649-1654.

44 Sanlaville D, Verloes A. CHARGE syndrome: an update. *Eur J Hum Genet* 2007;15(4):389-399.

45 Neveus T. Nocturnal enuresis-theoretic background and practical guidelines. *Pediatr Nephrol* 2011;26(8):1207-1214.

46 Danysz W, Kostowski W, Hauptmann M. Evidence for the locus coeruleus involvement in desipramine action in animal models of depression. *Pol J Pharmacol Pharm* 1985;37(6):855-864.

47 Holstege G, Griffiths D, de Wall H, Dalm E. Anatomical and physiological observations on supraspinal control of bladder and urethral sphincter muscles in the cat. *J Comp Neurol* 1986;250(4):449-461.

48 Kayama Y, Koyama Y. Brainstem neural mechanisms of sleep and wakefulness. *Eur Urol* 1998;33 Suppl 3:12-15.

49 Lightman SL, Todd K, Everitt BJ. Ascending noradrenergic projections from the brainstem: evidence for a major role in the regulation of blood pressure and vasopressin secretion. *Exp Brain Res* 1984;55(1):145-151.

50 Ornitz EM, Russell AT, Hanna GL, Gabikian P, Gehricke JG, Song D, et al. Prepulse inhibition of startle and the neurobiology of primary nocturnal enuresis. *Biol Psychiatry* 1999;45(11):1455-1466.

51 Hartshorne TS, Heussler HS, Dailor AN, Williams GL, Papadopoulos D, Brandt KK. Sleep disturbances in CHARGE syndrome: types and relationships with behavior and caregiver well-being. *Dev Med Child Neurol* 2009;51(2):143-150.

52 Pickard GE, Sollars PJ, Rinchik EM, Nolan PM, Bucan M. Mutagenesis and behavioral screening for altered circadian activity identifies the mouse mutant, Wheels. *Brain Res* 1995;705(1-2):255-266.

53 De Leersnyder H. Inverted rhythm of melatonin secretion in Smith-Magenis syndrome: from symptoms to treatment. *Trends Endocrinol Metab* 2006;17(7):291-298.

54 Braam W, Didden R, Smits MG, Curfs LM. Melatonin for chronic insomnia in Angelman syndrome: a randomized placebo-controlled trial. *J Child Neurol* 2008;23(6):649-654.

55 Wirojanan J, Jacquemont S, Diaz R, Bacalman S, Anders TF, Hagerman RJ, et al. The efficacy of melatonin for sleep problems in children with autism, fragile X syndrome, or autism and fragile X syndrome. *J Clin Sleep Med* 2009;5(2):145-150.

56 Feret H, McDonald-McGinn DM, Santani A, Zackai EH. Expanding the phenotypic overlap between CHARGE and Kallmann syndromes due to *CHD7* mutations. 2010;Abstract of poster presented at the 60th Annual ASHG Meeting, Washington DC, 2-6 Nov. 2010. No.1671:www.ashg.org/cgi-bin/2010/ashg10s.

57 Wessels MW, Kuchinka B, Heydanus R, Smit BJ, Dooijes D, de Krijger RR, et al. Polyalanine expansion in the *ZIC3* gene leading to X-linked heterotaxy with VACTERL association: a new polyalanine disorder? *J Med Genet* 2010;47(5):351-355.

Summary

The aim of this thesis was to gain more insight into CHARGE syndrome and Kallmann syndrome, with a special focus on the *CHD7* gene. For this purpose, we collected all the *CHD7* mutations reported in the literature or identified by the DNA diagnostic laboratories in Nijmegen and Copenhagen. In addition, olfaction and aspects of the reproductive system were studied in a mouse model for CHARGE syndrome. Smell and pubertal development and causes of death were studied in patients with CHARGE syndrome. Finally, the *CHD7* gene was analysed in patients with Kallmann syndrome.

Chapter 1: The background

Detailed background information on *CHD7*, CHARGE syndrome and Kallmann syndrome can be found in chapter 1. In short, CHARGE syndrome is a complex and variable multi-system disorder with an incidence of approximately 1/10,000 newborns. CHARGE is an acronym for ocular coloboma (closure defect of the eye), heart defects, atresia of choanae (blockage of the nasal airway), retardation of growth and/or development, genital hypoplasia (micropenis in boys) and ear anomalies. Other anomalies can also occur, for example, a decreased sense of balance (due to anomalies of the semicircular canals), cranial nerve dysfunction (which can result in hearing loss, facial palsy or swallowing problems) and a smell deficit. In addition, the majority of patients have hypogonadotropic hypogonadism (HH), which manifests as a lack of pubertal maturation and infertility. CHARGE syndrome is caused by autosomal dominant mutations in the *CHD7* gene; these are mostly *de novo* mutations.

CHARGE syndrome has considerable clinical overlap with another rare heritable disorder, Kallmann syndrome. The main features of Kallmann syndrome are HH and a smell deficit. Additional features may also occur, for example, hearing loss, kidney anomalies and missing teeth. A mutation can be identified in only 25-35% of patients, in genes displaying different modes of inheritance: X-linked recessive (caused by mutations in *KAL1*), autosomal dominant (caused by mutations in *FGFR1*, *FGF8* and *CHD7*), and autosomal recessive (caused by mutations in *PROK2* and *PROKR2*). However, it has been postulated that many patients with Kallmann syndrome may have mutations in several different genes (oligogenic inheritance).

Chapter 2: The *CHD7* gene

In **chapter 2.1**, a complete overview is given of all pathogenic *CHD7* mutations reported in the literature or identified by the DNA diagnostic laboratories in Nijmegen and Copenhagen (in total, 531 different mutations per June 15th 2011). The *CHD7* mutations were found to be equally distributed along the coding region of the *CHD7* gene. Most mutations were unique, but 96 recurrent mutations were identified (predominantly arginine to stop mutations). Nonsense and frameshift mutations were the main mutations found, with splice site mutations, missense mutations and deletions being present only in a minority of patients. We built a locus-specific database that contains all the *CHD7* mutations and relevant clinical data (available at www.CHD7.org). This database is a valuable source of information for clinicians as well as researchers. In addition, we summarize the latest data on

expression studies, animal models and functional studies.

In **chapter 2.2**, we present a novel classification system that can help to predict whether a certain missense variant in the *CHD7* gene is either pathogenic (disease-causing) or benign (not associated with the disease). Correct classification of missense variants is essential for genetic counselling of patients and their families. Our classification system combines the outcome of three computational algorithms (SIFT, PolyPhen and align-GVGD) with information about the segregation of the variant of interest (*de novo* versus inherited). This combination of different variables is expected to lead to a more confident prediction of pathogenicity than was previously possible. We have used our system to classify 145 *CHD7* missense variants and have stored the data in the locus-specific database presented in chapter 2.1. Our classification of missense variants was confirmed by studies in a structural model of the CHD7 chromo- and helicase domains, which showed that the variants that we classified as 'probably pathogenic' were predicted to reduce the stability of the CHD7 protein and were frequently located in the protein core. Furthermore, the pathogenic missense mutations were mainly located in the middle of the *CHD7* gene, whereas the benign missense variants were predominantly clustered in the 5' and 3' regions. Finally, we showed that missense mutations are, in general, associated with a milder phenotype than mutations that lead to a truncated protein.

In **chapter 2.3**, we describe our investigations into how often partial deletions of the *CHD7* gene are present in patients with features of CHARGE syndrome. Partial deletions cannot be detected by routine DNA analysis of the *CHD7* gene (sequence analysis), but can be identified with multiplex ligation-dependent probe amplification (MLPA). We therefore performed MLPA in 54 patients with features of CHARGE syndrome, who did not show a *CHD7* mutation upon sequence analysis. Only one partial deletion of the *CHD7* gene was identified in a patient with a clinical diagnosis of CHARGE syndrome. We concluded that partial deletions of the *CHD7* gene are rarely found in patients with features of CHARGE syndrome.

Chapter 3: CHARGE syndrome

In **chapter 3.1**, we present the results of our studies in a mouse model for CHARGE syndrome, the *Whirligig* mouse, which harbours a nonsense mutation in the *Chd7* gene. We set out to investigate whether these mice have a smell deficit and genital anomalies, in analogy to patients with CHARGE syndrome. We designed a smell test and showed that the *Whirligig* mice performed less well than the wild-type mice. Their poor performance could be the result of a smell deficit, but could also be due to a severe balance disturbance. Next, we studied the area of the brain that is involved in olfaction and found that some mice had abnormal olfactory bulbs. We also identified abnormalities of the reproductive organs in some of the mice (testes hypoplasia in males and uterine abnormalities in females). The fertility of the *Whirligig* mice was also mildly decreased. It was remarkable that the *Whirligig* mice showed a very variable phenotype, with genital anomalies, heart defects, cleft palate

and choanal anomalies being present in only some of the mice, even though they were genetically identical. This may indicate that slight local modulations in the reduced CHD7 level, rather than variants in modifier genes, underlie the wide variations in the phenotypes seen between individuals.

In **chapter 3.2**, smell and pubertal development were studied in 35 adolescent patients with CHARGE syndrome. Anosmia (completely absent sense of smell) was present in 81% of patients and HH was seen in 67% of girls and 89% of boys with CHARGE syndrome. In our cohort, anosmia was always correlated with HH, whereas patients with a normal sense of smell always had a normal pubertal development. We therefore proposed using a smell test to predict the occurrence of HH in patients with CHARGE syndrome. This provides the opportunity to start with hormone replacement therapy at an early age in affected patients, leading to age-appropriate puberty. This will in turn reduce socio-emotional problems and also lower the risk of osteoporosis and cardiovascular disease, which are related to low sex hormone levels.

In **chapter 3.3**, the clinical data are presented of seven patients with CHARGE syndrome who died unexpectedly after the neonatal period. Respiratory aspiration (food or stomach acid entering the lungs) probably contributed to death in five patients, one patient choked while eating, and another died of post-operative airway complications. Unfortunately, no autopsies were performed on these patients. Based on our findings and a literature review, we suggested that gastro-oesophageal reflux disease, poor coordination of swallowing and breathing, respiratory aspiration, and post-operative airway events are important contributors to early death in CHARGE syndrome. We proposed cranial nerve dysfunction as the underlying mechanism and advised examining patients with CHARGE syndrome at a multidisciplinary specialist clinic. Assessment of swallowing and cranial nerve function is important and timely treatment of swallowing problems and gastro-oesophageal reflux disease is required. In order to prevent post-operative airway events, we advised combining surgical procedures whenever possible and monitoring patients with CHARGE syndrome for a longer period after surgery.

Chapter 4: Kallmann syndrome

In **chapter 4.1**, we studied the overlap between CHARGE and Kallmann syndromes by performing *CHD7* analysis in 36 patients with Kallmann syndrome and 20 patients with solely HH and a normal sense of smell. The patients were from Japan and North America. Three *CHD7* mutations were identified in the patients with Kallmann syndrome, whereas none of the patients with solely HH had a *CHD7* mutation. Thorough re-evaluation of the *CHD7*-positive patients with Kallmann syndrome revealed that they all had additional features of CHARGE syndrome. We concluded that patients with Kallmann syndrome should be carefully screened for features of CHARGE syndrome and recommended performing *CHD7* analysis in patients who have any additional CHARGE features.

In **chapter 4.2**, we describe the results of *CHD7* analysis in a cohort of Dutch Kallmann patients. We conducted this study because another group presented data that conflicted with the previous study (chapter 4.1). The other group had identified *CHD7* mutations in patients with HH or Kallmann syndrome who did not have additional features of CHARGE syndrome. We therefore decided to repeat our study in an independent cohort of clinically well characterised patients with Kallmann syndrome. We have identified three *CHD7* mutations in 36 Dutch patients. Upon re-evaluation, the *CHD7*-positive patients all had additional CHARGE features, which is in agreement with findings in our previous study. We conclude that the yield of *CHD7* analysis in patients with isolated Kallmann syndrome seems very low, but increases when additional CHARGE features are present. Our current advice is to evaluate all patients with Kallmann syndrome and to perform *CHD7* analysis if two or more additional CHARGE features, or semicircular canal anomalies, are present. Identification of a *CHD7* mutation has important clinical implications regarding the surveillance and genetic counselling of the patient.

Chapter 5: Implications and general discussion

In **chapter 5.1**, the expanding phenotypic spectrum of CHARGE syndrome is described and we show that some molecularly diagnosed patients have very few features of CHARGE syndrome. We present the clinical features of 280 *CHD7*-positive patients and show that four features are almost universally present: external ear anomalies, cranial nerve dysfunction, semicircular canal hypoplasia, and delayed motor development. CHARGE syndrome is primarily a clinical diagnosis, but *CHD7* analysis can help to establish the diagnosis in patients who do not fulfil the clinical criteria. We propose a guideline for conducting *CHD7* analysis, which also indicates when imaging of the semicircular canals is helpful in the diagnostic process. Finally, we present updated recommendations for the clinical surveillance of patients with a *CHD7* mutation.

In **chapter 5.2**, the results of the work presented in this thesis are discussed in relation to the literature and we highlight the clinical implications of our research. In addition, we present plans for future research. For example, our group will perform exome sequencing in a group of patients with some features of CHARGE syndrome who do not have a mutation or deletion in the *CHD7* gene.

The major accomplishments of the research presented in this thesis are as follows:

- We built a locus-specific database that contains all the *CHD7* mutations (www.CHD7.org)
- We created a classification system that leads to more confident prediction of the pathogenicity of *CHD7* missense variants
- We gained more insight into the pathogenesis of anosmia and hypogonadotropic hypogonadism in CHARGE syndrome
- We presented recommendations to improve the clinical surveillance on pubertal development and to prevent sudden death in patients with CHARGE syndrome

- We compiled a guideline for *CHD7* analysis in patients with features of CHARGE or Kallmann syndrome.

Nederlandse samenvatting

Dit promotieonderzoek was erop gericht meer inzicht te verkrijgen in CHARGE syndroom en Kallmann syndroom met een focus op het *CHD7* gen. Hiertoe hebben we alle *CHD7* mutaties verzameld die eerder in de literatuur waren beschreven of die geïdentificeerd waren in de moleculair genetische laboratoria van Nijmegen en Kopenhagen. Daarnaast hebben we het reukvermogen en aspecten van het voortplantingssysteem in een muismodel voor CHARGE syndroom bestudeerd. Vervolgens hebben we bij patiënten met CHARGE syndroom het reukvermogen, de puberteitsontwikkeling en mogelijke oorzaken van overlijden onderzocht. Ten slotte is het *CHD7* gen geanalyseerd in patiënten met Kallmann syndroom.

Hoofdstuk 1: Achtergrondinformatie

Gedetailleerde achtergrondinformatie over het *CHD7* gen, over CHARGE syndroom en over Kallmann syndroom is te vinden in **hoofdstuk 1**. Kort samengevat is CHARGE syndroom een complexe en variabele multisysteem aandoening die voorkomt bij ongeveer 1 op de 10.000 pasgeborenen. CHARGE is een Engels acroniem, waarbij de zes letters staan voor zes kenmerken: colobomen (aangeboren oogafwijkingen), aangeboren hartafwijkingen, atresie van de choanen (blokkade van de opening tussen neus en mond), retardatie van groei en/of ontwikkeling, onderontwikkeling van de genitaliën (micropenis bij jongens) en oorafwijkingen. Daarnaast kunnen ook andere problemen optreden, zoals een verminderd evenwicht (ten gevolge van afwijkingen aan het evenwichtsorgaan), hersenzenuwdisfunctie (wat kan leiden tot gehoorverlies, aangezichtsverlamming of slikproblemen) en een afwezig reukvermogen. Verder heeft de meerderheid van de patiënten een hypogonadotroop hypogonadisme (HH), wat het uitblijven van de puberteit en onvruchtbaarheid tot gevolg heeft.

CHARGE syndroom wordt veroorzaakt door autosomaal dominante mutaties in het *CHD7* gen. Dat wil zeggen dat CHARGE syndroom even vaak voorkomt bij jongens als bij meisjes (autosomaal) en dat de kans dat iemand met CHARGE syndroom dit doorgeeft aan zijn of haar kind 50% is (dominant). Het kind met CHARGE syndroom is echter vaak de eerste in de familie, omdat de *CHD7* mutatie meestal nieuw (*denovo*) ontstaat.

CHARGE syndroom lijkt klinisch gezien op een andere zeldzame ziekte, Kallmann syndroom. Bij beide syndromen komen HH en een verminderd reukvermogen voor. Deze twee kenmerken zijn de hoofdkenmerken van Kallmann syndroom en zijn dus, per definitie, altijd aanwezig bij patiënten met Kallmann syndroom. Patiënten met Kallmann syndroom hebben soms ook nog andere verschijnselen, zoals gehoorverlies, nierafwijkingen en missende tanden. Slechts bij 25 tot 30% van de patiënten met Kallmann syndroom kan een mutatie worden aangetoond, in genen met verschillende overervingpatronen: X-gebonden recessief (veroorzaakt door mutaties in het *KAL1*-gen), autosomaal dominant (veroorzaakt door mutaties in het *FGFR1*-, *FGF8*- en *CHD7*-gen) en autosomaal recessief (veroorzaakt door mutaties in het *PROK2*- en *PROKR2*-gen). Het meest recente inzicht is echter dat in het merendeel van de patiënten met Kallmann syndroom meerdere genen gemuteerd zijn (oligogene overerving).

Hoofdstuk 2: Het *CHD7* gen

In **hoofdstuk 2.1** wordt een volledig overzicht gegeven van alle pathogene *CHD7* mutaties die zijn beschreven in de literatuur of die zijn geïdentificeerd door de moleculair genetische laboratoria in Nijmegen en Kopenhagen (in totaal 531 verschillende mutaties op 15 juni 2011). De *CHD7* mutaties bleken gelijkmatig te zijn verdeeld over de coderende regio van het *CHD7* gen. De meeste mutaties waren uniek, maar 96 mutaties zijn meer dan eens gevonden (met name arginine naar stop mutaties). We hebben vooral nonsense en frameshift mutaties gevonden, terwijl splice site mutaties, missense mutaties en deleties slechts in een minderheid van de patiënten voorkwamen. We hebben een database gemaakt, die alle *CHD7* mutaties bevat met relevante klinische gegevens (beschikbaar op www.CHD7.org). Deze database is een waardevolle informatiebron voor artsen en onderzoekers. Daarnaast hebben we in dit hoofdstuk een up-to-date overzicht gegeven van alle expressiestudies, diermodellen en functionele studies betreffende CHD7.

In **hoofdstuk 2.2** presenteren we een nieuw classificatiesysteem dat kan helpen bij de voorspelling of een bepaalde missense variant in het *CHD7* gen pathogeen (ziekteverwekkend) of benigne (geen verband houdend met de ziekte) is. Een correcte classificatie van missense varianten is essentieel voor de erfelijkheidsvoorlichting aan patiënten en hun families. Ons classificatiesysteem combineert de output van drie algoritmes (SIFT, PolyPhen en Align-GVGD) met informatie over de oorsprong van een bepaalde missense variant (*de novo* versus overgeërfd). De combinatie van deze verschillende variabelen zal waarschijnlijk leiden tot een meer betrouwbare voorspelling van pathogeniteit dan voorheen mogelijk was. Met ons systeem hebben we 145 *CHD7* missense varianten geclassificeerd en we hebben deze data opgeslagen in de database die we hebben gepresenteerd in hoofdstuk 2.1. Onze classificatie van de missense varianten kwam overeen met de voorspelling gebaseerd op een structuurmodel van de chromo- en helicasedomeinen van het CHD7 eiwit. Dit model voorspelde dat de varianten die wij hebben geclassificeerd als 'waarschijnlijk pathogeen' de stabiliteit van het CHD7 eiwit verminderen en veelal in de kern van het eiwit liggen. Op DNA niveau bleken de pathogene missense varianten voornamelijk in het midden van het *CHD7* gen te liggen, terwijl de benigne missense varianten vooral aan het begin of aan het einde van het gen gelegen waren. Tot slot hebben we laten zien dat patiënten met een missense mutatie in het algemeen minder ernstige verschijnselen hebben dan patiënten met een mutatie die leidt tot een getrunceerd (verkort) eiwit.

In **hoofdstuk 2.3** hebben we onderzocht hoe vaak deleties van een gedeelte van het *CHD7* gen aanwezig zijn in patiënten met kenmerken van CHARGE syndroom. Gedeeltelijke deleties kunnen niet worden gedetecteerd met standaard DNA onderzoek van het *CHD7* gen (sequentieanalyse), maar kunnen wel worden gevonden met 'multiplex ligation-dependent probe amplification' (MLPA). Daarom hebben we MLPA analyse verricht in 54 patiënten met kenmerken van CHARGE syndroom, bij wie geen *CHD7* mutatie was gevonden met sequentieanalyse. Hierbij hebben we slechts één gedeeltelijke deletie van het *CHD7* gen gevonden in een patiënt met de klinische diagnose CHARGE

syndroom. Hieruit hebben we geconcludeerd dat gedeeltelijke deleties van het *CHD7* gen slechts zelden voorkomen bij patiënten met kenmerken van CHARGE syndroom.

Hoofdstuk 3: CHARGE syndroom

In **hoofdstuk 3.1** beschrijven we de resultaten van ons onderzoek in een muismodel voor CHARGE syndroom, de *Whirligig* muis, die een nonsense mutatie in het *CHD7* gen heeft. Ons doel was om te onderzoeken of deze muizen een reukstoornis en afwijkende genitaliën hebben, net als patiënten met CHARGE syndroom. De *Whirligig* muizen presteerden slechter bij de reuktest dan wildtype muizen. De slechte prestatie zou kunnen worden veroorzaakt door een reukstoornis, maar zou ook het gevolg kunnen zijn van ernstige evenwichtsstoornissen. Nader onderzoek wees uit dat enkele muizen afwijkende reukhersenen hadden. Sommige muizen hadden ook afwijkende geslachtsorganen (onderontwikkelde zaadballen bij mannetjes en baarmoederafwijkingen bij vrouwtjes). *Whirligig* muizen waren ook enigszins verminderd vruchtbaar. Erg opvallend was het sterk variabele fenotype van *Whirligig* muizen die genetisch identiek zijn. Deze grote variatie in fenotype wordt mogelijk veroorzaakt wordt door kleine lokale veranderingen in het door de mutatie toch al verminderde CHD7 niveau, en niet door variaties in modificerende genen.

In **hoofdstuk 3.2** staan de resultaten van ons onderzoek naar reukvermogen en puberteitsontwikkeling bij 35 adolescenten met CHARGE syndroom beschreven. Anosmie (volledig afwezig reukvermogen) was aanwezig bij 81% van de patiënten en HH (uitblijvende puberteit) werd gezien bij 67% van de meisjes en 89% van de jongens met CHARGE syndroom. Anosmie kwam altijd samen met HH voor, terwijl patiënten met een normaal reukvermogen ook altijd een normale puberteitsontwikkeling hadden doorgemaakt. We hebben daarom voorgesteld om een reuktest te gebruiken om te kunnen voorspellen bij welke patiënten met CHARGE syndroom de puberteitsontwikkeling niet spontaan op gang komt. Een hormoonbehandeling kan er vervolgens voor zorgen dat zij wel op de juiste leeftijd in de puberteit komen. Hierdoor zullen minder socio-emotionele problemen voorkomen en ook het risico op botontkalking en hart- en vaatziekten zal afnemen, omdat deze gerelateerd zijn aan lage hormoonspiegels.

In **hoofdstuk 3.3** worden de medische gegevens gepresenteerd van zeven patiënten met CHARGE syndroom die plotseling zijn overleden na de neonatale periode. Bij vijf patiënten heeft respiratoire aspiratie (voedsel of maagzuur dat in de longen terecht komt) waarschijnlijk bijgedragen aan het overlijden, één patiënt is gestikt tijdens het eten en een andere patiënt is overleden aan postoperatieve luchtwegproblemen. Helaas was bij geen van deze patiënten obductie verricht. Gebaseerd op onze bevindingen en een literatuuronderzoek, denken wij dat gastro-oesophageale reflux, verstoorde coördinatie van slikken en ademhalen, respiratoire aspiratie en postoperatieve luchtwegproblemen een belangrijke rol spelen bij vroegtijdig overlijden van patiënten met CHARGE syndroom. Hersenzenuwdisfunctie zou aan deze problemen ten grondslag kunnen liggen. Gezien

bovengenoemde risicofactoren adviseren we om patiënten met CHARGE syndroom te controleren op een multidisciplinaire polikliniek. Daarbij is controle van de slikfunctie en hersenzenuwfunctie belangrijk en snelle behandeling van slikproblemen en gastro-oesophageale reflux is raadzaam. Postoperatieve luchtwegproblemen zijn deels te voorkomen door operaties waarvoor narcose noodzakelijk is, indien mogelijk, te combineren en door patiënten met CHARGE syndroom na een operatie langer dan normaal op de uitslaapkamer te laten blijven.

Hoofdstuk 4: Kallmann syndroom

In **hoofdstuk 4.1** onderzoeken we de overlap tussen CHARGE syndroom en Kallmann syndroom door het *CHD7* gen te analyseren bij 36 patiënten met Kallmann syndroom en 20 patiënten met HH (uitblijvende puberteit en onvruchtbaarheid) en een normaal reukvermogen. Deze patiënten waren afkomstig uit Japan en Noord Amerika. We vonden drie *CHD7* mutaties bij patiënten met Kallmann syndroom, terwijl geen van de patiënten met alleen HH een *CHD7* mutatie had. De drie patiënten bij wie we een *CHD7* mutatie hadden gevonden, bleken bij herbeoordeling alledrie additionele CHARGE kenmerken te hebben. We concluderen dat patiënten met Kallmann syndroom goed moeten worden onderzocht op kenmerken van CHARGE syndroom en adviseren *CHD7* analyse als additionele CHARGE kenmerken aanwezig zijn.

In **hoofdstuk 4.2** beschrijven we de resultaten van *CHD7* analyse in een cohort Nederlandse patiënten met Kallmann syndroom. We hebben dit onderzoek verricht, omdat een andere onderzoeksgroep data had gepubliceerd die in strijd was met onze eerdere studie (hoofdstuk 4.1). Deze onderzoeksgroep had *CHD7* mutaties gevonden bij patiënten met HH of Kallmann syndroom die géén andere kenmerken van CHARGE syndroom hadden. Wij hebben daarom besloten om onze studie te herhalen in een onafhankelijk cohort van patiënten met Kallmann syndroom die klinisch goed onderzocht waren. We vonden drie *CHD7* mutaties bij 36 Nederlandse patiënten. Ook nu bleek dat alledrie patiënten additionele CHARGE kenmerken hadden, hetgeen in overeenstemming is met de resultaten van onze eerdere studie (hoofdstuk 4.1). We concluderen dat de opbrengst van *CHD7* analyse bij patiënten met Kallmann syndroom zonder andere CHARGE kenmerken erg laag is, maar dat deze analyse wel zinvol is bij patiënten met additionele CHARGE kenmerken. Ons huidige advies is om alle patiënten met Kallmann syndroom te evalueren en om *CHD7* analyse te verrichten als twee of meer additionele CHARGE kenmerken, of afwijkingen aan het evenwichtsorgaan, aanwezig zijn. Identificatie van een *CHD7* mutatie heeft belangrijke klinische consequenties voor de verdere controle van de patiënt en de erfelijkheidsvoorlichting aan de patiënt en zijn/haar familie.

Hoofdstuk 5: Implicaties en discussie

In **hoofdstuk 5.1** laten we zien dat sommige patiënten met een *CHD7* mutatie erg weinig kenmerken van CHARGE syndroom hebben, terwijl andere patiënten juist veel of ernstige afwijkingen hebben. We presenteren de klinische kenmerken van 280 patiënten met een *CHD7* mutatie en laten zien

dat vier kenmerken bijna altijd aanwezig zijn: een afwijkende oorschelp, hersenzenuwdisfunctie, een afwijkend evenwichtsorgaan en een vertraagde motorische ontwikkeling. CHARGE syndroom is voornamelijk een klinische diagnose, maar *CHD7* analyse kan helpen bij het stellen van de diagnose bij patiënten die niet aan de klinische criteria voldoen. We hebben een richtlijn opgesteld die, al dan niet na het verrichten van een scan van het evenwichtsorgaan, aangeeft bij welke patiënten *CHD7* analyse zinvol is. Tot slot presenteren we nieuwe adviezen voor de verdere controle van patiënten met een *CHD7* mutatie.

In **hoofdstuk 5.2** bediscussiëren we de resultaten van ons onderzoek in relatie tot de literatuur en we geven aan wat de klinische implicaties van ons onderzoek zijn. Daarnaast presenteren we onze plannen voor toekomstig onderzoek. Onze groep zal bijvoorbeeld exome sequencing (sequentieanalyse van het gehele coderende genoom) gaan verrichten in een groep patiënten met enkele kenmerken van CHARGE syndroom, bij wie geen mutatie of deletie van het *CHD7* gen is gevonden.

De belangrijkste resultaten van ons onderzoek zijn als volgt:

- We hebben een database gecreëerd die 531 *CHD7* mutaties bevatte op 15 juni 2011 (www.CHD7.org)
- We hebben een classificatiesysteem gemaakt waarmee de pathogeniteit van *CHD7* missense variaties beter kan worden voorspeld
- We hebben meer inzicht gekregen in de onderliggende mechanismen van anosmie en hypogonadotroop hypogonadisme in CHARGE syndroom
- We hebben adviezen gegeven voor de verdere controle van patiënten met CHARGE syndroom, met name gericht op puberteitsontwikkeling en op het voorkomen van plotse dood
- We hebben een richtlijn opgesteld die aangeeft wanneer *CHD7* analyse zinvol is bij patiënten met kenmerken van CHARGE syndroom of Kallmann syndroom.

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Jorieke

Curriculum Vitae

Jorieke van Kammen-Bergman werd op 12 september 1977 geboren in Oldenzaal. Nadat zij in 1995 haar atheneum opleiding voltooide aan het Thijcollege in Oldenzaal, begon zij met de studie biologie aan de Universiteit Utrecht. In 2004 behaalde zij cum laude haar bachelor diploma met als specialisatie moleculaire genetica/celbiologie. Inmiddels was ze ook begonnen met de opleiding geneeskunde aan de Universiteit Utrecht. Haar wetenschappelijke stage voerde haar naar de University of Western Australia in Perth, alwaar zij onderzoek deed naar de ziekte van Duchenne. In 2003 behaalde zij haar doctoraal met een cum laude vermelding en startte ze met haar co-schappen. Voor haar KNO en oogheelkunde co-schap ging zij naar het Kent & Sussex hospital in Tunbridge Wells, Engeland. In 2005 behaalde zij haar artsexamen en begon zij als ANIOS bij de afdeling genetica van het UMCG. In februari 2006 startte zij met de opleiding tot klinisch geneticus op dezelfde afdeling (opleiders dr. A.J. van Essen en dr. J.C. Oosterwijk) en in september 2007 begon zij met een AGIKO traject dat heeft geleid tot dit proefschrift. Een deel van haar promotieonderzoek vond plaats in het Wellcome Trust Sanger Institute in Hinxton, Engeland. In 2008 won zij drie prijzen voor presentatie van haar onderzoek op (inter)nationale congressen. Sinds augustus 2011 is zij klinisch geneticus en werkt zij bij EUROCAT (European Registration of Congenital Anomalies and Twins) in het UMCG.

List of publications

1. Janssen N*, **Bergman JEH***, Swertz MA, Tranebjærg L, Lodahl M, Schoots J, Hofstra RMW, van Ravenswaaij-Arts CMA, Hoefsloot LH. Mutation update on the *CHD7* gene involved in CHARGE syndrome. *Submitted* 2011.
2. **Bergman JEH***, Janssen N*, van der Sloot AM, de Walle HEK, Schoots J, Rendtorff ND, Tranebjærg L, Hoefsloot LH, van Ravenswaaij-Arts CMA, Hofstra RMW. A novel classification system to predict the pathogenic effects of *CHD7* missense variants in CHARGE syndrome. *Submitted* 2011.
3. **Bergman JEH**, de Ronde W, Jongmans MCJ, Wolffenbuttel BHR, Drop SLS, Hermus A, Bocca G, Hoefsloot LH, van Ravenswaaij-Arts CMA. The results of *CHD7* analysis in clinically well-characterised patients with Kallmann syndrome. *Submitted* 2011.
4. **Bergman JEH**, Janssen N, Hoefsloot LH, Jongmans MCJ, Hofstra RMW, van Ravenswaaij-Arts CMA. *CHD7* mutations and CHARGE syndrome: the clinical implications of an expanding phenotype. *J Med Genet* 2011;48(5):334-342.
5. **Bergman JEH**, Bocca G, Hoefsloot LH, Meiners LC, van Ravenswaaij-Arts CMA. Anosmia predicts hypogonadotropic hypogonadism in CHARGE syndrome. *J Pediatr* 2011;158(3):474-479.
6. Morava E, Wevers RA, Cantagrel V, Hoefsloot LH, Al-Gazali L, Schoots J, van Rooij A, Huijben K, van Ravenswaaij-Arts CMA, Jongmans MCJ, Sykut-Cegielska J, Hoffmann GF, Bluemel P, Adamowicz M, van Reeuwijk J, Ng BG, **Bergman JEH**, van Bokhoven H, Körner C, Babovic-Vuksanovic D, Willemsen MA, Gleeson JG, Lehle L, de Brouwer AP, Lefeber DJ. A novel cerebello-ocular syndrome with abnormal glycosylation due to abnormalities in dolichol metabolism. *Brain* 2010;133(11):3210-3220.
7. Batsukh T, Pieper L, Koszucka AM, von Velsen N, Hoyer-Fender S, Elbracht M, **Bergman JEH**, Hoefsloot LH, Paulis S. CHD8 interacts with CHD7, a protein which is mutated in CHARGE syndrome. *Hum Mol Genet* 2010;19(14):2856-2866.
8. **Bergman JEH**, Blake KD, Bakker MK, du Marchie Sarvaas GJ, Free RH, van Ravenswaaij-Arts CMA. Death in CHARGE syndrome after the neonatal period. *Clin Genet* 2010;77(3):232-240.
9. **Bergman JEH**, Bosman EA, van Ravenswaaij-Arts CMA, Steel KP. Study of smell and reproductive organs in a mouse model for CHARGE syndrome. *Eur J Hum Genet* 2010;18(2):171-177.
10. Wulffaert J, Scholte EM, Dijkxhoorn YM, **Bergman JEH**, van Ravenswaaij-Arts CMA, van Berckelaer-Onnes IA. Parenting stress in CHARGE syndrome and the relationship with child characteristics. *J Dev Phys Disabil* 2009;21(4):301-313.
11. **Bergman JEH**, van Ravenswaaij-Arts CMA, Bocca G, Wolffenbuttel BHR. Landelijk onderzoek gestart naar overlap Kallmann- en CHARGE-syndroom. *Endocrinologie* 2009;2:13.
12. Jongmans MCJ, van Ravenswaaij-Arts CMA, Pitteloud N, Ogata T, Sato N, Claahsen-van der Grinten HL, van der Donk K, Seminara S, **Bergman JEH**, Brunner HG, Crowley WF Jr, Hoefsloot LH. *CHD7* mutations in patients initially diagnosed with Kallmann syndrome – the clinical overlap with CHARGE syndrome. *Clin Genet* 2009;75(1):65-71.

13. Aalberts JJJ, van den Berg MP, **Bergman JEH**, du Marchie Sarvaas GJ, Post JG, van Unen H, Pals G, Boonstra PW, van Tintelen JP. The many faces of aggressive aortic pathology: Loeys-Dietz syndrome. *Neth Heart J* 2008;16(9):299-304.
14. **Bergman JEH**, de Wijs I, Jongmans MCJ, Admiraal RJ, Hoesloot LH, van Ravenswaaij CMA. Exon copy number alterations of the *CHD7* gene are not a major cause of CHARGE and CHARGE-like syndrome. *Eur J Med Gen* 2008;51(5):417-425.
15. **Bergman JEH**, Veenstra-Knol HE, van Essen AJ, van Ravenswaaij CMA, den Dunnen WF, van den Wijngaard A, van Tintelen JP. Two related Dutch families with a clinically variable presentation of cardioskeletal myopathy caused by a novel S13F mutation in the desmin gene. *Eur J Med Genet* 2007;50(5):355-366.

* these authors contributed equally

Addendum
Color figures

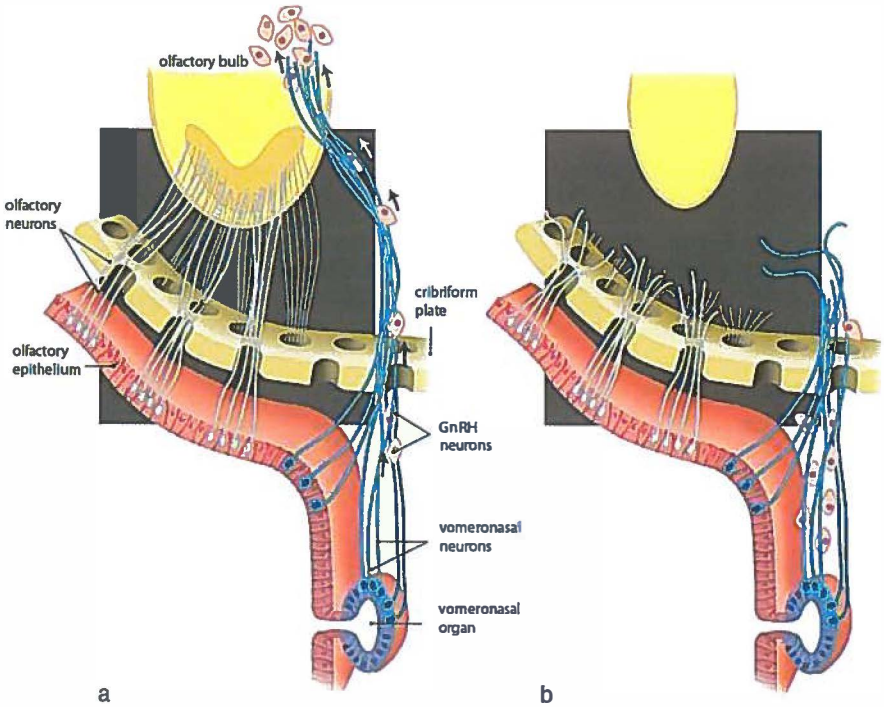
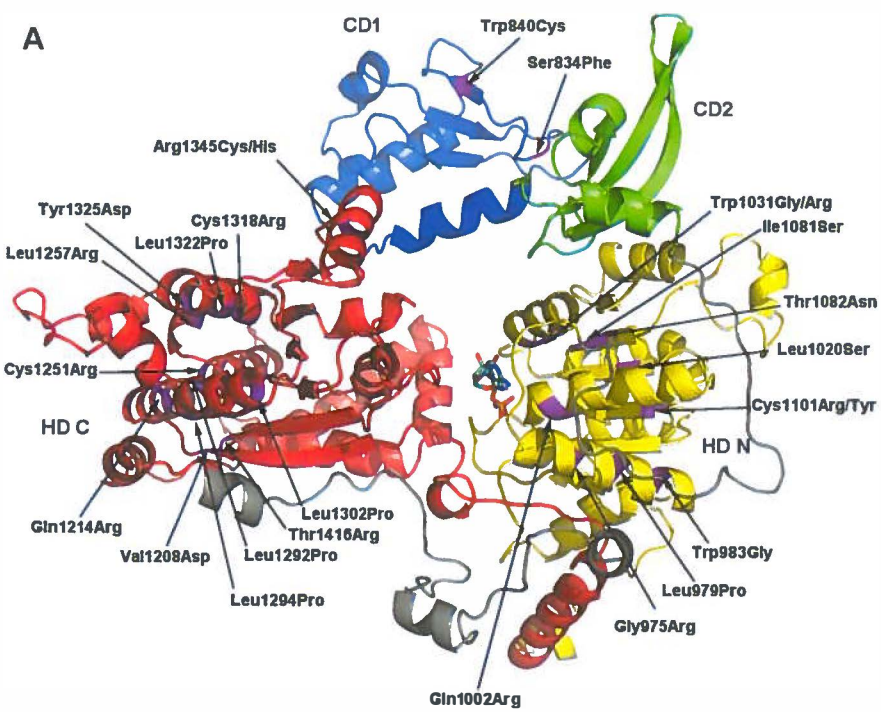


Figure 1. (Chapter 1, page 17)



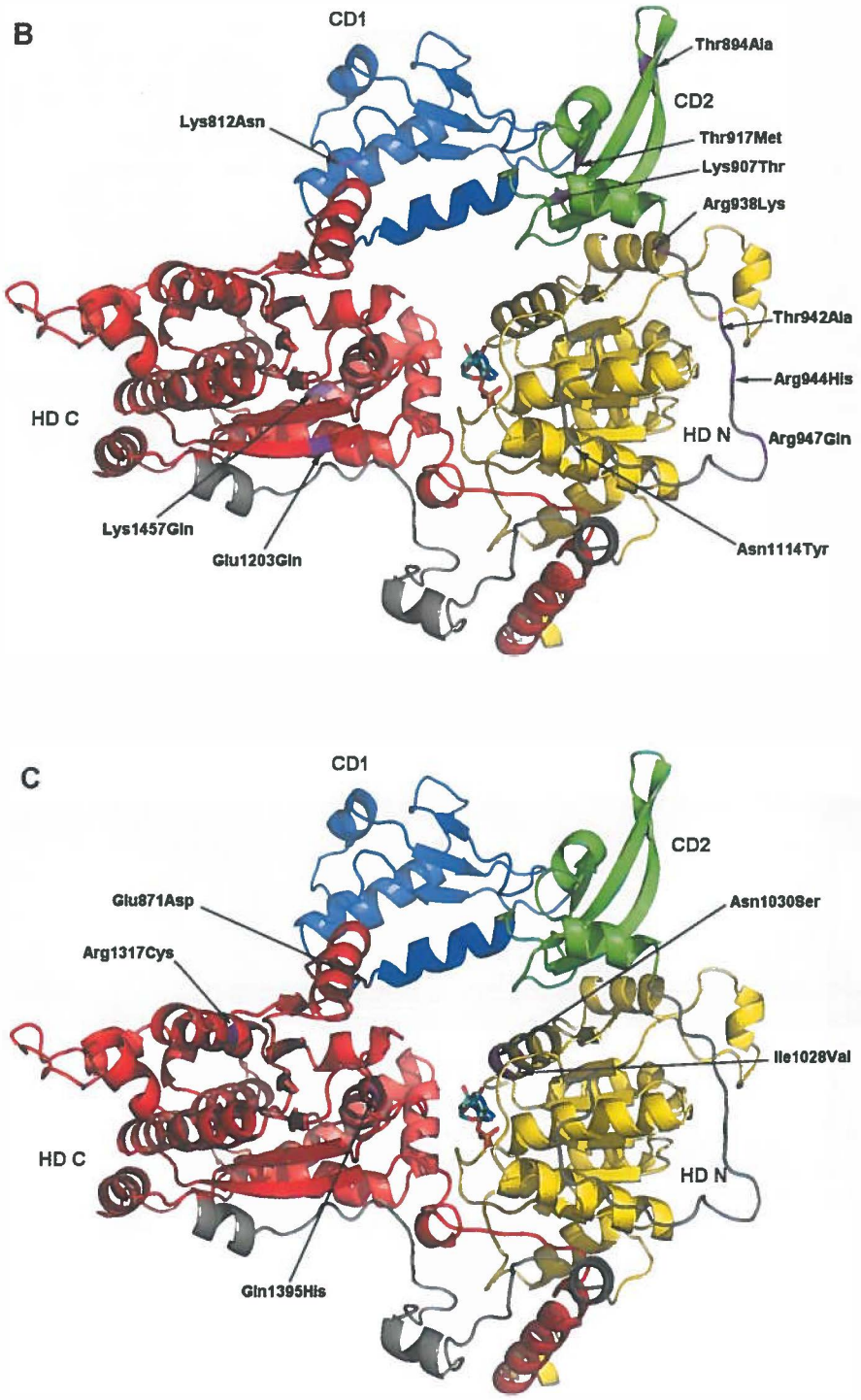


Figure 2 . (Chapter 2.2, page 77)

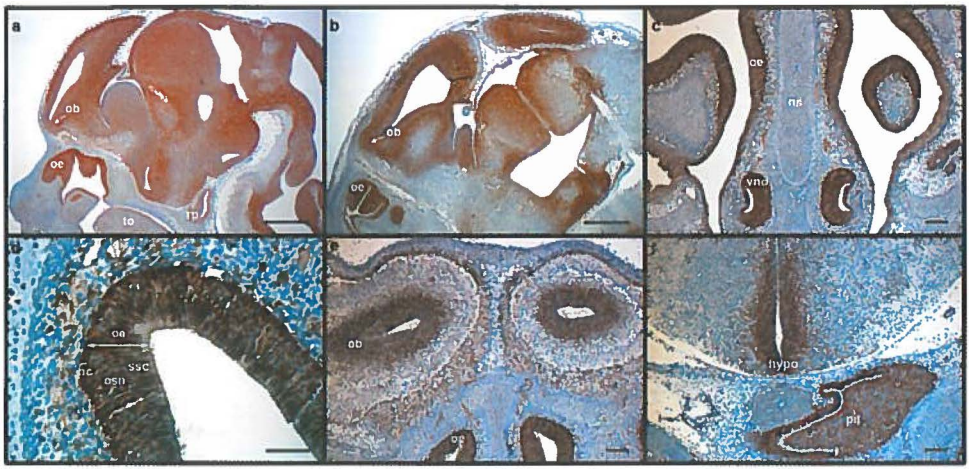


Figure 1. (Chapter 3.1, page 112)

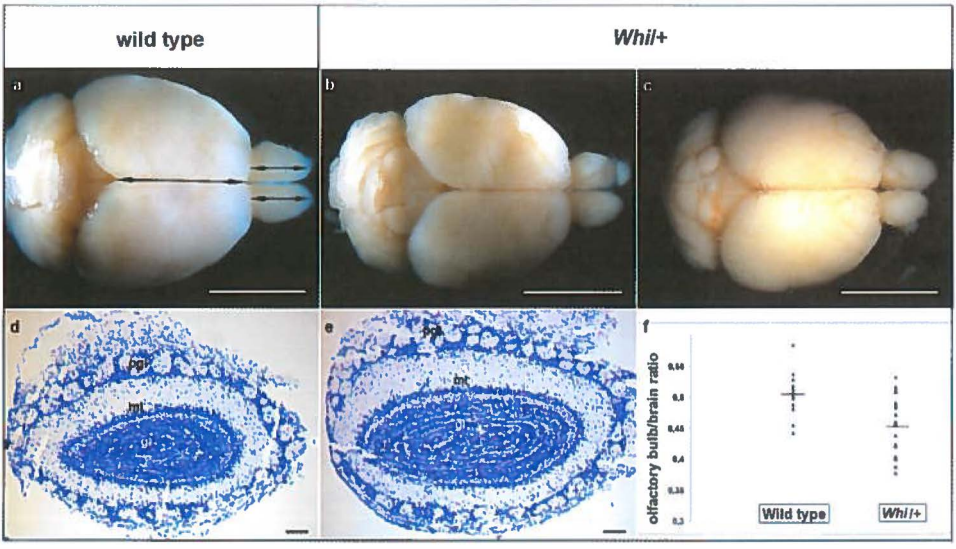


Figure 3. (Chapter 3.1, page 113)

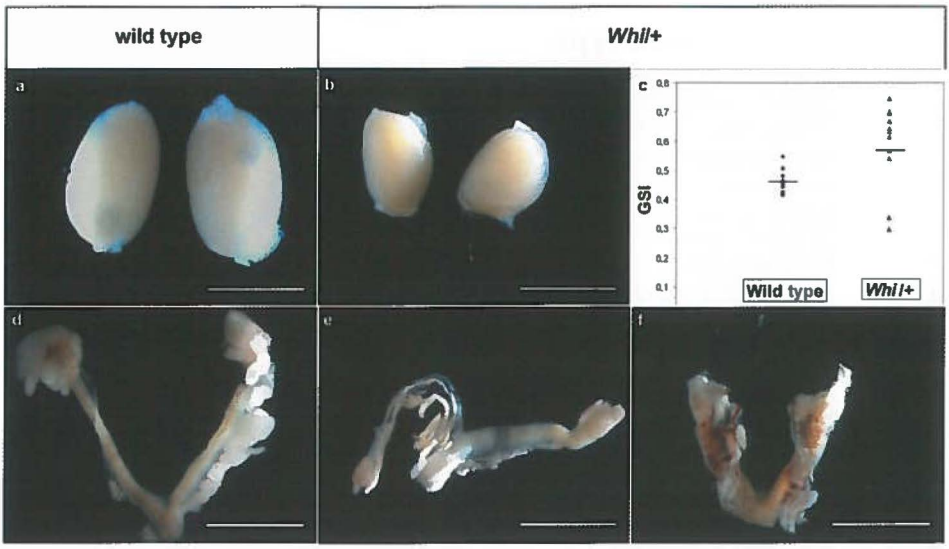


Figure 4. (Chapter 3.1, page 114)

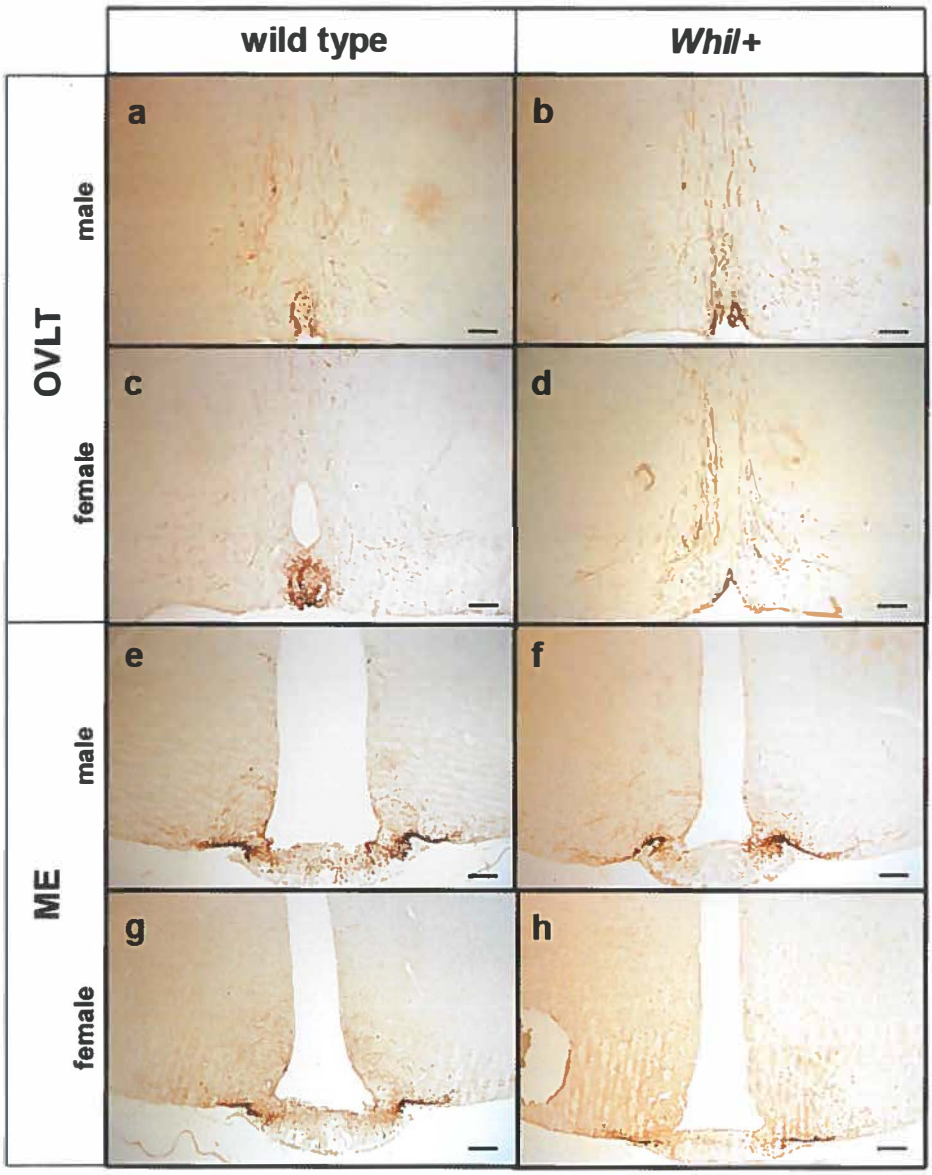
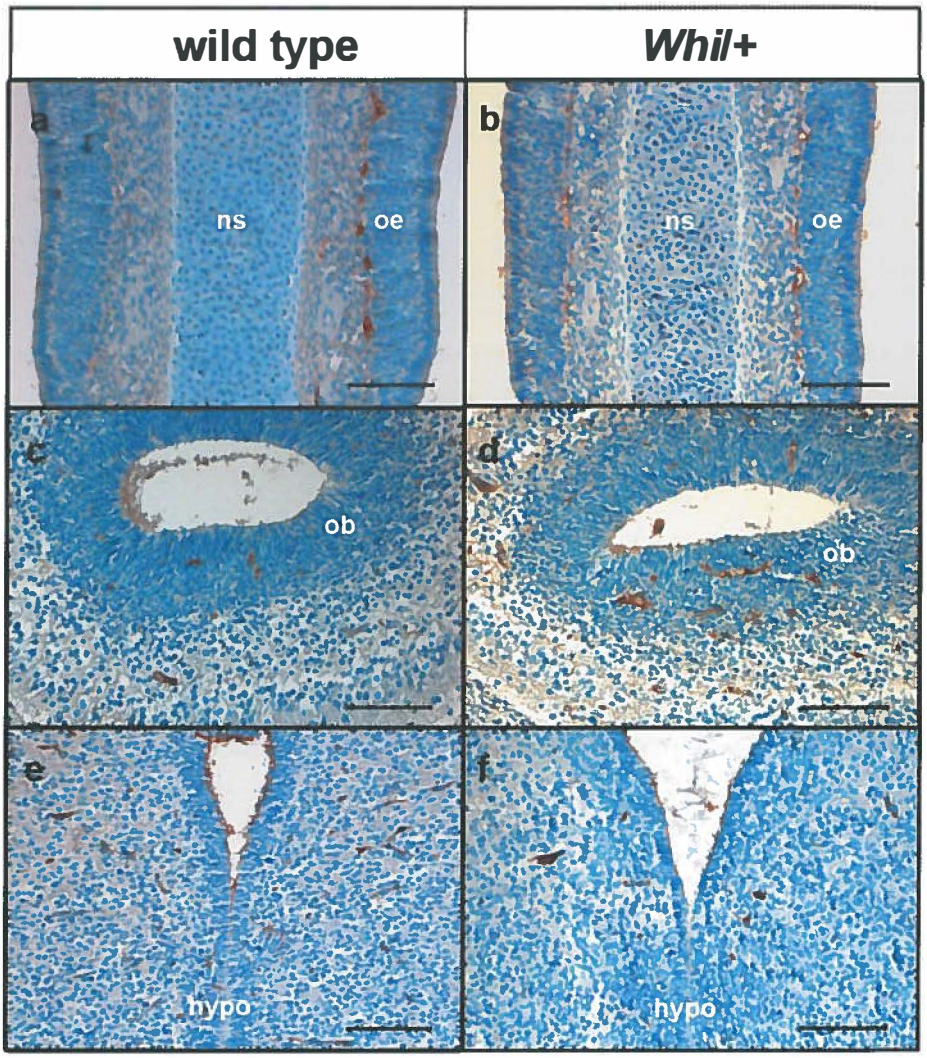


Figure 5. (Chapter 3.1, page 116)



Supplementary Figure 51. (Chapter 3.1, page 121)