CHARGE syndrome: CHD7 mutations, heart defects and overlapping syndromes

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LIST OF ABBREVIATIONS

ARSA: aberrant right subclavian artery
ATP: Adenosine triphosphate
ASD: atrial septal defect
AVSD: atrioventricular septal defects
CGH: comparative genomic hybridization
CHARGE: Coloboma, Heart disease, Choanal atresia, Retardation of growth and/or development, Genital hypoplasia and Ear abnormalities with or without deafness
BRK: Brahma and Kismet
CHD: Chromodomain Helicase DNA-binding
Chromo: chromatin organization modifier
CLP: cleft lip and/or palate
dB: decibel
Der: derivate
DNA: deoxyribonucleic acid
DORV: double outlet right ventricle
Dup: duplication
ES: embryonic stemcells
ESP: exome sequencing project
EUROCAT: European network of population-based registries for the epidemiologic surveillance of congenital anomalies
FISH: fluorescence in situ hybridization
IAA: interrupted aortic arch
Inv: inversion
H3K4: histone 3 lysine at position 4
HH: hypogonadotropic hypogonadism
KS: Kallmann syndrome
LVOTO: left ventricular outflow tract obstructions
Mb: Megabase
MCA: multiple congenital abnormalities
MIM: Mendelian Inheritance in Man
MLPA: multiplex ligation-dependent probe amplification
nIHH: normosmic idiopathic hypogonadotropic hypogonadism
PBAF: Polybromo- and BRGI-associated factor
PCR: polymerase chain reaction
PDA: patent ductus arteriosus
RAA: right-sided aortic arch

RNA: ribonucleic acid
rRNA: ribosomal RNA
RVOTO: right ventricular outflow tract obstructions
SANT: Switching-defective protein 3, Adaptor 2, Nuclear receptor corepressor, Transcription factor IIIB
SD or SDS: standard deviation score
SNP: single nucleotide polymorphism
SRO: shortest region of overlap
t: translocation
trp: triplication
TA: truncus arteriosus
TGA: transposition of the great arteries
TOF: tetralogy of Fallot
UV: unclassified variant
VSD: ventricular septal defect
WES: whole exome sequencing

GENETIC TERMINOLOGY

General

***De novo*:** an alteration in a gene that is present for the first time in one family member as a result of a mutation in a germ cell (egg or sperm) of one of the parents or in the fertilized egg itself.

Chromatine: is a complex of DNA and proteins that helps to compacting the DNA, strengthening the DNA during replication and regulating gene expression.

Codon: a sequence of three DNA or RNA nucleotides that corresponds with a specific amino acid or stop signal during protein synthesis.

Exome: the total of protein coding DNA (1-2% of total genome).

Exon: Any nucleotide sequence within a gene that is retained in the final mature RNA product, after the introns have been removed. The term exon refers to both the DNA sequence within a gene and to the corresponding sequence in RNA transcripts.

Expression: the ***clinical expression*** of a specific genetic predisposition is the way the disease presents. ***Variable expression*** means that people with a pathogenic mutation have different symptoms of a disease. ***Gene expression*** is the term used to show if a gene is used to synthesize gene products (for example proteins).

Genome: all genetic material in an organism.

Genotype: the particular type and arrangement of genes that an organism has.

Germline mosaicism: more than one set of genetic information is found specifically within the egg or spermcells.

Heterozygous: when different alleles of the gene are present on both homologous chromosomes

Homozygous: when identical alleles of the gene are present on both homologous chromosomes

Intron: any nucleotide sequence within a gene that is removed in the final mature RNA product. The term intron refers to both the DNA sequence within a gene and the corresponding sequence in RNA transcripts

Mendelian Inheritance in Man (MIM): a database that catalogues all the known diseases with a genetic component. The database is available online via www.omim.org.

Penetrance: the proportion of individuals carrying a particular variant of a gene (the genotype) that also express an associated trait (the phenotype).

Phenotype: all observable characteristics or traits in an organisms, such as its morphology, development, biochemical or physiological properties and behavior.

Sequence variants: The definitions are based on the Sequence Variant Nomenclature (http://varnomen.hgvs.org/)

Deletion: a sequence change where, compared to a reference sequence, one or more nucleotides are not present.

c.: coding DNA sequence.

Duplication: a sequence change where, compared to a reference sequence, a copy of one or more nucleotides are inserted directly after the original copy of that sequence.

Frameshift mutation: a sequence change between the translation initiation (start) and termination (stop) codon where, compared to a reference sequence, translation shifts to another reading frame.

Insertion: a sequence change where one or more nucleotides are inserted and where the insertion is not a copy of a sequence immediately prior.

Inversion: a sequence change where more than one nucleotide replacing the original sequence are the reverse complement of the original sequence.

Missense mutation: a variant changing one amino acid into another amino acid .

Nonsense mutation: a variant changing a amino acid to a translation termination (stop) codon.

p.: protein sequence.

Splice site mutation: a sequence change where, compared to a reference sequence, the normal RNA splicing pattern is altered.

Translocation: a translocation occurs when two chromosomes break and the fragments rejoin with the non-homologous chromosome.

Truncating mutation: result in a in a truncated, incomplete, and usually nonfunctional protein product.

Variants of unknown significance (VUS): refers to variants in the DNA of which the effect on protein function is unknown. VUS was previously known as 'unclassified variant (UV)'.

Some genetic techniques

Array-based comparative genomic hybridization (array CGH): Array CGH is a molecular technique which compares the amount of DNA at the different points in the genome to a reference genome. This allows the detection of small deletions and duplications in genetic material at random positions.

Chromatin immunoprecipitation sequencing (ChIP seq): is a method used to analyze protein interactions with DNA. ChIP-seq combines chromatin immunoprecipitation with massively parallel DNA sequencing to identify the binding sites of DNA-associated proteins. It can be used to map global binding sites precisely for any protein of interest.

Fluorescence in situ hybridization (FISH): Metaphase nuclei are hybridized with a specific probe labelled with a fluorescent dye. Fluorescent microscopy is used

to determine whether the two copies of a specific DNA sequence are present or not. FISH can be used to identify a deletions or duplication at a specific, known position at the chromosome.

Karyotyping: Karyotyping is a chromosomal analysis that describes the number and appearance of chromosomes in metaphase nuclei under a normal light microscope. It detects numerical chromosomal anomalies and large structural anomalies.

Multiplex ligation-dependent probe amplification (MLPA): Multiplex refers to the amplification of several different DNA sequences simultaneously. The probe amplification is ligation dependent since PCR only amplifies correctly attached originally split probes, that need to be attached to each other and the target DNA by the enzyme ligase. MLPA is used to detect single or multiple deletions or duplications of a exon of a target gene.

Next generations sequencing (NGS): NGS is also called high-throughput sequencing. Sequencing refers to determining the order of nucleotides in the DNA molecule. NGS is a method for parallel sequencing large numbers of DNA templates reducing the amount of time and money needed for the test. It is used to refer to different modern sequencing technologies.

Sanger sequencing: Sequencing refers to determining the order of nucleotides in the DNA molecule. Sanger sequencing is the classical way to detect sequence alterations. DNA is analyzed by using small amounts of labelled dideoxynucleotide (ddNTPs) of the four normal nucleotides creating stops in the DNA string which can be detected by automated sequencing machines.

Single Nucleotide array (SNP) array: SNP array is a molecular technique that detects naturally occurring SNPs, variations at a single site, in DNA throughout the genome. This allows the detection of small deletions and duplications in genetic material at random positions. SNP array can also be used to identify loss of heterozygosity and perform genetic linkage analysis.

Whole exome sequencing (WES): Sequencing refers to determining the order of nucleotides in the DNA molecule. For whole exome sequencing the first step is to select only the exome. Next generation sequencing is then used to sequence the entire exome.

CHAPTER 1

General introduction, scope and outline of this thesis

1.1 GENERAL INTRODUCTION

CHARGE syndrome (MIM 214800) is characterized by a very variable combination of multiple congenital anomalies. It was first recognized in 1979 as a cluster of congenital anomalies by pediatrician Bryan Hall and ophthalmologist Helen Hittner and her colleagues.[1,2] Hall described 17 patients with choanal atresia and identified an association of this anomaly with multiple other congenital anomalies including coloboma, small ears, congenital heart defect and hypogenitalism. He also suggested there was a broader phenotypic spectrum because he recognized patients with the same association of congenital anomalies but without choanal atresia.[2] Hittner and her colleagues focused on the association of ocular coloboma with congenital heart defects, external ear anomalies, hearing loss and intellectual disability in ten patients.[1] In 1981, Roberta Pagon recognized the association described by Hall and by Hittner et al. as the same entity and included another 21 patients with either coloboma or choanal atresia to endorse the association. She introduced the acronym CHARGE, which stands for Coloboma, Heart defects, Atresia of chonae, Retardation of growth and/or development, Genital hypoplasia and Ear abnormalities and/or deafness to make it better recognizable to clinicians and to create awareness of the association.[3]

To make a clinical diagnosis of CHARGE syndrome, two sets of diagnostic criteria are still used in clinical practice: Blake's and Verloes' criteria. The criteria that Blake introduced in 1998 were last updated by a consortium in 2012.[4,5] This set of criteria uses the four C's as major features: Coloboma, Choanal atresia, Cranial nerve dysfunction and Characteristic ear abnormalities. A diagnosis of CHARGE syndrome can be made if a patient has all four of these major features, or has three major and three out of seven minor features (see Table 1 in chapter 3). Verloes also used coloboma and choanal atresia as major features, but added semicircular canal defects as a third major item.[6] He defined typical CHARGE syndrome as either all three major features or two major and two out of five minor features. Both clinical diagnostic criteria are very useful for diagnosing CHARGE syndrome and help clinicians provide the best care for their patients. The disadvantage of these criteria is that, for some features, specific tests like imaging for semicircular canal defects need to be done while other features have an age-dependent expression, like intellectual disability or hypogonadotropic hypogonadism in girls. Moreover, with the current clinical diagnostic criteria, patients who do not have a coloboma nor choanal atresia cannot receive the clinical diagnosis of typical CHARGE syndrome and thus may not be enrolled in appropriate care programs, e.g. screening for hearing loss, cardiac and renal abnormalities and endocrine dysfunction.

CHARGE was classified as an association for more than 20 years until Lisenka Vissers and colleagues identified the causative gene, *CHD7*, which allowed CHARGE to be re-classified as a syndrome.[7] Vissers and colleagues were very lucky to identify the *CHD7* gene using array-based comparative genomic hybridisation (array CGH) to identify copy-number-variations in two patients, since we now know such large deletions of *CHD7* occur in less than one percent of patients with CHARGE syndrome.[7,8] CHARGE syndrome is usually caused by a *de novo CHD7* mutation. Parents of a child with an apparently *de novo CHD7* mutation still have a recurrence risk of 1-2% due to somatic and germline mosaicism.[5] Patients with CHARGE syndrome have a 50% chance of transmitting the *CHD7* mutation to their offspring.[5]

Identification of the *CHD7* gene has helped in the diagnostic setting in several ways. It helped clinicians to identify the definitive cause of multiple congenital anomalies in clinically diagnosed CHARGE patients, knowledge which allows them to provide these patients with the best care. A genetic diagnosis can also relieve the feelings of guilt that parents of children with congenital anomalies often have, especially their worry that something they did during pregnancy caused the anomaly in their child. The identification of the *CHD7* gene also provided information on recurrence risk that can be used by patients and their family members, and led to expanded reproductive options with prenatal and pre-implantation genetic diagnosis. Finally, the identification of the causal nature of *CHD7* sequence variants created a lot of opportunities to perform new research on CHARGE syndrome and its phenotypes.

With the identification of *CHD7* mutations as the major cause of CHARGE syndrome, the clinical spectrum expanded, particularly on the milder end. Indeed, *CHD7* mutations have now been identified in patients who did not fulfill the clinical diagnostic criteria of CHARGE syndrome.[9,10] This molecular diagnosis has important implications for their own health and surveillance, but also leads to better genetic counseling for patients and their families about recurrence risk and prenatal diagnosis. More information on the expanding phenotype of *CHD7* mutations can be found in chapter 3 of this thesis. This expanding phenotype leads to the question: for which patients should *CHD7* analysis be performed? It's clear that patients may be missed when strictly using the current clinical diagnostic criteria as inclusion criteria for *CHD7* analysis. Furthermore, imaging of the semicircular canals may be difficult, so behavior suggestive of vestibular problems should also count as a major feature. In chapter 3 of this thesis, we therefore propose new guidelines for *CHD7* analysis based on clinical experience and phenotypic analysis of 280 patients with a pathogenic *CHD7* mutation.

Since its identification, many groups have analyzed the *CHD7* gene in clinically typical and in atypical CHARGE patients. This has expanded not only our knowledge of the phenotype, but has also increased our knowledge about the mutational spectrum of *CHD7* and made data available for phenotype-genotype correlation. Many unique mutations have been identified in the *CHD7* gene. No clear genotype-phenotype correlations have been identified, although pathogenic missense mutations, in general, lead to a milder phenotype. An overview of all the *CHD7* mutations identified up to July 2011 can be found in chapter 2.1. A more recent update is available at the online database www.CHD7.org. Since CHARGE syndrome is caused by haploinsuffciency of CHD7, the interpretation of truncating nonsense mutations and frameshift mutations is often clear. However, interpreting the effect of missense variants in the *CHD7* gene is still difficult. We therefore designed a classification system, which can be found in chapter 2.2 and that uses the results of two computational algorithms, the prediction of a newly developed structural model of two important CHD7 domains together with segregation and phenotypic data to make a prediction on pathogenicity of a missense variant.

The identification of the *CHD7* gene as a cause of the variable CHARGE syndrome also interested basic scientific researchers. *CHD7* codes for the highly conserved CHD7 protein that consists of 2997 amino-acids. The current idea is that CHD7 functions as a regulator of gene expression during embryonic development in a tissue-specific and time-specific manner.[11] Knowledge of basic research on the *CHD7* gene and CHD7 protein helps our understanding of how a change in one single gene can cause such a variable syndrome. A review on CHD7 function up to July 2011 can be found in chapter 2.1.

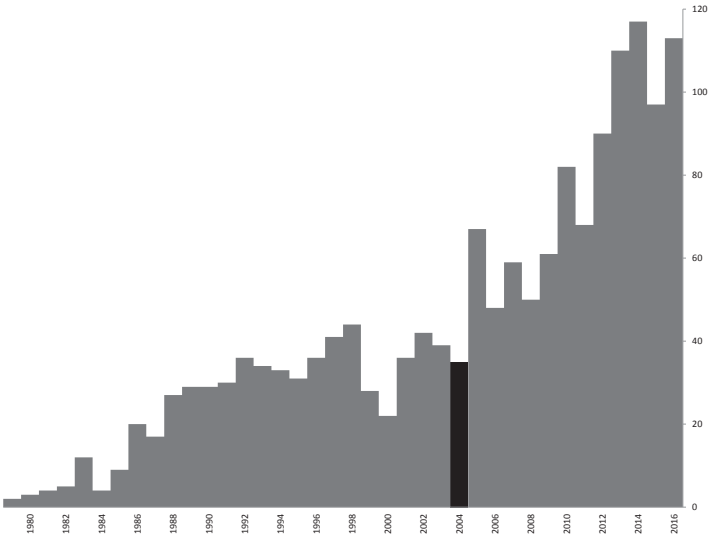
CHARGE syndrome is a very variable syndrome and the phenotype can even differ between monozygotic twins.[9,12] Before the molecular cause of CHARGE syndrome was known, patients could only be included in studies based on their clinical phenotype. By selecting patients on their pathogenic *CHD7* mutation, it is possible to select a genetically homogeneous group in which a specific phenotype can be studied. Studying a phenotype is not only interesting from a clinical point of view (for example see chapter 4.2), but the kinds of defects that are revealed may also provide information about the function of CHD7 in the specific organ affected. In chapter 4.1, we carefully studied congenital heart defects in 299 patients with pathogenic *CHD7* mutations. Although we saw variability in the heart defects, the cluster of conotruncal or outflow tract anomalies and atrioventricular septal defects were over-represented compared to non-syndromic heart defects.

Congenital heart defects are the most frequent congenital malformations with a prevalence of 0.8% in the general population. They may have a huge impact on the quality of life of patients and their family. The exact cause of a congenital heart defect is usually unknown. Congenital heart defects can occur as an isolated feature, but may also occur in combination with other features as they do in CHARGE syndrome. Identifying the cause of a congenital heart defect is essential for optimal clinical management of patients and counseling about recurrence risk and reproductive options for patients and their families. For example, if the heart defect is caused by a *CHD7* mutation, a patient's hearing, vision and balance also needs to be screened. The guidelines for *CHD7* analysis we propose in chapter 3 are based on the known phenotypic spectrum of *CHD7* mutations, while the actual phenotypic spectrum of *CHD7* mutation may not yet be fully known. This made us analyze *CHD7* in a cohort of patients with specific congenital heart defects and other features of CHARGE syndrome in chapter 4.3 to see in which patients *CHD7* analysis is warranted.

A clinical diagnosis is not the same as a molecular diagnosis. After analyzing the *CHD7* gene using Sanger sequencing techniques and MLPA, no molecular cause is identified in 5-10% of clinically typical CHARGE patients.[13] Therefore the question remains what causes the CHARGE phenotype in these patients. First, these patients may still harbor a *CHD7* mutation that was not identified with the current knowledge and techniques. Standard *CHD7* analysis, for example, usually does not include the promoter or deep intronic regions of *CHD7*. Second, another, not yet identified, gene may cause the CHARGE phenotype in these patients. Third, we know there are other syndromes that have overlapping features with CHARGE syndrome, and which are caused by other genomic alterations (for examples of these see chapters 5.1 and 5.2). Thus, some patients presenting with a CHARGE phenotype may actually have another clinically overlapping syndrome.

The identification of the *CHD7* gene as the major cause of CHARGE syndrome resulted in a renewed interest in this syndrome with its complex and highly variable phenotype, exemplified by a significant increase in yearly publications (see Figure 1). Current research focuses on understanding the function of CHD7 and how its haploinsufficiency can result in such a variable multi-organ involvement. The recognition of the CHARGE association as a syndrome and thus as a single disease entity instead of a group of disorders, boosted clinical research aiming at improving care and guidelines. This thesis explores the clinical spectrum of *CHD7* variants with a focus of heart defects and the role of CHD7 in organ development

Figure 1. Pubmed search on CHARGE syndrome or CHD7



Year of publication on X-axis Number of articles on Y-axis. The black bar indicated the year 2004 when Visser et al. published their article that identified *CHD7*mutations as a major cause of CHARGE syndrome.7 A search in biomedical literature on the terms CHARGE syndrome or *CHD7* using Pubmed, shows the number of published articles has enormously increased from 34 in 2004 to 113 in 2016.

from a clinical point of view by comparing the CHARGE phenotype with overlapping syndromes due to other developmental gene defects.

1.2 SCOPE AND OUTLINE OF THIS THESIS

The main aim of this thesis was to contribute to the knowledge on the phenotype of CHARGE syndrome caused by *CHD7* mutations, with a special focus of heart defects, and to learn more about other molecular causes of clinically diagnosed CHARGE patients.

In **chapter 2** we provide an update on mutations of the *CHD7* gene. **Chapter 2.1** is an overview of mutations in the *CHD7* gene and knowledge about the function of CHD7. Since not all mutations in genes are published and some patients are published in more than one paper, we have made an online open-access *CHD7* mutation database presenting a more realistic overview of *CHD7* variants. We have also used the detection of *CHD7* mutations together with birth numbers to estimate a new birth incidence of CHARGE syndrome in the Netherlands. In **chapter**

2.2, we provide a classification system to predict the chance that a given missense variant is pathogenic. In **chapter 2.3,** we identify the cause of CHARGE syndrome in five typical CHARGE patients, who were negative for *CHD7* variants on routine diagnostic testing, using exome sequencing.

Chapter 3 focuses on the clinical implications of the identification of the *CHD7* gene. Our aim was to gain more insight into the phenotype of *CHD7* mutations, especially at the milder end of the spectrum, by studying 280 patients with a *CHD7* mutation. Based on this information, we developed guidelines for determining when *CHD7* analysis should be done.

In **chapter 4,** we specifically focus on the relation between *CHD7* variants and congenital heart defects. **Chapter 4.1** provides insight into the cardiac phenotype associated with mutations in the *CHD7* gene. In **chapter 4.2,** we identify the prevalence of arch vessel anomalies in CHARGE syndrome to create awareness of the morbidity they might cause. In **chapter 4.3,** we investigate whether *CHD7* analysis is warranted in patients with both a CHARGE-typical heart defect and one other feature of CHARGE syndrome. In **chapter 4.4,** we summarize the knowledge on congenital heart disease in CHARGE syndrome from a clinical and molecular perspective.

Chapter 5 describes the clinical overlap of CHARGE syndrome with two microdeletion syndromes that both have heart defects as a feature. In **chapter 5.1,** we focus on the clinical overlap between CHARGE syndrome and 22q11.2 deletion syndrome in several ways e.g. by comparing clinical features between a cohort of patients with a *CHD7* mutation and a cohort of patients with 22q11.2 deletion, by describing case reports of patients diagnosed with CHARGE syndrome, but carrying a 22q11.2 deletion, by *CHD7* analysis in a cohort of patients with clinical features of 22q11.2 deletion syndrome without 22q11.2 deletion. **Chapter 5.2** describes the clinical phenotype of the 5q11.2 microdeletion syndrome and its clinical overlap with CHARGE syndrome and 22q11.2 deletion syndrome.

Chapter 6 provides an overview of this thesis and discusses future perspectives.. **Chapter 6.1** summarizes the results described in this thesis. In **chapter 6.2** a reflection is given of what we have achieved and the knowledge that we added to the field of CHARGE syndrome and *CHD7* mutations. This is discussed within a wider perspective of current research on CHD7, CHARGE and overlapping syndromes, to address what is known and which questions still need to be answered.

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CHAPTER 2

CHD7 mutations

CHAPTER 2.1

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

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Adapted from: 'Mutation update on the CHD7 gene involved in CHARGE syndrome'. Human Mutation 2012; 33(8):1149-1160. With permission from Wiley.

ABSTRACT

CHD7 is a member of the chromodomain helicase DNA-binding (CHD) protein family that plays a role in transcription regulation by chromatin remodeling. 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 analyzed 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.

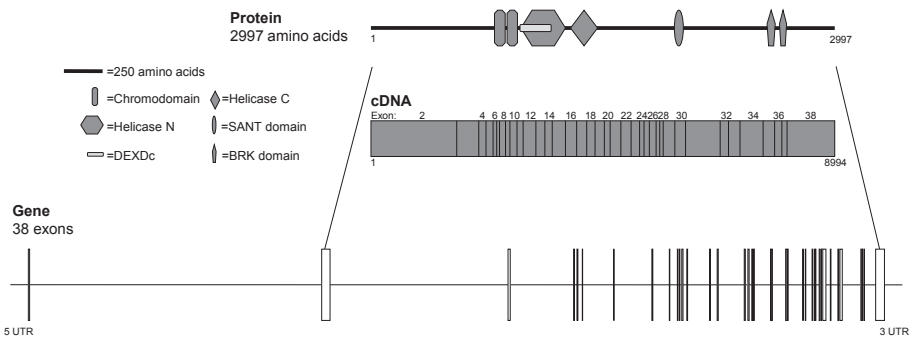
Keywords: *CHD7* gene, CHARGE syndrome, Kallmann syndrome, mutation spectrum, *CHD7* database

INTRODUCTION

Chromodomain **h**elicase **D**NA-binding (CHD) proteins play a role in transcription activation and repression by chromatin remodeling. For this function, all members of the CHD protein family possess two chromodomains (**chr**omatin **o**rganization **mo**difier 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 harbor paired N-terminal PHD (**p**lant **h**omeo **d**omain) Zinc-finger-like domains. Members of subfamily III are characterized by C-terminal paired BRK (**Br**ahma and **K**ismet) domains and a SANT-like domain (**s**witching-defective protein 3, **a**daptor 2, **n**uclear receptor co-repressor, **t**ranscription factor IIIB). CHD7 is one of the CHD proteins of subfamily III.[1,2]

CHD7 (MIM 608892) 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

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



Overview of *CHD7* with its 38 exons and introns (bottom). 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 (middle). The *CHD7* protein consists of 2997 amino acids and has several conserved domains which are drawn to scale (top).
Chromodomain, chromatin organization modifier; 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.

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 remodeling in embryonic stem cells and other cell types.[3,5]

Heterozygous mutations and deletions of *CHD7* result in CHARGE syndrome (MIM 214800), a complex of multiple congenital malformations involving the central nervous system, eye, ear, nose and mediastinal.[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 or agenesis.[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 (Supp. 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 discuss the clinical implications of identifying a *CHD7* mutation. The interpretation of missense variants is discussed in another paper in this issue.[21]

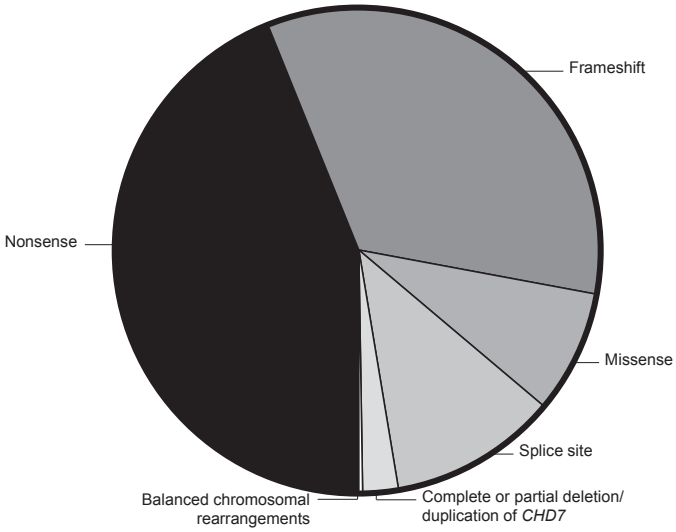
MUTATION SPECTRUM

Intragenic *CHD7* mutations in CHARGE syndrome

Per June 15th 2011, a total of 528 pathogenic and unique *CHD7* alterations had been identified in 802 index patients with CHARGE syndrome, including 183

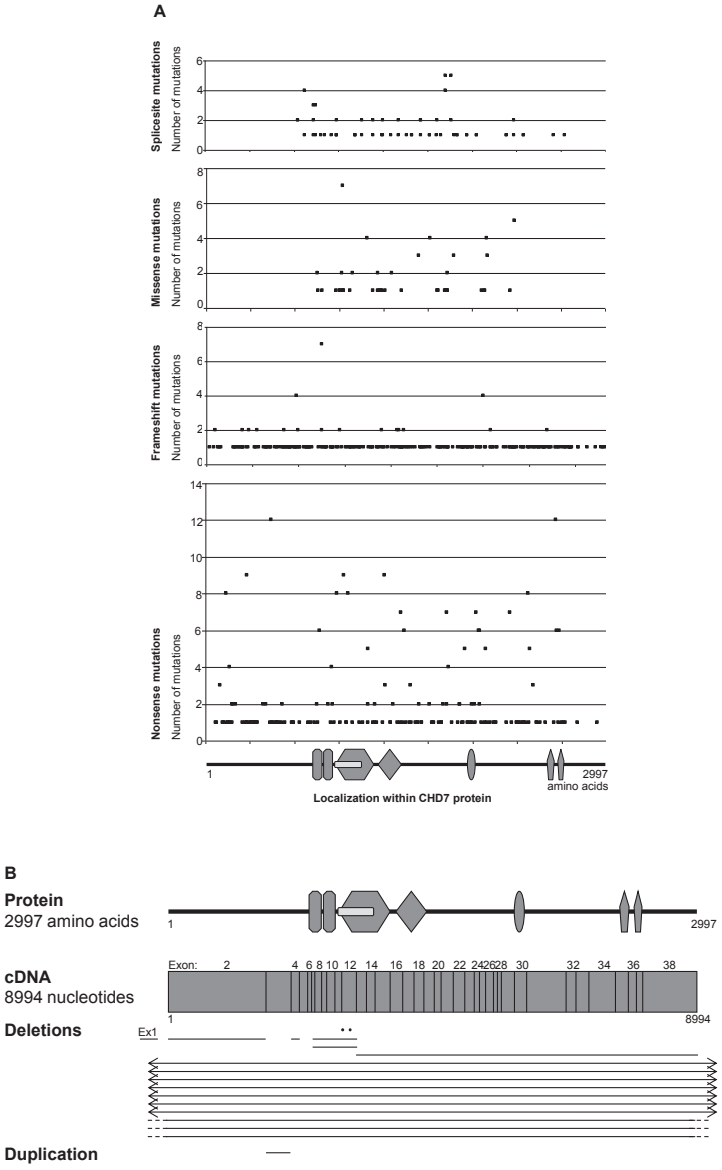
new mutations identified by the DNA diagnostic laboratories of the RUNMC and ICMM.[8,9,12,13,17-20,22-69] The majority of the pathogenic *CHD7* variants are intragenic mutations (Figure 2). A schematic presentation of *CHD7* and the locations of the unique pathogenic mutations within the gene are presented in Figure 3, grouped by mutation type. In addition to the pathogenic mutations, 91 unique unclassified variants have been described in 114 patients in the literature and from our laboratories; these are mostly missense variants and intronic variants near the splice sites. In Supp. 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).

Figure 2. Distribution of pathogenic 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.

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 *CHD7*, 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.

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 (44%), and frameshift deletions or insertions (34%). Splice site and missense mutations are found in 11% and 8%, 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% size of the total coding sequences).

Approximately 30% of the mutations in index patients (including recurrent mutations) are found in the regions of *CHD7* that encode for the functional domains. The encoded region of these domains is approximately 23% of *CHD7*, so the frequency of mutations within these domains is only slightly higher than would be expected if the mutations were distributed equally (Supp. 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 that include the chromo-, helicase- and SANT domains, while they were not found in the first seven or last five exons of the gene that contain the BRK domains (Figure 3A). In contrast, benign missense variants occurred more often in the first and last exons, which are non-conserved regions.[21] 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, 94 different recurrent mutations were found in 356 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.[27] and is in agreement with previous observations that the CG-nucleotide pair is hyper-mutable to TG.[70] 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.[8,9,25,27,45,56,64] In addition, we recently identified two

whole gene deletions of *CHD7*. A loss of 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.[23,27,30,59,64,65] 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%).[23,27,30,59,61,64,65]

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.[8,65] 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*.[9,45] The second *de novo* translocation t(8;13)(q11.2;q22) was reported in monozygotic twins and disrupted *CHD7*.[47] We report here an additional translocation t(2;8)(q11.2;q12.2), 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.[9,13,17,23,26,38,50,58,61,63,64] 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),[27] 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.[12,13,23,26,50,63,64]

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.gov/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.[17,20,24,27,38,44,55,61,64,71] 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,[17,27] but was proven to be a benign variant.[20] In Supp. 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.[12,13,17,35,48,54,62,64] 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.[72] In all CHARGE families, a remarkable clinical variability is seen. Especially the parents are relatively mildly affected, and do not fulfill 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 (germ-line 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.[21]

Germ-line and somatic mosaicism have been suggested in some families in the literature.[13,17,27,48,64] More recently, germ-line 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.[54] 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);[48] and in a father whose child had CHARGE syndrome (c.7636G>T; p.Glu2546X).[27] 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 characterized 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.[73,74] Two groups have analyzed *CHD7* in patients with normosmic idiopathic hypogonadotropic hypogonadism (nIHH) or KS. The first study analyzed 197 patients and identified seven *CHD7* mutations.[20] Four patients with a *CHD7* mutation were diagnosed with nIHH 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 nIHH 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 nIHH/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. CHARGE syndrome is under diagnosed in patients presenting with Kallmann syndrome if no careful clinical work-up is performed after the detection of a *CHD7* mutation. Recently, *CHD7* analysis was performed in a third cohort of 30 Finnish patients with Kallmann syndrome, but no pathogenic mutations were identified.[75]

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.[76] In 53 families with isolated scoliosis, a genome-wide scan showed linkage and association with 8q12 loci.[71] Further analysis revealed a potentially functional polymorphism in *CHD7* (c.1666-3238A>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 (CLP) occurs in 30-48% of patients with CHARGE syndrome with a *CHD7* mutation.[12,13,17,77] In 184 cases with non-syndromic CLP, a role for *CHD7* could not be proven, although some variants were found.[38]

Congenital heart disease occurs in approximately 75% of CHARGE patients.[12,13,17,77] Analysis of *CHD7* in 67 patients with a congenital heart defect and in 100 controls revealed seven intronic variants.[78] Remarkably, one variant was detected in patients only (c.3523-35C>G). Variant c.3523-35C>G is now known to be a benign variant (Supp. Table S3). Another variant (c.3202-5T>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, intra-

genic 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 (MIM 608166), 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.[79] No mutation in *CHD7* was found in these patients, but deletions were not excluded.[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* (MIM 601542) and *PAX2* (MIM 167409) in 29 and 34 patients with CHARGE syndrome, respectively.[80,81] *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.[28]

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.[82,83] However, our review of the clinical features revealed that these patients had neither choanal atresia nor coloboma, and thus did not fulfill the clinical diagnostic criteria for CHARGE syndrome.[15,16]

Table 1. Unique chromosomal imbalances mimicking CHARGE syndrome

Chromosomal imbalance	Reference
der(2)t(2;21)(q37;qter)	Fernandez-Rebollo et al., 2009[114]
der(3)t(3;22)(p25.1;q11.1)	Clementi et al., 1991[115]
del(3)(p12p21.2)	Wieczorek et al., 1997[116]
der(4)t(4;8)(q34,3;q22,1)	Khalifa et al., 2011[117]
der(6)t(4;6)(q34;q25)	Sanlaville et al., 2002[118]
der(9)t(9;13)(p23;q33)	Sanlaville et al., 2002[118]
inv dup(14)(q22q24.3)	North et al., 1995[119]
der(18)t(2;18)(q37.3;q22.3)	Clementi et al., 1991[115]
trisomy 18	Lee et al., 1995[120]
der(21)t(19;21)(q13.1q22.3)	De Krijger et al., 1999[121]
der(X)t(X;2)(p22.1;q33)	Lev et al., 2000[122]

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.[8,12,41,46,57,67,84,85] The overlapping clinical features include cleft palate, cardiac malformations, ear abnormalities, hearing loss, growth deficiency, developmental delay, renal abnormalities, hypocalcaemia and immune deficiency.[8,46,57,67,84,86,87] *CHD7* mutations are more often, but not exclusively, associated with coloboma, choanal atresia, facial nerve palsy, tracheo-esophageal fistula and micropenis compared to 22q11.2 deletions.[87] Hypoplastic semicircular canals are suggestive for CHARGE syndrome, as they are present in almost all patients with CHARGE syndrome.[12,16,88,89] 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.[12,90] 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.

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.[21]

The database software was constructed by the Genomics Coordination Center, a joint venture of the Department of Genetics, UMCG, and the Groningen Bioinformatics Center, University of Groningen, the Netherlands. The software is based on the online patient registry for dystrophic epidermolysis bullosa.[91] All the software has been built using the open-source MOLGENIS framework and is freely available to others working on locus-specific databases at http://www.molgenis.org.[92,93]

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.[6,7,17,58,94-97] In all species, Chd7 expression patterns correlate with the developmental abnormalities observed in CHARGE syndrome.[4,6,17,58] 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.[4-7,17,58] 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.[4,6,7,17,58,94,95] 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 animal models for CHARGE syndrome 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 behavior 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 estrus 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-esophageal 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.[77]

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 remodeling 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.[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 hetero-

zygous 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 (**p**olybromo- and **B**RG1-**a**ssociated **f**actor 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* (MIM 601622) and *SOX9* (MIM 608160). 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 of $Chd7^{Gt/+}$ mice *Otx2* and *GnRH1* expression were diminished.[100] These results suggest that Chd7 affects GnRH neurogenesis and signaling 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 (*SOX2*, MIM 184429) 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*, MIM 165240), tracheo-esophageal anomalies are seen in Feingold syndrome

(*MYCN*, MIM 164840), and semicircular canal anomalies are present in Alagille syndrome (*JAG1*, MIM 601920).

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 live born children in the Netherlands between 1 January 2006 and 31 December 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.[13,17,64] 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.[12,77] In addition, most patients have some degree of developmental delay, but their cognitive function can be normal.[12,77] Coloboma and choanal atresia are found in 75-81% and 38-55% of patients with a *CHD7* mutation, respectively.[12,77] 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-esophageal anomalies in 19-29%.[12,77] No clear genotype-phenotype correlations have been found for *CHD7* mutations, although missense mutations can be associated with a milder phenotype.[12,13,18,21] 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 counseling about reproductive options. In patients who do not completely fulfill 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 counseling 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.[55] In these cases, the recurrence risk for the parents is 2-3%, as both germline and somatic mosaicism have been described.[13,48,54] 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. Fetal 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 proteins is emerging, indicating that it regulates the expression of genes in a cell type- and embryonic stage-dependent manner.

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SUPPLEMENTARY MATERIAL

Supplementary methods section

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).

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.

					Phenotypic information[f]												
CHD7 c[a]	CHD7 p[b]	Exon	Mutation type[c]	Segregation[e]	C	H	A	R G	R D	G	E	SCC	C(L)P	HL	FD	VII	TE
del		2-38	del	U	no	yes	yes	U	U	U	U	yes	U	U	U	U	U
del		2-38	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	2	fs	U	yes	yes	U	yes	U	yes	yes	yes	U	yes	U	no	U
191_194del	Thr64fs	2	fs	U	yes	yes	U	U	U	U	U	U	U	yes	U	U	U
232C>T	Gln78X	2	non	U	U	no	U	U	poss	yes	yes	U	yes	yes	poss	yes	U
257del	Pro86fs	2	fs	de novo	no	no	no	U	yes	No	yes	U	yes	yes	yes	yes	no
282del	Asn96fs	2	fs	de novo	U	no	yes	U	poss	yes	U	U	U	yes	U	U	U
317delA	His106fs	2	fs	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
334C>T	Gln112X	2	non	U	yes	yes	yes	yes	U	yes	U	no	U	yes	U	yes	U
388C>T	Gln130X	2	non	not maternal	yes	U	no	yes	U	yes	U	yes	U	yes	yes	U	U
406C>T	Gln136X	2	non	U	U	U	U	U	yes	U	U	U	U	U	U	U	U
469C>T	Arg157X	2	non	U	yes	yes	yes	U	U	yes	yes	yes	U	U	U	U	yes
469C>T	Arg157X	2	non	U	yes	U	U	U	U	U	yes	U	yes	U	U	U	U
550C>T	Gln184X	2	non	U	no	yes	yes	U	U	U	U	U	U	U	U	U	yes
601C>T	Gln201X	2	non	U	yes	yes	no	yes	yes	U	yes	U	no	yes	U	U	U
604C>T	Gln202X	2	non	de novo	yes	yes	yes	U	U	U	yes	U	U	U	U	U	no
608dup	His203fs	2	fs	U	yes	yes	yes	U	U	U	yes	yes	U	U	U	U	yes
619C>T	Gln207X	2	non	de novo	yes	yes	U	U	yes	U	yes	U	U	yes	yes	yes	U
619C>T	Gln207X	2	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
627del	Met210X	2	fs	U	yes	yes	yes	U	yes	yes	yes	U	U	yes	U	yes	U

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. (continued)

					Phenotypic information[f]												
CHD7 c[a]	CHD7 p[b]	Exon	Mutation type[c]	Segregation[e]	C	H	A	R G	R D	G	E	SCC	C(L)P	HL	FD	VII	TE
635delA	Gln212fs	2	fs	U	yes	yes	U	no	yes	yes	yes	U	U	yes	poss	yes	no
674del	Pro225fs	2	fs	U	yes	yes	yes	U	U	U	yes	U	U	U	U	U	U
689C>G	Ser230X	2	non	de novo	yes	no	U	U	U	yes	yes	U	yes	U	U	U	U
718del	Gln240fs	2	fs	U	no	yes	yes	no	U	yes	yes	yes	no	yes	U	U	yes
781del	Ser261fs	2	fs	de novo	U	yes	U	U	U	yes	yes	U	yes	U	U	U	U
791_792del	Leu264fs	2	fs	U	U	yes	yes	U	U	U	yes	U	U	U	U	U	U
844C>T	Gln282X	2	non	U	U	yes	U	U	U	U	yes	U	yes	yes	U	U	yes
921_922del	Gly308fs	2	fs	U	U	yes	yes	U	U	U	yes	U	U	yes	U	yes	U
934C>T	Arg312X	2	non	U	yes	yes	yes	yes	U	U	yes	U	U	U	U	U	U
934C>T	Arg312X	2	non	U	U	U	U	U	U	U	yes	U	U	yes	yes	U	U
995T>G	Leu332X	2	non	de novo	yes	yes	U	U	poss	yes	yes	U	U	yes	poss	yes	U
1066_1069 dup	Ser357fs	2	fs	U	yes	no	no	yes	yes	yes	yes	yes	U	yes	U	yes	no
1093C>T	Gln365X	2	non	U	yes	no	U	U	poss	yes	yes	U	yes	yes	poss	U	yes
1123C>T	Gln375X	2	non	de novo	yes	no	U	yes	yes	yes	yes	yes	yes	yes	yes	U	U
1135C>T	Gln379X	2	non	U	yes	yes	U	U	U	yes	U	yes	U	yes	U	yes	U
1159C>T	Gln387X	2	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
1170T>G	Tyr390X	2	non	U	U	yes	yes	U	U	U	yes	U	U	U	U	U	U
1247insG	Ser417fs	2	fs	U	yes	yes	yes	U	U	U	yes	U	U	U	U	U	no
1312C>T	Gln438X	2	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
1480C>T	Arg494X	2	non	U	yes	yes	no	no	U	yes	yes	yes	no	yes	yes	U	no
1480C>T	Arg494X	2	non	U	yes	yes	yes	no	yes	yes	yes	yes	U	yes	U	U	yes

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. (continued)

					Phenotypic information[f]												
CHD7 c[a]	CHD7 p[b]	Exon	Mutation type[c]	Segregation[e]	C	H	A	R G	R D	G	E	SCC	C(L)P	HL	FD	VII	TE
1480C>T	Arg494X	2	non	U	U	yes	yes	U	U	U	yes	U	U	U	yes	U	U
1480C>T	Arg494X	2	non	de novo	yes	yes	yes	yes	U	U	yes	yes	U	yes	U	U	no
1480C>T	Arg494X	2	non	not maternal	U	yes	no	U	U	No	yes	U	no	U	U	U	U
1528del	Gln510fs	2	fs	U	yes	yes	U	U	U	yes	U	U	yes	yes	U	U	U
1645C>T	Gln549X	2	non	U	yes	yes	U	yes	yes	U	U	U	U	U	U	U	U
1678delG	Glu560fs	3	fs	U	yes	yes	U	U	yes	yes	U	U	yes	yes	U	U	U
1683_1684 delCT	Phe562fs	3	fs	U	yes	U	yes	yes	yes	yes	U	yes	U	yes	U	U	U
1735C>T	Gln579X	3	non	U	yes	yes	yes	U	U	U	yes	U	U	U	U	yes	U
1735C>T	Gln579X	3	non	U	yes	U	no	U	U	yes	yes	U	U	U	poss	U	yes
1740del	Val581fs	3	fs	U	yes	U	U	U	yes	U	yes	U	yes	yes	poss	yes	U
1926_1929 del	Lys643fs	3	fs	U	poss	U	U	no	yes	yes	yes	U	no	yes	yes	yes	U
1932_1935 delGAAA	Lys645fs	3	fs	not maternal	yes	no	yes	U	yes	yes	yes	U	yes	no	yes	U	yes
1953dup	Asp652fs	3	fs	de novo	yes	yes	no	yes	U	U	yes	U	no	yes	yes	no	yes
1953dup	Asp652fs	3	fs	U	U	yes	U	no	yes	yes	yes	U	U	yes	yes	U	U
1953dup	Asp652fs	3	fs	U	yes	no	U	yes	yes	yes	yes	U	U	yes	poss	U	U
1953dup	Asp652fs	3	fs	de novo	yes	yes	no	no	U	no	yes	yes	yes	yes	no	yes	no
1972G>T	Glu658X	3	non	U	yes	yes	U	yes	yes	yes	yes	yes	U	yes	yes	yes	U
1983dup	Lys662fs	3	fs	U	yes	yes	U	U	yes	U	yes	U	U	yes	U	U	U
1988_1989dup	Glu664fs	3	fs	de novo	U	yes	U	U	U	U	U	U	U	U	U	U	U
1989dup	Glu664fs	3	fs	U	yes	no	U	U	yes	yes	yes	yes	U	yes	U	U	U
1990G>T	Glu664X	3	non	U	yes	no	U	U	yes	U	U	yes	yes	yes	yes	U	U

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. (continued)

					Phenotypic information[f]												
CHD7 c[a]	CHD7 p[b]	Exon	Mutation type[c]	Segregation[e]	C	H	A	R G	R D	G	E	SCC	C(L)P	HL	FD	VII	TE
2019_2023 delinsGGG	Pro674fs	3	fs	U	yes	yes	no	U	yes	U	yes	U	U	yes	yes	U	yes
2096+2T>A	U	intron 3	splice	de novo	yes	yes	no	yes	U	yes	yes	yes	U	U	U	yes	no
2097-1G>A	U	intron 3	splice	U	yes	yes	U	U	yes	U	yes	yes	U	U	yes	yes	U
2118del	Ala707fs	4	fs	de novo	no	yes	yes	U	poss	U	yes	U	U	U	U	U	U
2157del	Glu1720fs	4	fs	de novo	yes	yes	yes	yes	yes	U	U	U	U	yes	yes	U	U
2181dup	Asp728fs	4	fs	de novo	U	yes	U	U	poss	U	U	yes	U	U	U	yes	yes
2196dup	Pro733fs	4	fs	de novo	U	yes	U	U	U	U	U	U	yes	U	U	U	yes
2236delC	Gln746fs	4	fs	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
2238+1G>T	U	intron 4	splice	U	yes	no	no	no	yes	no	yes	yes	no	yes	no	yes	no
2238+2T>G	U	intron 4	splice	U	U	U	U	U	U	U	U	U	U	U	U	U	U
2238+1G>A	U	intron 4	splice	U	U	yes	yes	U	U	U	yes	U	U	U	U	U	U
2247_2260 del	Ser750fs	5	fs	U	yes	yes	yes	U	U	U	U	yes	U	U	U	U	U
2374C>T	Gln792X	5	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
2443-2A>T	U	intron 6	splice	de novo	no	yes	no	yes	yes	yes	yes	U	no	yes	U	yes	no
2443-1G>A	U	intron 6	splice	U	U	U	U	U	U	U	U	U	U	U	U	U	U
2498+2T>C	U	intron 7	splice	U	yes	yes	U	yes	poss	U	yes	U	yes	yes	U	U	U
2498+1G>T	U	intron 7	splice	de novo	U	yes	yes	U	U	U	U	U	U	yes	U	U	U
2498+2dup	U	intron 7	splice	U	U	yes	U	U	U	U	yes	U	yes	U	U	U	poss
2504_2508 del	Tyr835fs	8	fs	de novo	U	yes	yes	U	U	no	yes	yes	U	yes	poss	U	U
2504_2508 del	Leu836fs	8	fs	U	yes	yes	U	U	U	U	yes	U	U	U	U	U	U
2504_2508 del	Tyr835fs	8	fs	U	U	yes	U	U	U	U	U	U	yes	U	U	U	no

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. (continued)

					Phenotypic information[f]												
CHD7 c[a]	CHD7 p[b]	Exon	Mutation type[c]	Segregation[e]	C	H	A	R G	R D	G	E	SCC	C(L)P	HL	FD	VII	TE
2520G>C	Trp840Cys	8	mis	de novo	yes	no	no	no	yes	yes	yes	U	no	no	U	no	U
2571insA	Arg858fs	8	fs	de novo	yes	yes	no	yes	U	yes	U	yes	U	yes	U	U	yes
2572C>T	Arg858X	8	non	de novo	U	yes	yes	U	U	yes	yes	U	U	U	U	U	yes
2577dup	Lys860X	8	fs	U	yes	yes	no	U	yes	U	yes	U	U	yes	U	yes	U
2601_2605del	Lys867fs	8	fs	U	U	yes	U	U	U	U	yes	U	U	U	U	U	U
2620delG	Asp874fs	9	fs	U	yes	yes	U	yes	yes	yes	yes	U	no	yes	yes	yes	no
2707_2710 delCACT	His903fs	10	fs	de novo	yes	yes	U	U	U	U	U	U	U	U	U	U	U
2815G>T	Glu939X	10	non	U	no	yes	no	U	yes	yes	yes	yes	yes	yes	yes	yes	no
2829del	glu943fs	10	fs	U	yes	yes	yes	U	U	U	yes	U	U	U	U	U	U
2839C>T	Arg947X	11	non	U	U	no	yes	U	U	U	U	yes	U	U	U	U	U
2859del	Trp953X	11	fs	U	yes	yes	no	yes	yes	U	yes	yes	U	yes	yes	U	no
2887A>T	Lys963X	11	non	not maternal	U	U	U	U	U	U	U	U	U	U	U	U	U
2905_2906 del	Arg969fs	11	fs	U	U	U	U	U	U	U	U	U	U	U	U	U	U
2957+2T>C	U	intron 11	splice	U	yes	yes	no	U	yes	U	yes	U	U	yes	poss	U	U
2957+5G>A	U	intron 11	splice	de novo	yes	yes	no	yes	no	yes	yes	U	yes	yes	U	yes	no
2959C>T	Arg987X	12	non	not maternal	yes	yes	U	U	U	yes	yes	U	U	U	poss	U	U
3023_3024 del	Tyr1008X	12	fs	de novo	yes	yes	no	yes	U	yes	yes	U	U	yes	poss	U	no
3024T>A	Tyr1008X	12	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
3059T>C	Leu1020Ser	12	mis	U	U	yes	yes	U	U	U	U	U	U	U	U	U	yes

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					Phenotypic information[f]												
CHD7 c[a]	CHD7 p[b]	Exon	Mutation type[c]	Segregation[e]	C	H	A	R G	R D	G	E	SCC	C(L)P	HL	FD	VII	TE
3082A>G	Ile1028Val	12	mis	de novo	yes	no	U	U	yes	yes	yes	U	yes	yes	U	U	U
3082A>G	Ile1028Val	12	mis	de novo	yes	yes	no	U	yes	U	U	U	U	U	yes	U	U
3082A>G	ile1028Val	12	mis	U	no	yes	U	U	no	yes	U	yes	yes	yes	yes	U	no
3091T>C	Trp1031Arg	12	mis	de novo	yes	yes	U	U	U	yes	yes	U	U	U	U	yes	U
3106C>T	Arg1036X	12	non	de novo	yes	yes	yes	U	yes	U	yes	yes	yes	U	U	U	yes
3106C>T	Arg1036X	12	non	de novo	yes	yes	yes	U	U	U	U	U	U	U	U	U	U
3117dup	Glu1040fs	12	fs	U	yes	U	U	U	U	U	U	U	U	U	U	poss	yes
3165del	Ile1056fs	12	fs	U	U	U	U	U	U	U	U	U	U	U	U	U	U
3173T>A	Leu1058X	12	non	de novo	yes	yes	no	no	U	yes	yes	yes	no	yes	yes	yes	no
3205C>T	Arg1069X	13	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
3205C>T	Arg1069X	13	non	U	no	yes	U	U	poss	yes	yes	U	yes	yes	U	U	U
3222_3223 ins5	Tyr1075fs	13	fs	de novo	no	yes	yes	no	U	U	yes	U	U	U	U	U	U
3245C>A	Thr1082Asn	13	mis	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
3301T>C	Cys1101Arg	13	mis	de novo	U	U	U	U	U	U	yes	U	U	yes	U	U	U
3318del	Ala1107fs	13	fs	de novo	U	yes	U	U	U	U	U	U	yes	U	U	U	U
3322delC	His1108fs	13	fs	de novo	U	U	yes	U	U	yes	U	U	U	yes	U	U	U
3336del	Asn1112fs	13	fs	U	yes	yes	yes	yes	U	U	U	U	U	yes	U	U	U
3378+5G>C	U	intron 13	splice	de novo	yes	yes	yes	yes	yes	U	yes	U	no	yes	U	U	U
3379-1G>A	U	intron 13	splice	U[°]	yes	yes	yes	U	poss	U	U	U	U	U	U	U	yes

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. (continued)

					Phenotypic information[f]												
CHD7 c[a]	CHD7 p[b]	Exon	Mutation type[c]	Segregation[e]	C	H	A	R G	R D	G	E	SCC	C(L)P	HL	FD	VII	TE
3379-3C>G	U	intron 13 and exon 34	splice	U	yes	U	yes	U	yes	yes	U	U	yes	yes	poss	U	U
3514_3515 del	Glu1172fs	14	fs	de novo	yes	no	U	yes	poss	yes	yes	U	yes	yes	yes	U	U
3522+2T>G	U	intron 14	splice	U	yes	U	U	U	U	U	yes	yes	U	U	U	yes	U
3522+2T>C	U	intron 14	splice	de novo	U	no	U	U	U	U	U	U	U	yes	U	U	U
3523-1G>C	U	intron 14	splice	U	yes	yes	no	yes	yes	yes	yes	U	no	yes	U	yes	no
3573dup	Glu1192fs	15	fs	U	yes	yes	yes	U	yes	U	U	U	U	yes	U	U	U
3616dup	Ile1206fs	15	fs	de novo	yes	yes	U	U	U	yes	yes	yes	yes	yes	U	U	U
3641A>G	Gln1214Arg	15	mis	de novo	yes	no	no	yes	yes	yes	yes	U	U	yes	U	U	no
3641A>G	Gln1214Arg	15	mis	de novo	U	U	U	U	yes	U	yes	U	U	yes	U	U	U
3655C>T	Arg1219X	15	non	de novo	yes	yes	U	U	U	U	U	U	U	U	U	U	U
3655C>T	Arg1219X	15	non	U	yes	yes	U	yes	U	U	U	U	U	yes	U	U	U
3734del	Met1245fs	15	fs	de novo	yes	yes	yes	no	yes	yes	yes	yes	yes	yes	yes	U	no
3807del	Phe1269fs	16	fs	U	yes	no	no	yes	yes	yes	yes	U	no	yes	U	poss	no
3847C>T	Gln1283X	16	non	U	U	U	yes	U	U	yes	yes	U	U	yes	U	U	U
3875T>C	Leu1292Pro	16	mis	de novo	U	yes	U	U	U	U	U	U	U	U	U	U	U
3937del	Ser1313fs	16	fs	de novo	yes	no	no	no	yes	U	yes	U	yes	yes	poss	U	no
3952T>C	Cys1318Arg	16	mis	de novo	yes	yes	no	U	poss	yes	yes	U	no	U	yes	yes	no

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. (continued)

					Phenotypic information[f]												
CHD7 c[a]	CHD7 p[b]	Exon	Mutation type[c]	Segregation[e]	C	H	A	R G	R D	G	E	SCC	C(L)P	HL	FD	VII	TE
3990-2G>A	U	intron 16	splice	de novo	yes	yes	no	no	U	yes	yes	yes	U	yes	yes	yes	no
3993C>G	Tyr1331X	17	non	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
4015C>T	Arg1339X	17	non	de novo	U	yes	U	U	U	U	U	U	U	U	U	U	U
4034G>A	Arg1345His	17	mis	de novo	no	no	U	U	yes	no	yes	U	yes	yes	poss	U	yes
4113_4114 insCA	Gly1372fs	17	fs	de novo	yes	yes	no	yes	yes	U	yes	U	no	yes	U	yes	U
4213C>T	Gln1405X	18	non	U	yes	no	yes	U	U	U	yes	no	U	yes	poss	U	U
4213C>T	Gln1405X	18	non	de novo	yes	no	U	yes	yes	U	yes	U	U	yes	poss	U	U
4253del	Asn1418fs	18	fs	not maternal	yes	no	U	U	yes	U	yes	U	yes	yes	yes	U	U
4257del	Tyr1420fs	18	fs	U	yes	yes	U	U	U	U	yes	U	yes	U	poss	U	U
4270del	Met1424fs	18	fs	U	yes	yes	yes	U	U	yes	yes	yes	U	U	U	U	yes
4295_4296 del	Leu1432fs	18	fs	U	U	U	U	U	U	U	U	U	U	U	U	U	U
4353+2T>C	U	intron 18	splice	U	U	yes	yes	U	U	U	U	U	U	yes	U	U	U
4357_4358 dupCA	Gln1453fs	19	fs	not maternal	U	no	U	U	yes	yes	yes	U	U	yes	yes	yes	U
4375G>T	Glu1459X	19	non	de novo	yes	yes	yes	U	U	yes	U	U	no	U	U	no	no
4393del	Arg1465fs	19	fs	not maternal	yes	yes	yes	yes	yes	U	yes	U	U	U	U	U	no
4393C>T	Arg1465X	19	non	U	yes	yes	no	no	yes	no	yes	yes	yes	yes	yes	U	no
4393C>T	Arg1465X	19	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
4393C>T	Arg1465X	19	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
4424del	Glu1475fs	19	fs	U	U	U	U	U	U	U	U	U	U	U	U	U	U

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. (continued)

					Phenotypic information[f]												
CHD7 c[a]	CHD7 p[b]	Exon	Mutation type[c]	Segregation[e]	C	H	A	R G	R D	G	E	SCC	C(L)P	HL	FD	VII	TE
4480C>T	Arg1494X	19	non	de novo	yes	yes	U	yes	yes	U	yes	U	U	yes	yes	yes	U
4480C>T	Arg1494X	19	non	U	U	U	U	U	U	U	U	yes	U	U	U	U	U
4644+5G>A	U	intron 20	splice	de novo	no	no	yes	no	yes	U	no	yes	U	yes	no	no	no
4665del	Pro1556fs	21	fs	U	U	yes	U	U	U	yes	yes	U	yes	U	U	yes	U
4731del	Asp1578fs	21	fs	de novo	no	no	yes	U	U	yes	yes	yes	no	yes	yes	U	U
4783C>T	Gln1595X	21	non	U	no	no	yes	U	U	yes	yes	yes	no	yes	yes	yes	no
4850+1dup	Trp1618fs	intron 21	fs	U	no	yes	yes	U	U	U	yes	no	no	U	U	yes	no
4850+2T>A	U	intron 21	splice	de novo	U	U	U	U	U	U	U	U	U	U	U	yes	U
4850+1G>A	U	intron 21	splice	not maternal	U	U	U	U	U	U	U	U	U	U	U	U	U
4851-2A>G	U	intron 21	splice	U	yes	no	no	yes	yes	yes	yes	yes	U	yes	yes	yes	poss
4851-2A>T	U	intron 21	splice	U	yes	yes	no	yes	U	yes	yes	yes	U	yes	yes	yes	U
4854G>A	Trp1618X	22	non	de novo	yes	yes	U	U	no	U	yes	U	yes	yes	U	yes	U
5012_5018 del	Thr1671fs	22	fs	U	U	yes	U	U	U	U	U	U	U	U	U	U	U
5050G>A	Gly1684Ser	22	mis	de novo	no	yes	no	yes	yes	U	U	yes	yes	yes	U	U	no
5069dup	Arg1691fs	23	fs	de novo	yes	yes	yes	U	U	U	yes	yes	no	yes	no	yes	U
5136G>A	Trp1712X	23	non	not maternal	U	yes	U	U	U	U	U	U	U	U	U	U	U
5205_5206 insT	Asn1736X	23	non	U	yes	poss	yes	yes	yes	U	U	U	U	yes	yes	U	U

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. (continued)

					Phenotypic information[f]												
CHD7 c[a]	CHD7 p[b]	Exon	Mutation type[c]	Segregation[e]	C	H	A	R G	R D	G	E	SCC	C(L)P	HL	FD	VII	TE
5211-1G>C	U	intron 23	splice	U	yes	yes	yes	U	U	yes	yes	U	U	yes	yes	U	no
5241_5244del	Tyr1747X	24	fs	de novo	no	yes	U	no	yes	yes	yes	yes	no	yes	yes	yes	no
5297C>G	Ser1766X	24	non	de novo	yes	yes	U	yes	yes	U	yes	yes	U	yes	yes	yes	no
5316G>A	Trp1772X	25	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
5390G>T	Gly1797Val	25	mis	de novo	yes	no	U	no	yes	yes	yes	yes	U	yes	yes	yes	U
5405-17G>A	U	intron 25	splice	de novo	no	no	no	yes	yes	yes	yes	yes	yes	yes	yes	U	U
5405-17G>A	U	intron 25	splice	U	no	no	no	no	yes	U	yes	yes	no	yes	yes	U	U
5405-17G>A	U	intron 25	splice	U	yes	no	no	U	U	U	yes	U	yes	yes	U	U	no
5405-7G>A	U	intron 25	splice	U	yes	no	prob	yes	yes	No	yes	yes	U	yes	U	yes	U
5405-7G>A	U	intron 25	splice	de novo	no	no	no	U	U	yes	no	yes	yes	yes	U	U	no
5405-7G>A	U	intron 25	splice	U	U	yes	U	U	yes	yes	U	yes	yes	yes	U	U	U
5418C>G	Tyr1806X	26	non	U	yes	yes	yes	U	U	yes	yes	U	no	U	U	yes	U
5428C>T	Arg1810X	26	non	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
5434G>C	Asp1812His	26	mis	de novo	no	no	yes	no	poss	U	yes	yes	no	yes	yes	U	no
5435A>G	Asp1812Gly	26	mis	de novo	yes	yes	U	no	no	yes	U	U	U	yes	poss	U	yes
5444T>C	Leu1815Pro	26	mis	U	yes	yes	U	U	U	U	yes	U	yes	U	U	yes	U

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. (continued)

					Phenotypic information[f]												
CHD7 c[a]	CHD7 p[b]	Exon	Mutation type[c]	Segregation[e]	C	H	A	R G	R D	G	E	SCC	C(L)P	HL	FD	VII	TE
5453dup	Glu1819fs	26	fs	de novo	U	U	yes	U	U	U	U	U	U	U	U	U	U
5458C>T	Arg1820X	26	non	de novo	yes	yes	yes	U	yes	U	yes	U	U	yes	U	U	yes
5458C>T	Arg1820X	26	non	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
5534+1G>T	U	intron 26	splice	de novo	yes	yes	no	U	U	U	yes	U	yes	U	U	U	U
5534+1G>A/T	U	intron 26	splice	de novo	U	yes	prob	U	U	U	yes	U	U	U	U	U	U
5539G>T	Glu1847X	27	non	de novo	U	yes	U	U	yes	yes	U	U	U	yes	U	U	U
5592del	Phe1864fs	27	fs	U	yes	yes	U	yes	yes	yes	yes	U	yes	yes	U	U	U
5607+1G>A	U	intron 27	splice	de novo	yes	yes	no	U	U	U	yes	U	no	U	yes	yes	U
5706C>A	Tyr1902X	29	non	U	yes	yes	U	U	yes	U	U	U	U	yes	U	U	U
5709G>A	Trp1903X	29	non	U	U	yes	yes	U	yes	U	U	U	U	yes	U	yes	yes
5866dup	Glu1956fs	29	fs	U	U	U	U	U	U	U	U	U	U	U	U	U	U
5898G>A	Trp1966X	30	non	U	yes	yes	yes	yes	yes	yes	yes	yes	U	yes	U	yes	no
5908G>T	Glu1970X	30	non	de novo	yes	yes	yes	U	U	U	U	U	U	U	U	U	U
5910dup	Glu1971fs	30	fs	not maternal	yes	yes	no	yes	yes	U	yes	yes	U	yes	U	U	yes
5944_5989 dup	Phe1997fs	30	fs	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
5968C>T	Gln1990X	30	non	de novo	yes	yes	no	U	yes	yes	yes	U	yes	yes	yes	U	U
6018_6019insG	Ser2007fs	30	fs	U	no	yes	yes	yes	yes	U	yes	U	no	yes	poss	U	no
6018dup	Ser2007fs	30	fs	U	yes	no	no	yes	yes	yes	yes	U	no	U	U	yes	no
6070del	Arg2024fs	30	fs	U	yes	yes	U	U	yes	U	U	yes	yes	yes	poss	U	U

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. (continued)

					Phenotypic information[f]												
CHD7 c[a]	CHD7 p[b]	Exon	Mutation type[c]	Segregation[e]	C	H	A	R G	R D	G	E	SCC	C(L)P	HL	FD	VII	TE
6070C>T	Arg2024X	30	non	U	yes	no	yes	yes	yes	U	U	U	no	U	poss	yes	no
6070C>T	Arg2024X	30	non	U	yes	yes	yes	U	yes	U	yes	U	U	U	U	U	yes
6070C>T	Arg2024X	30	non	U	yes	yes	U	U	yes	U	yes	U	U	yes	U	poss	U
6070C>T	Arg2024X	30	non	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
6079C>T	Arg2027X	30	non	de novo	yes	no	no	U	yes	U	yes	yes	no	yes	yes	yes	U
6079C>T	Arg2027X	30	non	U	yes	yes	yes	no	yes	U	yes	U	U	yes	poss	yes	no
6079C>T	Arg2027X	30	non	U	yes	yes	U	U	U	U	yes	U	yes	U	U	yes	U
6079C>T	Arg2027X	30	non	U	yes	yes	yes	U	yes	yes	yes	no	no	yes	U	yes	no
6079C>T	Arg2027X	30	non	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
6103+1G>A	U	intron 30	splice	U	yes	U	yes	U	U	U	yes	yes	U	U	U	U	U
6104-2A>T	U	intron 30	splice	U	yes	yes	U	U	U	yes	U	U	yes	U	poss	yes	U
6148C>T	Arg2050X	31	non	U	yes	yes	U	U	yes	yes	yes	U	no	yes	yes	U	poss
6148C>T	Arg2050X	31	non	de novo	yes	yes	yes	U	U	U	yes	U	yes	yes	U	yes	U
6148C>T	Arg2050X	31	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
6157C>T	Arg2053X	31	non	U	no	yes	no	yes	yes	U	yes	U	no	yes	yes	U	no
6157C>T	Arg2053X	31	non	U	yes	U	U	U	U	U	yes	U	U	U	yes	yes	yes
6157C>T	Arg2053X	31	non	U	yes	yes	yes	yes	yes	U	yes	yes	U	yes	U	yes	no
6165_6166 delGT	Tyr2056fs	31	fs	de novo	yes	yes	U	U	U	U	yes	U	U	yes	U	yes	U
6165_6166 delGT	Tyr2056fs	31	fs	de novo	U	yes	U	U	U	U	U	U	U	yes	yes	U	U
6179del	Leu2060fs	31	fs	U	yes	yes	U	U	U	U	yes	U	U	U	U	yes	U

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. (continued)

					Phenotypic information[f]												
CHD7 c[a]	CHD7 p[b]	Exon	Mutation type[c]	Segregation[e]	C	H	A	R G	R D	G	E	SCC	C(L)P	HL	FD	VII	TE
6196G>T	Glu2066X	31	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
6224del	Gly2075fs	31	fs	U	yes	yes	yes	U	U	U	yes	yes	U	yes	U	U	U
6271T>C	Trp2091Arg	31	mis	de novo	yes	no	no	yes	poss	U	yes	U	no	yes	yes	U	no
6292C>T	Arg2098X	31	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
6316A>T	Lys2106X	31	non	U	yes	yes	no	U	yes	U	yes	yes	U	yes	U	U	no
6322G>A	Gly2108Arg	31	mis	de novo	yes	no	U	U	no	yes	yes	U	yes	yes	poss	U	U
6473C>G	Ser2158X	31	non	U	yes	yes	U	U	yes	U	U	U	U	yes	U	U	U
6520A>T	Lys2174X	31	non	U	U	U	U	U	U	U	U	U	U	U	U	U	U
6526G>T	Glu2176X	31	non	U	yes	yes	U	U	yes	U	yes	yes	yes	yes	U	yes	no
6620dup	Cys2207fs	31	fs	U	yes	yes	U	U	U	U	yes	U	no	yes	poss	U	no
6651del	Glu2217fs	31	fs	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
6652_6656 del	Leu2218fs	31	fs	U	U	U	U	U	U	yes	yes	U	U	U	U	U	U
6667del	Val2223fs	31	fs	U	U	yes	U	U	U	U	yes	yes	yes	U	U	U	U
6681_6685 dup	Ser2229fs	31	fs	de novo	yes	yes	U	U	U	yes	yes	yes	yes	U	U	U	U
6688delA	Lys2230fs	31	fs	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
6705del	Gly2236fs	31	fs	U	yes	yes	yes	U	U	U	U	U	U	yes	U	U	U
6857dup	Phe2287fs	32	fs	de novo	no	no	yes	U	yes	yes	yes	yes	yes	yes	yes	yes	no
6857G>C	Gly2286Ala	32	mis	de novo	yes	yes	no	no	yes	U	yes	no	U	yes	U	yes	no
6888_6889 insT	Ala2297fs	32	fs	U	U	yes	yes	U	yes	U	U	U	U	yes	U	U	U
6937-1G>C	U	intron 32	splice	de novo	yes	yes	no	yes	yes	U	yes	U	no	U	poss	yes	no

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. (continued)

					Phenotypic information[f]												
CHD7 c[a]	CHD7 p[b]	Exon	Mutation type[c]	Segregation[e]	C	H	A	R G	R D	G	E	SCC	C(L)P	HL	FD	VII	TE
6937-2A>G	U	intron 32	splice	U	yes	yes	no	yes	U	yes	yes	U	U	no	U	yes	U
6937-2A>G	U	intron 32	splice	U	yes	no	no	yes	yes	yes	U	yes	yes	U	U	U	no
7075C>T	Gln2359X	33	non	U	no	yes	yes	no	U	yes	yes	yes	no	yes	yes	U	no
7106del	Val2369fs	33	fs	U	yes	yes	no	yes	U	No	yes	yes	no	yes	yes	yes	no
7160C>A	Ser2387X	33	non	de novo	yes	yes	yes	no	U	No	yes	yes	no	yes	yes	yes	yes
7164+1G>A	U	intron 33	splice	de novo	U	yes	yes	no	no	No	yes	yes	U	yes	U	U	no
7252C>T	Arg2418X	34	non	de novo	yes	yes	no	U	yes	U	yes	U	yes	yes	U	yes	U
7252C>T	Arg2418X	34	non	not maternal[°]	yes	U	U	U	U	U	U	yes	U	U	U	U	U
7252C>T	Arg2418X	34	non	de novo	U	yes	U	U	U	yes	yes	U	U	yes	U	U	U
7252C>T	Arg2418X	34	non	de novo	yes	yes	no	yes	yes	yes	yes	U	yes	yes	U	U	no
7276C>T	Gln2426X	34	non	de novo	yes	U	U	U	U	U	U	U	U	yes	U	U	U
7282C>T	Arg2428X	34	non	U	yes	yes	U	U	poss	U	yes	U	yes	yes	poss	U	U
7282C>T	Arg2428X	34	non	de novo	U	U	U	U	U	U	U	U	U	U	U	U	U
7282C>T	Arg2428X	34	non	U	yes	U	U	U	U	U	yes	yes	yes	U	U	U	U
7320del	Val2441fs	34	fs	U	yes	no	no	U	U	U	no	yes	U	yes	U	U	U
7344_7345 del	Glu2450fs	34	fs	U	yes	yes	yes	U	U	U	yes	yes	U	U	U	yes	U
7400del	Leu2467fs	34	fs	U	U	U	U	U	U	U	U	U	U	U	U	U	U
7441C>T	Gln2481X	34	non	de novo	yes	yes	yes	U	yes	yes	yes	U	U	yes	U	U	U
7451dupT	Leu2485fs	34	fs	U	no	yes	no	yes	yes	yes	yes	yes	no	yes	yes	U	no

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. (continued)

					Phenotypic information[f]												
CHD7 c[a]	CHD7 p[b]	Exon	Mutation type[c]	Segregation[e]	C	H	A	R G	R D	G	E	SCC	C(L)P	HL	FD	VII	TE
7454dup	Asn2486fs	34	fs	U	U	U	U	U	U	U	U	U	U	U	U	U	U
7577del	Phe2526fs	34	fs	U	yes	yes	U	U	U	U	U	U	U	U	U	yes	U
7593dup	Thr2532fs	34	fs	U	no	yes	no	yes	yes	yes	yes	yes	yes	yes	yes	U	U
7717_7720 del	Asn2573fs	35	fs	U	yes	no	U	yes	yes	yes	U	U	U	U	yes	U	U
7879C>T	Arg2627X	36	non	U	U	no	U	U	yes	yes	yes	U	U	U	yes	yes	U
7879C>T	Arg2627X	36	non	de novo	yes	yes	U	yes	yes	U	yes	U	no	U	U	U	yes
7879C>T	Arg2627X	36	non	U	yes	yes	yes	U	U	yes	U	U	yes	U	U	U	yes
7879C>T	Arg2627X	36	non	de novo	U	yes	U	U	U	U	yes	yes	U	yes	U	U	U
7879C>T	Arg2627X	36	non	de novo	yes	no	yes	U	U	yes	yes	yes	no	yes	no	U	no
7879C>T	Arg2627X	36	non	U	yes	U	yes	no	no	U	yes	U	U	no	U	yes	U
7879C>T	Arg2627X	36	non	U	U	yes	U	U	poss	U	yes	yes	yes	yes	poss	yes	U
7879C>T	Arg2627X	36	non	U	yes	yes	yes	U	U	U	yes	U	U	U	U	U	U
7891C>T	Arg2631X	36	non	U	yes	yes	U	U	yes	No	U	no	yes	yes	U	yes	no
7895del	Asn2632fs	36	fs	U	U	U	U	U	U	U	U	U	U	U	U	U	U
7933G>T	Glu2645X	36	non	U	yes	yes	U	yes	U	U	yes	U	yes	yes	U	U	U
7957C>T	Arg2653X	36	non	U	yes	yes	yes	yes	yes	No	yes	yes	no	yes	yes	U	no
7957C>T	Arg2653X	36	non	U	U	yes	U	U	U	yes	yes	U	yes	U	U	U	U
7957C>T	Arg2653X	36	non	U	yes	yes	U	U	yes	U	U	U	U	yes	U	U	U
8077-1G>A	U	intron 37	splice	de novo	no	yes	no	no	yes	U	yes	U	U	yes	yes	yes	U
8356G>T	Gly2786X	38	non	U	yes	yes	no	no	yes	yes	no	U	no	yes	U	yes	yes

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. (continued)

					Phenotypic information[f]												
CHD7 c[a]	CHD7 p[b]	Exon	Mutation type[c]	Segregation[e]	C	H	A	RG	RD	G	E	SCC	C(L)P	HL	FD	VII	TE
8613_8622 delins14	Asn2871fs	38	fs	de novo	yes	yes	no	U	U	U	yes	yes	yes	yes	U	U	U
8630_8634 del	Ala2877fs	38	fs	U	yes	yes	yes	U	U	yes	yes	U	no	U	yes	U	yes
8737dup	Leu2913fs	38	fs	U	yes	U	yes	U	poss	yes	yes	no	U	yes	poss	U	U
8962dup	Asp2988fs	38	fs	de novo	yes	yes	yes	U	U	yes	U	U	yes	yes	yes	U	U

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

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

					Phenotypic information[f]												
CHD7 c[a]	CHD7 p[b]	Exon	Domain[d]	Segregation[e]	C	H	A	R D	R G	G	E	SCC	C(L)P	HL	FD	VII	TE
-380C>T[$]	U			U	U	yes	yes	U	U	U	yes	U	U	U	U	U	U
-360C>T	U			U	U	yes	U	U	U	U	U	U	U	yes	U	U	U
215A>G*	Tyr72Cys	2		U	yes	yes	no	yes	yes	U	yes	U	no	no	U	U	U
295G>C*	Ala99Pro	2		paternal	U	yes	no	yes	U	yes	yes	U	no	yes	U	poss	U
760C>G*	Gln254Glu	2		U	U	yes	yes	yes	U	U	yes	yes	U	yes	U	U	yes
1315C>T*	Pro439Ser	2		U	U	yes	U	U	U	U	U	U	U	U	U	U	U
1672C>G*	Pro558Ala	3		U	U	U	U	U	U	U	U	U	U	U	U	U	U
2095A>G*	Ser699Gly	3		U	yes	yes	yes	yes	U	U	U	U	U	U	U	U	U
2824A>G*	Thr942Ala	10	CD2	paternal	yes	no	U	U	no	yes	yes	U	yes	yes	U	U	U
2923G>A	Gly975Arg	11	HD	U	U	U	yes	yes	U	U	U	yes	U	yes	U	U	U
3242T>G	Ile1081Ser	13		U	U	U	U	U	U	U	U	U	U	U	U	U	U
3751T>C	Cys1251Arg	15	HD	U	yes	yes	U	U	U	U	yes	yes	U	yes	U	yes	U
3949C>T	Arg1317Cys	16		U	yes	U	U	U	U	U	U	U	U	U	U	U	U
4850G>A	Gly1617Asp	21		U	U	yes	yes	U	U	U	U	U	U	U	U	yes	U
4850G>A	Gly1617Asp	21		not maternal	yes	yes	U	yes	yes	yes	yes	U	U	yes	poss	yes	U
4856G>T	Gly1619Val	22		U	no	yes	yes	yes	yes	U	yes	yes	U	U	U	U	U
5597A>G	Asp1866Gly	27		de novo	yes	yes	U	U	U	U	U	U	U	U	U	U	U
5894+5G>A	U	intron 29		U	U	U	U	U	U	U	U	U	U	U	U	U	U
6221T>C	Leu2074Pro	31		not maternal[#]	no	U	U	U	U	yes	U	U	U	yes	U	U	U
6221T>C	Leu2074Pro	31		not maternal[#]	yes	U	U	U	U	yes	U	U	U	yes	U	U	U
6290A>G	Asp2097Gly	31		U	U	U	U	U	U	U	U	U	U	U	U	U	U

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

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

					Phenotypic information[f]												
CHD7 c[a]	CHD7 p[b]	Exon	Domain[d]	Segregation[e]	C	H	A	R D	R G	G	E	SCC	C(L)P	HL	FD	VII	TE
6775G>A*	Ala2259Thr	31		U	U	U	U	U	U	U	U	U	U	U	U	U	U
6777A>G	Ala2259Ala	32		not maternal	yes	U	U	no	U	yes	yes	U	yes	yes	U	U	U
6935A>C	Lys2312Thr	32		not maternal	no	U	yes	U	yes	U	yes	U	U	yes	U	yes	U
7097T>G	Leu2366Arg	33		U	U	U	no	yes	U	U	U	yes	U	yes	U	U	U
7390A>G	Lys2464Glu	34		maternal	U	U	U	no	U	yes	yes	U	yes	yes	no	U	no

[a] NCBI Reference Sequence: NM_017780.3. cDNA numbering followed the rule of A from ATG transcript start is nucleotide 1. [$], UV found together with a frameshift mutation (c.791_792del; p.Leu264fs); * Classified as benign variant in paper of Bergman et al,[21] but less reliable prediction because data were missing.

[b] NCBI Reference sequence: NP_060250.2. U, unknown.

[c] del, deletion; fs, frameshift mutation; mis, missense mutation; non, nonsense mutation; splice, splice site mutation; transloc, translocation

[d] CD, chromodomain; HD, helicase domain; SANT, SANT domain;

[e] *, sib with features of CHARGE syndrome but unknown *CHD7* status; [#], sibs with *CHD7* mutation, father was not available for DNA analysis, but had features of Kallmann syndrome; U, unknown;

[f] VII, facial palsy; A, atresia of choanae; C, coloboma; C(L)P, cleft lip and/or palate; E, ear anomaly; FD, feeding difficulties necessitating tube feeding; G, genital hypoplasia; H, heart defect; HL, hearing loss; poss, possibly; prob, probably; RG, retarded growth; RD, retarded development; SCC, semicircular canal hypoplasia; TE, tracheo-esophageal anomaly; U, unknown.

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

A. Localization 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*
Chromo1	800-867	799-867	801-862	801-861	800-867	799-864	801-861	799-867
Chromo2	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.

Supplementary Table S2. The domains of CHD7: localization, sizes and mutations (continued)
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 (%)*	
Chromo1	2.0	-2.3	38	-39	4.8	-4.9
Chromo2	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 index patient each domain / the total number of pathogenic mutations in *CHD7* in index patients (excluding whole or partial gene deletion and translocation, but including recurrent mutations, N=790)
Chromo= chromodomain, chromatin organization modifier domain; Helicase N = helicase N-lobe; DEXDc = DEAD-like helicases 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 c	CHD7 p	Exon	NCBI SNP database	MAF[b]	Inherited[c]	Other mutation[d]	Phenotype of index[e]	Number of controls[f]	Reference[g]
109A>T	Met37Leu	2			+		CH		
216T>C	Tyr72Tyr	2	rs16926453	0.0048	U	fs	CH		1
277A>G	Thr93Ala	2			+		CH		
307T>A	Ser103Thr	2	rs41272435	0.0155	+	fs, mis, hom	CH	12	1,2,3
309G>A	Ser103Ser	2	rs115293759	0.0055	U		CH		1
350G>A	Gly117Asp	2			+		CH		3
363T>G	Gly121Gly	2	rs79158412		U		CH		
500C>T	Pro167Leu	2	rs61742851		U		CH		
657C>T	Gly219Gly	2	rs113483301	0.0201	U		CH		1
712G>A	Val238Met	2			U		CH		1
712G>C	Val238Leu	2			U	fs	CH		
715C>G	Leu239Ala	2			U	fs	CH		
856A>G	Arg286Gly	2	rs61995713		U		CH		
1018A>G	Met340Val	2	rs41305525	0.0053	+	non	CH, SC	3, 2/69 SC	1,2,4,5
1105C>G	Pro369Ala	2			+		CH		1
1179A>G	Pro393Pro	2	rs111238892		U		CH		
1323T>G	Gly441Gly	2			U		CLP	2	6
1397C>T	Ser466Leu	2	rs71640285		U		CH	6	1,2,6
1467A>G	Gln489Gln	2	rs71640286		U		C	1	2
1536A>G	Pro512Pro	2			U		CH		1
1565G>T	Gly522Val	2			+	hom	CLP, CH	12	1,6
1571A>C	His524Pro	2	rs78962949		U		CH		
1579T>G	Ser527Ala	2			U		CLP	2, 1/92 CLP	6
1632C>G	Pro544Pro	2	rs45536935		U		SC	4/69 SC	4

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

CHD7 c	CHD7 p	Exon	NCBI SNP database	MAF[b]	Inherited[c]	Other mutation[d]	Phenotype of index[e]	Number of controls[f]	Reference[g]
1665+34G>A	?	intron 2	rs7836586	0.2114	+	fs, non, del	SC, CH	13/69 SC	4,7,8,9
1786C>A	Gln596Lys	3	rs75653665		U		CH		
1803G>A	Lys601Lys	3	rs74999330		U		CH		
1907G>T	Gly636Val	3			+	non	CH		1
2049G>A	Lys683Lys	3	rs78107494		U		CH		
2050_2055dupAAAGCA	Lys684_Ala685dup	3			+	del	CH	3	3
2053_2058dupGCAAAA	Ala685_Lys686dup	3			+	del, non	CH	3	1
2067G>A	Thr689Thr	3	rs34979623	0.0024	U		CH		
2096+15insA	?	intron 3	rs111577577		U		U		
2124T>C	Ser708Ser	4	rs79302359	0.0127	U		CH		1
2230G>A	Gly744Ser	4			U	mis	CH		1,5
2238+39G>A	?	intron 4	rs4540437	0.1351	U		SC, CH	13/69 SC	4,7,8
2361C>A	Ser787Ser	5			U		CH		1
2376+49insTGGACT	?	intron 5	rs33909822		U		CH		
2376+41G>A	?	intron 5	rs74407310		U		U		
2376+42insTGGACT	?	intron 5	rs112021443		U		U		
2376+43insTGGACT	?	intron 5	rs35504039		+	fs	CH		9
2376+48insTGGACT	?	intron 5	rs5891777		+	non, fs	CH		
2376+49A>T	?	intron 5	rs77952475		U		U		
2377-3dupT	?	intron 5			U		U		5
2436A>T	Lys812Asn	6	rs61978638	0.0060	U		CH		
2442+38A>T	?	intron 6	rs41272438	0.0163	U		CH	17	2,7
2443-43C>T	?	intron 6	rs74693288	0.0052	U		U		
2614-48C>G	?	intron 8	rs79276682	0.0690	U		U		

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

CHD7 c	CHD7 p	Exon	NCBI SNP database	MAF[b]	Inherited[c]	Other mutation[d]	Phenotype of index[e]	Number of controls[f]	Reference[g]
2614-45G>A	?	intron 8	rs6471902	0.1852	U		CH		7
2831G>A	Arg944His	10	rs117506164	0.0077	+		CH		1
2957+20C>T	?	intron 11			U		CH		
3201+21T>G	?	intron 12		0,0075	U		CH		8
3202-5T>C	?	intron 12		0,1505	U		CH		8
3379-33A>G	?	intron 13	rs45461501		U		CH		1
3522+13T>A	?	intron 14			U	fs	CH		
3523-39C>G	?	intron 14		0,011	U		CH		7
3523-35C>G	?	intron 14	rs41272442	0.0588	+	non	CHD		
3778+17C>T	?	intron 15	rs111863846	0.0044	U		U		
3879G>A	Val1293Val	16			U		CH		
4533+34T>C	?	intron 19			U	fs	CH		
4533+46A>G	?	intron 19	rs7844902	0.1987	U	non, fs, del	CH		7
4534-13G>T	?	intron 19	rs114996731		U		U		
4614T>C	Ala1535Ala	20			U	mis	CH		10
4644+26C>T	?	intron 20	rs115999896		U		U		
4644+36C>T	?	intron 20	rs71640287		U		CH	1	2
4780C>T	Pro1594Ser	21			U	fs	CH		
5015C>T	Ala1672Val	22	rs61737194		U		CH		
5051-4C>A	?	intron 22			U		CH		5
5051-4C>T	?	intron 22	rs71640288		+	mis	CH	2	1,2,5
5147insGCCAGCTG		23			U	fs	CH		1
5307C>T	Ala1769Ala	25	rs16926499	0.0246	U		CH		1
5404+41C>T	?	intron 25	rs115544727		U		U		

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

CHD7 c	CHD7 p	Exon	NCBI SNP database	MAF[b]	Inherited[c]	Other mutation[d]	Phenotype of index[e]	Number of controls[f]	Reference[g]
5607+27A>T	?	intron 27	rs77988197		U		U		
5754T>C	Thr1918Thr	29	rs61746542		U		CH		
5757C>G	Ala1919Ala	29	rs79203206	0.0086	U		CH		
5894+32C>G/T	?	intron 29	rs41265252	0.0100	U		U		
5895-23A>G	?	intron 29			U		CH		5
5915C>G	Ala1972Gly	30			+		CH		
6103+8C>T	?	intron 30	rs3763592	0.1391	+	fs	CH		1,2,7
6111C>T	Pro2037Pro	31	rs41312170	0.0053	U		CH	2	1,2
6135G>A	Pro2045Pro	31	rs6999971	0.0304	+	non, fs	CH	8	1,2,3
6167C>A	Arg2062Arg	31			U	non	CH		11
6184C>T	Arg2062Trp	31			U	non	CH		
6276G>A	Glu2092Glu	31	rs2068096	0.1184	+	non,fs	CH	12	1,2,3,7
6282A>G	Gly2094Gly	31	rs41312172	0.0032	U	non	CH	1	1,2
6304G>A	Val2102Ile	31			U		CH		6
6335C>T	Thr2112Met	31			+		CLP		6
6352A>G	Asn2118Asp	31			+		CH		
6478G>A	Ala2160Thr	31	rs61753399	0.0014	U	mis	CH		1,5
6513C>T	Ala2171Ala	31			U		CH		
6672C>T	Gly2224Gly	31			U		CLP	1	6
6673G>A	Ala2225Thr	31			U		CH		1
6738G>A	Glu2246Glu	31	rs61729627	0.0632	U		CH		1,5
6822T>C	Ala2274Ala	32	rs61743849		U		CH		1
6843C>G	Asp2281Glu	32			U		CH		5
6924G>A	Ser2308Ser	32	rs61733338	0.0086	+	non	CH		9

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

CHD7 c	CHD7 p	Exon	NCBI SNP database	MAF[b]	Inherited[c]	Other mutation[d]	Phenotype of index[e]	Number of controls[f]	Reference[g]
6936+29G>T	?	intron 32	rs78582735		U		U		
6936+35C>T	?	intron 32	rs112592072		U		U		
6989G>C	Gly2330Ala	33	rs77704609		U		CH		1
6990C>T	Gly2330Gly	33	rs116523071		U		CH		
7107C>T	Val2369Val	33			U		C	1	6
7209G>A	Arg2403Arg	34	rs61746518		U		U		
7243G>T	Als2415Ser	34	rs41315633		U		U		
7278G>A	Gln2426Gln	34	rs115546145		U		U		
7356A>G	Thr2452Thr	34	rs2272727	0.0382	+	non, fs, del	CH		1,2,3,7
7463G>A	Gly2488Asp	34			+		CH		
7471C>T	Arg2491Cys	34			U	spl	CH		
7579A>C	Met2527Leu	34			+		CH		1
7590A>G	Lys2530Lys	34	rs61742801	0.0086	U		CH		1
7830+44A>G	?	intron 35	rs115428154	0.0143	U		U		
7958G>A	Arg2653Gln	36			+	fs	CH		
8173A>G	Ile2725Val	38	rs113877656		U		U		
8199T>G	Ala2733Ala	38			U		CH		
8250T>G	Phe2750Leu	38	rs3750308		U		U		
8339C>T	Ala2780Val	38			+		CH		
8355C>T	Ala2785Ala	38			U	mis	CH		1
8365G>A	Ala2789Thr	38			U		IHH		2
8416C>G	Leu2806Val	38	rs45521933	0.0045	+		CH		1,5
8569T>G	Ser2857Ala	38			U		CH		1
8661G>A	Pro2887Pro	38			U	fs	CH		3

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

CHD7 c	CHD7 p	Exon	NCBI SNP database MAF[b]	Inherited[c]	Other mutation[d]	Phenotype of index[e]	Number of controls[f]	Reference[g]
8790C>T	Ala2930Ala	38	rs61736186	U		CH		
8950C>T	Leu2984Phe	38		+	fs	CH		1,12

[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] Allele frequency from NCBI SNP database (25 May 2011) or literature

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

[d] The type of pathogenic mutation that was found in at least one index patient next to the benign variant. fs, frameshift; mis, missense; hom, homozygous; non, nonsense; spl, splice

[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.

[g] References

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CHAPTER 2.2

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

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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 counseling. 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 two computational algorithms (PolyPhen-2 and Align-GVGD) and the prediction of a newly developed structural model of the chromo- and helicase domains of CHD7 with segregation and phenotypic data. The combination of different variables will lead to a more confident prediction of pathogenicity than was previously possible. We have used our system to classify 145 *CHD7* missense variants. Our data show that pathogenic missense mutations are mainly present in the middle of the *CHD7* gene, whereas benign variants are mainly 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.

Keywords: CHARGE syndrome, *CHD7*, missense mutation, classification system, prediction pathogenicity, genotype-phenotype correlation

INTRODUCTION

CHARGE syndrome (MIM 214800) is a clinically heterogeneous syndrome that is characterized by the occurrence of ocular **c**oloboma, **h**eart defects, **a**tresia of choanae, **r**etardation of growth and/or development, **g**enital anomalies, and **e**ar anomalies often combined with deafness.[1-4] It is inherited in an autosomal dominant fashion. Most cases are sporadic due to *de novo* mutations but familial recurrence has also been described.[1] CHARGE syndrome has an estimated incidence of 1 in 16,000 newborns.[5] The major gene involved in CHARGE syndrome is *CHD7* (MIM 608892) and heterozygous *CHD7* mutations are found in more than 90% of the patients with typical CHARGE syndrome based on the clinical diagnostic criteria.[6-9] Nonsense and frameshift mutations are most prevalent with a frequency of 44% and 34%, respectively.[5] Splice site mutations are found in 11% of patients, while missense mutations are present in 8% of patients. Deletions and genomic rearrangements occur in 3% of cases. Although missense mutations in the *CHD7* gene are found in only a minority of patients, their interpretation may be problematic, thus resulting in difficulties in genetic counseling.

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 the consequences of a missense variant is more difficult, especially in a rare disease 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 two computational algorithms, PolyPhen-2[20,21] and Align-GVGD,[22,23] and the prediction of a newly developed structural model of the CHD7 chromo- and helicase domains, with segregation and phenotypic data. We

have classified all the *CHD7* missense variants known to us (n=145). 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

Inclusion of *CHD7* missense variants

In this paper we give an overview of all *CHD7* missense variants reported in the literature before June 15th 2011[1,7,9,14,24-41] and the variants that were reported in the NCBI Single Nucleotide Polymorphism database (http://www.ncbi.nlm.nih.gov/SNP, dbSNP build 132) with frequency data (n=104, Supp. Table S1). In addition, we show all unpublished missense variants that were found in the DNA diagnostic laboratories of the Radboud University Nijmegen Medical Center (RUNMC), Nijmegen, the Netherlands and the Department of Cellular and Molecular Medicine (ICMM), the Panum Institute, University of Copenhagen, Denmark (n=41).

CHD7 analysis

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.[42] The GenBank accession number NM_017780.2 was used as reference sequence for the *CHD7* gene. The A of ATG was designated number 1. The intron sequences of the *CHD7* gene can be found in NG_007009.1. Segregation of the *CHD7* variant was studied whenever possible.

Development of a classification system for *CHD7* missense variants

We first screened all *CHD7* missense variants for possible splice effects using the splicing module of Alamut version 1.5 (http://www.interactive-biosoftware.com/alamut.html). This module contains four splice prediction programs; SpliceSiteFinder-Like,[43] MaxEntScan,[44] NNSPLICE[45] and GeneSplicer.[46] In addition, all 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. The alignments of the computational algorithms were provided by Alamut version 1.5.

SIFT

The SIFT algorithm, **S**orting **I**ntolerant **F**rom **T**olerant, 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 .[47,48] 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 (probably pathogenic) and scores greater or equal to 0.05 are predicted to be tolerated (benign). Native SIFT alignments were used.

PolyPhen-2

PolyPhen-2, **Poly**morphism **Phen**otyping program version 2, is available at http://genetics.bwh.harvard.edu/pph2/. PolyPhen-2 is an update from PolyPhen[21] and relies on sequence-based and structure-based features.[20] 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'. 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).[22,23] A grade, varying from C0 to C65, is given to estimate the probability that a certain variant is pathogenic. We interpreted C0 as 'probably benign', C15, C25 and C35 as 'possibly pathogenic', and C45, C55 and C65 as 'probably pathogenic' in agreement with McGee et al.[12] We used the native alignments of Align-GVGD.

Structural model of the CHD7 chromo- and helicase domains

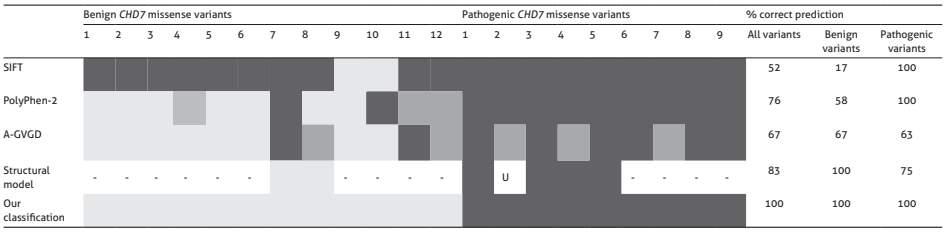
No experimentally derived structures of the CHD7 chromo- and helicase domains are available as yet and we therefore constructed a structural model for these

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 modeling of the CHD7 chromo- and helicase domains were selected from the protein database using BLAST (Supp. 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] A low percentage sequence identity (approximately 30%) between the target sequence and most of the template sequences was observed. 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. 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. 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] Modeling of the *CHD7* variants and the assessment of the effect on CHD7 stability was performed using the FoldX protein design algorithm,[60-62] as described previously.[13,15-17]

Performance of the computational algorithms and our structural model

In order to test whether SIFT, PolyPhen-2, Align-GVGD and our structural model gave correct predictions, we examined whether their predictions were correct for 12 surely benign and 9 surely pathogenic *CHD7* missense variants (Table 1; Supp. Table S1). The surely benign variants had been found in two or more controls. The surely pathogenic variants had occurred at least twice *de novo* in a patient with CHARGE syndrome, or had occurred *de novo* once and were found in at least two patients with CHARGE syndrome (Table 1; Supp. Table S1). Furthermore, none of the surely pathogenic *CHD7* missense variants was predicted to influence splicing.

Table 1. Performance of the computational algorithms (SIFT, PolyPhen-2 and Align-GVGD), our structural model and our classification system which integrates the predictions of PolyPhen-2, Align-GVGD and the structural model with segregation and phenotypic data.



- Variant predicted to be pathogenic
- Variant predicted to be possibly pathogenic (by PolyPhen and Align-GVGD)
- Variant predicted to be benign
- \- Variant located outside the chromo- and helicase domains that were modeled by our structural model
- U Variant predicted to have an undetermined effect by our structural model

Benign *CHD7* missense variants
(present in two or more controls and/or found in homozygous state)

1: p.Ser103Thr
2: p.Met340Val
3: p.Ser466Leu
4: p.Gly522Val
5: p.Ser527Ala
6: p.Gln596Lys
7: p.Lys812Asn
8: p.Arg944His
9:p.Ala2160Thr
10: p.Gly2330Ala
11: p.Ala2415Ser
12: p.Phe2750Leu

Pathogenic *CHD7* missense variants
(occurred *de novo* in at least two patients with CHARGE syndrome, or had occurred *de novo* once and were found in at least two patients with CHARGE syndrome, none of the variants were predicted to influence splicing)

1: p.Leu1020Ser
2: p.Ile1028Val
3: p.Cys1101Arg
4: p.Gln1214Arg
5: p.Leu1294Pro
6: p.Leu1815Pro
7: p.His2096Arg
8: p.Gly2108Arg
9: p.Ile2116Asn

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 35 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. Fisher's exact test was performed to identify significant differences between the two groups of patients (significance level $p<0.05$).

RESULTS

Development of our classification system

The performance of the computational algorithms and our structural model is shown in Table 1. SIFT gave a correct prediction for only 2 of 12 benign variants, whereas PolyPhen-2 and especially Align-GVGD performed much better for the benign variants. PolyPhen-2 was better than Align-GVGD in correctly predicting the pathogenic variants. Therefore, we included PolyPhen-2 and Align-GVGD in our classification system, but did not include SIFT (Table 2). The output of PolyPhen-2 and Align-GVGD was scored as 0 (benign), +0.5 (possibly pathogenic), or +1 (probably pathogenic) and was then summed as previously suggested by McGee et al.[12]

Table 2. Novel classification system for *CHD7* missense variants

Computational algorithms (summed score between 0 and +2)
Polyphen-2: benign = 0, possibly damaging = +0.5 and probably damaging = +1
Align-GVGD: C0 = 0, C15/C25/C35 = +0.5 and C45/C55/C65 = +1
Structural model (summed score between -1 and +1)
Minor effect = -1, undetermined effect = 0, detrimental effect or located close to the ATP binding site = +1
Segregation analysis (summed score between -10 and +4)
Variant occurred *de novo* in one patient with features of CHARGE syndrome = +3 OR Variant occurred at least twice *de novo* in patients with features of CHARGE syndrome = +4
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 -11 and +7)
Probably benign: total score 0 or less
Unknown: total score between 0 and +4
Probably pathogenic: total score +4 or more

* this means that the variant is present in one or more clinically well characterized persons without features of CHARGE syndrome, or the variant is found in two or more persons reported to be normal, but for whom no detailed clinical information is 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)

Two surely benign and five surely pathogenic missense variants were located in the chromo- or helicase domain and could therefore be modeled. Our structural model gave a correct prediction for all variants, although one variant had an undetermined effect (Table 1). As our structural model did predict correctly almost all variants, we decided to integrate our structural model in our classification system. Variants that were predicted to have a minor effect on the stability of the CHD7 protein were scored as -1, variants that had an undetermined effect received a score of 0 and variants that were predicted to have a detrimental effect or were located in the ATP-binding domain were scored as +1 (Table 2).

In addition to the scores of the algorithms and our structural model, we integrated data from segregation analysis in our classification system (Table 2). 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 at least twice *de novo*, in patients with features of CHARGE syndrome, 4 points were added. In contrast, 2 points were subtracted if the variant was found in at least one clinically well characterized person without features of CHARGE syndrome, or if the variant was found in at least two 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). Five points were subtracted if the variant was found in a homozygous state (this because homozygous *CHD7* 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 -11 and +7. 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 2; Supp. Table S1).

Classification of *CHD7* missense variants

A complete overview of all 145 missense variants in the *CHD7* gene is supplied in Supp. 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 classification system (see Table 2), 40 variants had a score ≥+4 and were classified as 'probably pathogenic' (27%, with four variants possibly affecting splicing), 46 variants had a score between 0 and +4

and were classified as 'UV' (32%, with six variants possibly affecting splicing), and 59 variants had a score ≤0 and were classified as 'probably benign' (41%, with 2 variants possibly affecting splicing) (Supp. Table S1). Our classification agreed well with most of the classifications of the 104 previously reported variants (Table 3). However, it was discordant for five variants; p.His55Arg, p.Pro732Ala, p.Val2102Ile, p.Ala2789Thr and p.Lys2948Glu. We had classified these five variants as 'probably benign' based solely on *in silico* data, but they had previously been reported as pathogenic[7,14,30] Supp. Table S1).

Table 3. Our classification of 104 *CHD7* missense variants versus the classification as reported in the literature

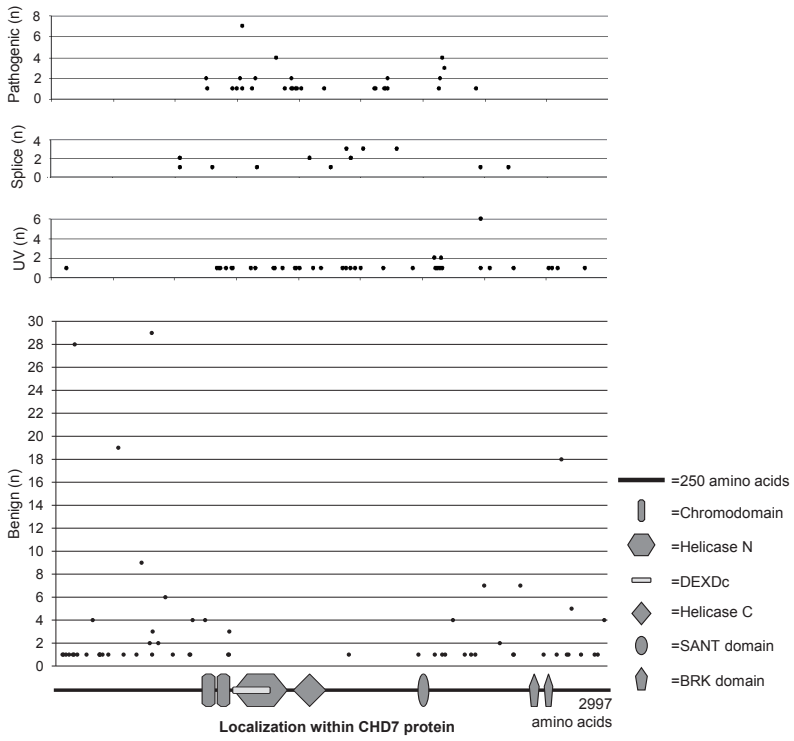
		Classification as reported in the literature				
		Pathogenic	UV	dbSNP	Benign	Total
Our classification	Pathogenic	29	0	0	0	29
	UV	8	24	1	2	35
	Benign	5	11	8	16	40
	Total	42	35	9	18	104

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, 13/38 were classified as 'probably pathogenic' (including three variants possibly affecting splicing), 5/38 as 'UV' (including two variants possibly affecting splicing) and 20/38 as 'probably benign' (with 1 variant possibly affecting splicing). Four benign variants (p.Ser103Thr, p.Met340Val, p.Gly522Val and p.Phe2750Leu) were found in more than 15 index persons. 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[63] and homozygous *CHD7* mutations have never been found in a patient with CHARGE syndrome). The three most frequently occurring pathogenic missense mutations were each found in more than three index patients (p.Ile1028Val, p.Gln1214Arg and p.Gly2108Arg). Three pathogenic missense mutations were possibly implicated in familial CHARGE syndrome: p.Ser834Phe,[29] p.His2096Arg[31] and p.Gly2108Arg.[35]

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,

Figure 1. The distribution of missense variants over the coding region of the *CHD7* gene



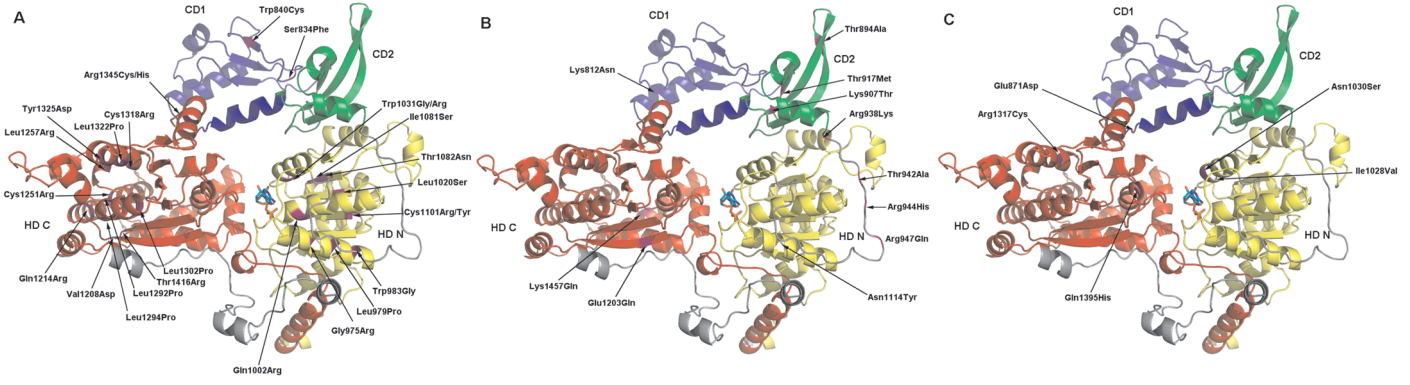
The distribution of 145 different missense variants over the coding region of the *CHD7* gene (the variants were found in 322 index patients or controls). The missense variants are divided into four categories: 'probably pathogenic', 'possible effect on splicing', 'UV', and 'probably benign'. The variants 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

functional domains of CHD7; 11 in the chromodomains, 31 in the helicase domain, and only three in the SANT and BRK domains.

Structural model of CHD7

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

Figure 2. Structural model of the CHD7 chromo- and helicase domains



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 (Hauk et al., 2010) and additional template structures (Supp. 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.

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 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 unfavorable 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?

Of the 42 variants that were located in the chromo- or helicase domain, 11 variants were predicted to have a minor effect (26%), 28 variants were predicted to have a detrimental effect (67%) and for three variants the effect could not be determined (7%). The variants that were predicted to decrease the stability of the CHD7 protein were frequently located in the core of the protein (24/28), whereas the variants with a predicted minor effect were often located at the surface or in linker/loop regions (9/11). Two variants were considered to have a detrimental effect due to their close location to the ATP binding site; p.Asn1030Ser in the Helicase N-lobe and p.Gln1395His in the Helicase 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'. No direct effects on phosphorylation or interaction motives were found for any of the variants.

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=35) with that of patients with a truncating mutation in the *CHD7* gene (n=315) (Table 4). The patients with a truncating mutation more often fulfilled the clinical criteria of Blake et al.[6] and Verloes[8] (p=0.017 and p=0.031, respectively). In addition, cleft lip and/or palate (p=0.042), choanal anomalies (p=0.015) 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. 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 4. Phenotypic comparison of patients carrying a *CHD7* missense mutation with patients carrying a *CHD7* truncating mutation

	Patients with a CHD7 missense mutation (n=35)*	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	31.8% (7/22)	55.6% (80/144)	*0.042*
Choanal anomaly	30.0% (6/20)	60.4% (110/182)	*0.015*
Heart defect	46.7% (14/30)	82.5% (212/257)	*<0.001*
Tracheo-esophageal anomaly	13.6% (3/22)	33.6% (43/128)	0.080
Coloboma and/or microphthalmia	75.9% (22/29)	86.9% (199/229)	0.154
Cranial nerve dysfunction	78.9% (15/19)	90.8% (119/131)	0.123
Semicircular canal anomaly and/or balance disturbance	95.0% (19/20)	100% (121/121)	0.142
External ear anomaly	96.4% (27/28)	98.2% (217/221)	0.452
Kidney anomaly	30.0% (6/20)	37.6% (44/117)	0.620

* 35 missense variants that we classified as 'probably pathogenic'
^ 315 truncating mutations; 5 deletions, 145 frameshift mutations and 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 harboring 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 Bergman et al.[1]). Early detection and treatment of hypogonadotropic hypogonadism is important, because this will reduce the risk of osteoporosis.[27] In addition, genetic counseling 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 the results of PolyPhen-2, Align-GVGD and our structural model with segregation and phenotypic data (Table 2). 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 40 variants as 'probably pathogenic', 46 as 'unclassified variant', and 59 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 frequently in agreement with predictions from the literature (Table 3).

According to our classification system, a variant is considered 'probably pathogenic' when it has occurred at least twice *de novo* 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 have a deleterious effect according to either PolyPhen-2, Align-GVGD or our structural model. 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 feel that one *de novo* occurrence is not enough for classifying a variant as surely pathogenic, because a benign polymorphism can also by chance occur *de novo* in a sporadic patient with CHARGE syndrome (*e.g.* c.6103+8C>T, which had occurred *de novo* in a CHARGE patient,[37] but was later conclusively proven to be benign).[14] We classify a variant as 'probably benign' when there are no clues to suggest pathogenicity from either PolyPhen-2, Align-GVGD, our structural model 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 at least twice *de novo* in patients with features of CHARGE syndrome. Five variants that we classified as 'probably benign', p.His55Arg, p.Pro732Ala, p.Val2102Ile, p.Ala2789Thr and p.Lys2948Glu, were previously reported as pathogenic missense mutations.[14,307] Because all five variants were predicted to be 'benign' by PolyPhen-2 and Align-GVGD and segregation data (including phenotypic 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 modeling 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] Hopefully, segregation data concerning these five variants will become available in the future, leading to a more reliable classification of pathogenicity.

Unfortunately, we had to classify one third of the *CHD7* variants as 'UV' (46/145), due to a lack of segregation data (n=25) or of phenotypic data of the carrier parent (n=17). For only four variants, these data were available, but we still had to classify the variant as 'UV'. Three of these variants had been identified in an affected family member, but unfortunately it was unknown whether the variant had occurred *de novo* in the affected parent. 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) provides a valuable source of information, as it contains both segregation and clinical data. Clinical data are important, because the phenotype of patients who undergo *CHD7* analysis in a clinical diagnostic laboratory is not always highly suggestive of CHARGE syndrome.26,65 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.

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.

Distribution of *CHD7* missense variants

The *CHD7* missense variants were present in the entire coding region of the *CHD7* gene (Figure 1, Supp. 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: 47/59 'probably benign' variants were found in amino acids 1-820 and 2320-2997 (Figure 1). The 5' end of the *CHD7* gene is only weakly to moderately conserved among species and both the N- and C-terminal 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, Supp. Table S2). 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.

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 (Supp. Table S1). For every new *CHD7* variant that is submitted to the *CHD7* database, we will provide the prediction of our structural model.

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 4). 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 *CHD7* truncating mutation: cleft lip/palate, choanal anomalies, and congenital heart defects. This is consistent with a previous study that showed that ten severely affected fetuses with CHARGE syndrome were all carrying a *CHD7* truncating mutation.[67] 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.[1,29,35,37,68]

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 PolyPhen-2 and Align-GVGD and the prediction of our structural model with segregation 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. 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 or those that were classified as 'probably benign' solely based on *in silico* data. *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 mainly 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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SUPPLEMENTARY MATERIAL

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

CHD7 variant[a]	Domain[b]	SIFT[c]	Poly Phen-2[d]	Align-GVGD[e]	Structural model of CHD7[f]	# controls[g]	# index patients[h]	Segregation[i]	Other mutation found in index patient[j]	Total summed score ; classification[k]	References[l]
c.109A>T p.Met37Leu	-	tolerated/0.23	benign /0.001	C0		0	1 CH	unaffected parent carrier	-	-2 ; benign	CS
c.123G>A p.Met41Ile	-	not tolerated	benign /0.002	C0		0	1 CH	U	-	0 ; benign*	1
c.164A>G p.His55Arg	-	not tolerated	benign /0.023	C0		0	1 KS	U	-	0 ; benign*	2
c.215A>G p.Tyr72Cys	-	not tolerated	benign /0.247	C0		0	1 CH	U	-	0 ; benign*	CS
c.257C>G p.Pro86Arg	-	not tolerated	poss /0.253	C0		0	1 CH	parent carrier	-	0.5 ; UV	1
c.277A>G p.Thr93Ala	-	tolerated/1.0	benign /0	C0		0	1 CH	parent carrier	-	0 ; benign*	CS
c.295G>C p.Ala99Pro	-	not tolerated	benign /0.156	C0		0	1 CH	parent carrier	-	0 ; benign*	CS
c.307T>A p.Ser103Thr	-	not tolerated	benign /0.013	C0		15	10 CH, 3 KS/ HH	parent carrier	+, homo	-10 ; benign	1, 2, 3, dbSNP, CS
c.350G>A p.Gly117Asp	-	not tolerated	poss /0.545	C0		0	1 CH	unaffected parent carrier	+	-4.5 ; benign	3
c.500C>T p.Pro167Leu	-	not tolerated	benign /0	C0		1	0	U	-	0 ; benign*	dbSNP
c.602A>G p.Gln201Arg	-	not tolerated	benign /0	C0		0	4 CH	parent carrier	-	0 ; benign*	1
c.712G>A p.Val238Met	-	tolerated/0.25	benign /0.002	C0		0	1 CH	U	-	0 ; benign*	1
c.712G>C p.Val238Leu	-	tolerated/0.16	benign /0	C0		0	1 CH	U	+	-3 ; benign	CS
c.715C>G p.Pro239Ala	-	not tolerated	benign /0.066	C0		0	1 CH	U	+	-3 ; benign	CS
c.760C>G p.Gln254Glu	-	not tolerated	benign /0.004	C0		0	1 CH	U	-	0 ; benign*	CS
c.856A>G p.Arg286Gly	-	not tolerated	benign /0.043	C0		1	0	U	-	0 ; benign*	dbSNP
c.1018A>G p.Met340Val	-	not tolerated	benign /0	C0		5	11 CH, 1 HH/ KS, 2 SC/C	poss unaffected parent carrier	+	-5 ; benign	1, 2, 4, 5, dbSNP, CS
c.1105C>G p.Pro369Ala	-	tolerated/0.13	benign /0.066	C0		0	1 CH	3 family members carrier	-	-2 ; benign	1
c.1315C>T p.Pro439Ser	-	not tolerated	benign /0.112	C0		0	1 CH	U	-	0 ; benign*	CS

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

CHD7 variant[a]	Domain[b]	SIFT[c]	Poly Phen-2[d]	Align-GVGD[e]	Structural model of CHD7[f]	# controls[g]	# index patients[h]	Segregation[i]	Other mutation found in index patient[j]	Total summed score ; classification[k]	References[l]
c.1397C>T p.Ser466Leu	-	not tolerated	benign /0.001	C0		4	3 CH, 1 CLP, 1 KS/HH	parent carrier	-	-2 ; benign	1, 2, 6, CS
c.1531T>G p.Leu511Val	-	not tolerated	benign /0.052	C0		1	1 CLP	U	-	0 ; benign*	6
c.1565G>T p.Gly522Val	-	not tolerated	poss /0.622	C0		13	3 CH, 13 CLP	U	-, homo	-6.5 ; benign	1, 6, CS
c.1571A>C p.His524Pro	-	not tolerated	benign /0.045	C0		1	0	U	-	0 ; benign*	dbSNP
c.1579T>G p.Ser527Ala	-	not tolerated	benign /0.008	C0		2	1 CLP	U	-	-2 ; benign	6
c.1672C>G p.Pro558Ala	-	not tolerated	benign /0.066	C0		0	2 CH	U	-	0 ; benign*	1, CS
c.1786C>A p.Gln596Lys	-	not tolerated	benign /0.008	C0		6	0	U	-	-2 ; benign	dbSNP
c.1907G>T p.Gly636Val	-	tolerated/0.40	poss /0.622	C0		0	1 CH	U	+	-2.5 ; benign	1
c.2095A>G p.Ser699Gly	-	not tolerated	benign /0.013	C0		0	2 CH	U	-	0 ; benign*	CS
c.2096G>C p.Ser699Thr	-	not tolerated	benign /0.013	C0		0	1 CH	U	-	0 ; benign*	1
c.2182G>A p.Asp728Asn	-	not tolerated	benign /0.139	C0		0	1 CH	U	-	0 ; benign*	1
c.2194C>G p.Pro732Ala	-	not tolerated	benign /0.136	C0		0	1 CH	parent carrier	-	0 ; benign*	7
c.2230G>A p.Gly744Ser	-	not tolerated	prob /0.968	C0		0	4 CH	poss unaffected parent carrier	poss	-1 or -2 ; benign	1, 5
c.2436A>T p.Lys812Asn	CD1 loop	not tolerated	prob /0.988	C65	minor	2	4 CH	unaffected parent carrier	-	-1 ; benign	dbSNP, CS
c.2501C>T p.Ser834Phe	CD1 core	not tolerated	prob /0.998	C65	detrimental	0	1 CH, 1 HH	de novo in affected parent	-	6 ; pathogenic	2, 8
c.2520G>C p.Trp840Cys	CD1 core	not tolerated	prob /0.999	C65	detrimental	0	1 CH	de novo	-	6 ; pathogenic	CS
c.2613G>T p.Glu871Asp	CD1 surface*	not tolerated	poss /0.560	C35	undeterm	0	1 CH	de novo	-	4 ; pathogenic	1
c.2680A>G p.Thr894Ala	CD2 surface	not tolerated	poss /0.850	C55	minor	0	1 CH	parent carrier	-	0.5 ; UV	1
c.2720A>C p.Lys907Thr	CD2 surface*	not tolerated	prob /0.999	C65	minor	0	1 CH	U	-	1 ; UV	1

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

CHD7 variant[a]	Domain[b]	SIFT[c]	Poly Phen-2[d]	Align-GVGD[e]	Structural model of CHD7[f]	# controls[g]	# index patients[h]	Segregation[i]	Other mutation found in index patient[j]	Total summed score ; classification[k]	References[l]
c.2750C>T p.Thr917Met	CD2 core	not tolerated	prob /1.000	C65	minor	0	1 CH	parent carrier	-	1 ; UV	1
c.2813G>A p.Arg938Lys	CD2 linker surface*	not tolerated	benign /0.211	C25	minor	0	1 CH	U	-	-0.5 ; benign*	1
c.2824A>G p.Thr942Ala	CD2 linker loop surface	not tolerated/0.04	benign /0.021	C0	minor	0	1 CH	parent carrier	-	-1 ; benign*	CS
c.2831G>A p.Arg944His	CD2 linker loop surface	not tolerated	benign /0.185	C25	minor	2	1 CH	parent carrier	-	-2.5 ; benign	1, dbSNP
c.2840G>A p.Arg947Gln	CD2 linker loop surface	not tolerated	prob /0.986	C35	minor	0	1 CH	parent carrier	-	0.5 ; UV	1
c.2923G>A p.Gly975Arg	HD N core	not tolerated	prob /1.000	C65	detrimental	0	1 CH	U	-	3 ; UV	CS
c.2936T>C p.Leu979Pro	HD N core	not tolerated	prob /0.994	C65	detrimental	0	1 CH	de novo	-	6 ; pathogenic	9
c.2947T>G p.Trp983Gly	HD N core	not tolerated	prob /0.968	C65	detrimental	0	1 CH	U	-	3 ; UV	5
c.3005A>G p.Gln1002Arg	HD N core	not tolerated	prob /0.990	C35	detrimental	0	1 CH	de novo	-	5.5 ; pathogenic	1
c.3059T>C p.Leu1020Ser	HD N core	not tolerated	prob /0.991	C65	detrimental	0	2 CH	de novo	-	6 ; pathogenic	10, CS
c.3082A>G p.Ile1028Val	HD N core	not tolerated	prob /0.981	C25	undeterm	0	7 CH	>1 de novo	-	5.5 ; pathogenic	1, 5, CS, 7/11/12 (same patient)

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

CHD7 variant[a]	Domain[b]	SIFT[c]	Poly Phen-2[d]	Align-GVGD[e]	Structural model of CHD7[f]	# controls[g]	# index patients[h]	Segregation[i]	Other mutation found in index patient[j]	Total summed score ; classification[k]	References[l]
c.3089A>G p.Asn1030Ser	HD N ATP binding cleft	not tolerated	prob /0.995	C45	ATP binding	0	1 CH	de novo	-	6 ; pathogenic	14
c.3091T>C p.Trp1031Arg	HD N core	not tolerated	prob /0.999	C65	detrimental	0	1 CH	de novo	-	6 ; pathogenic	CS
c.3091T>G p.Trp1031Gly	HD N core	not tolerated	prob /0.998	C65	detrimental	0	1 CH	de novo	-	6 ; pathogenic	15
c.3242T>G p.Ile1081Ser	HD N core	not tolerated	prob /0.998	C65	detrimental	0	1 CH	U	-	3 ; UV	CS
c.3245C>A p.Thr1082Asn	HD N core	not tolerated	prob /0.999	C55	detrimental	0	1 CH	de novo	-	6 ; pathogenic	CS
c.3301T>C p.Cys1101Arg	HD N core	not tolerated	prob /0.998	C65	detrimental	0	2 CH	>1 de novo	-	7 ; pathogenic	12
c.3302G>A p.Cys1101Tyr	HD N core	not tolerated	prob /0.998	C65	detrimental	0	1 CH	U	-	3 ; UV	7/12 (same patient)
c.3340A>T p.Asn1114Tyr	HD N loop surface	not tolerated	prob /0.999	C65	minor	0	1 CH	U	-	1 ; UV	12
c.3607G>C p.Glu1203Gln	HD C core	not tolerated	prob /0.998	C25	minor	0	1 CH	U	-	0.5 ; UV	1
c.3623T>A p.Val1208Asp	HD C core	not tolerated	prob /0.995	C65	detrimental	0	1 CH	U	-	3 ; UV	1
c.3641A>G p.Gln1214Arg	HD C core	not tolerated	prob /0.959	C35	detrimental	0	4 CH	>1 de novo	-	6.5 ; pathogenic	3, 15, CS
c.3751T>C p.Cys1251Arg	HD C core	not tolerated	prob /0.998	C65	detrimental	0	1 CH	U	-	3 ; UV	CS
c.3770T>G p.Leu1257Arg	HD C core	not tolerated	prob /0.997	C65	detrimental	0	1 CH	de novo	-	6 ; pathogenic	7/11/12 (same patient)

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

CHD7 variant[a]	Domain[b]	SIFT[c]	Poly Phen-2[d]	Align-GVGD[e]	Structural model of CHD7[f]	# controls[g]	# index patients[h]	Segregation[i]	Other mutation found in index patient[j]	Total summed score ; classification[k]	References[l]
c.3875T>C p.Leu1292Pro	HD C core	not tolerated	prob /0.997	C65	detrimental	0	1 CH	de novo	-	6 ; pathogenic	CS
c.3881T>C p.Leu1294Pro	HD C core	not tolerated	prob /0.997	C65	detrimental	0	2 CH	de novo	-	6 ; pathogenic	1, 15
c.3905T>C p.Leu1302Pro	HD C core	not tolerated	prob /0.962	C65	detrimental	0	1 CH	de novo	-	6 ; pathogenic	3
c.3949C>T p.Arg1317Cys	HD C surface	not tolerated	prob /0.978	C65	undeterm	0	1 CH	parent carrier	-	2 ; UV	CS
c.3952T>C p.Cys1318Arg	HD C core	not tolerated	prob /0.951	C65	detrimental	0	1 CH	de novo	-	6 ; pathogenic	CS
c.3965T>C p.Leu1322Pro	HD C core	not tolerated	prob /0.976	C65	detrimental	0	1 CH	U	-	3 ; UV	1
c.3973T>G p.Tyr1325Asp	HD C core	not tolerated	prob /0.942	C65	detrimental	0	1 CH	de novo	-	6 ; pathogenic	12
c.4033C>T p.Arg1345Cys	HD C surface*	not tolerated	prob /0.999	C65	detrimental	0	1 CH	parent carrier	-	3 ; UV	1
c.4034G>A p.Arg1345His	HD C surface*	not tolerated	prob /0.999	C25	detrimental	0	1 CH	de novo	-	5.5 ; pathogenic	CS
c.4185G>C p.Gln1395His	HD C ATP binding cleft	not tolerated	prob /0.997	C15	ATP binding	0	2 CH	>1 de novo	-	6.5 ; pathogenic	1, 5
c.4247C>G p.Thr1416Arg	HD C core	not tolerated	prob /0.965	C65	detrimental	0	1 CH	U	-	3 ; UV	1
c.4369A>C p.Lys1457Gln	HD C surface	not tolerated	prob /0.993	C45	minor	0	1 CH	parent carrier	-	1 ; UV	1
c.4406A>G p.Tyr1469Cys	-	not tolerated	prob /0.996	C65		0	1 CH	de novo	-	5 ; pathogenic	12/13 (same patient)
c.4529C>G p.Ala1510Gly	-	not tolerated	poss /0.901	C55		0	1 CH	U	-	1.5 ; UV	16

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

CHD7 variant[a]	Domain[b]	SIFT[c]	Poly Phen-2[d]	Align-GVGD[e]	Structural model of CHD7[f]	# controls[g]	# index patients[h]	Segregation[i]	Other mutation found in index patient[j]	Total summed score ; classification[k]	References[l]
c.4727T>G p.Phe1576Cys	-	not tolerated	benign /0.106	C65		0	1 CH	U	-	1 ; UV	1
c.4774C>T p.Arg1592Trp	-	not tolerated	prob /0.974	C65		0	1 CH	parent carrier	-	2 ; UV	3
c.4780C>T p.Pro1594Ser	-	not tolerated	poss /0.757	C65		0	1 CH	U	+	-1.5 ; benign	CS
c.4787A>G p.Asp1596Gly	-	not tolerated	prob /0.941	C65		0	3 CH	de novo	-	5 ; pathogenic	5, 17, 7/12 (same patient)
c.4849G>A p.Gly1617Ser	-	not tolerated	prob / 0.969	C55		0	1 CH	U	-	2 ; UV	1
c.4850G>A p.Gly1617Asp	-	not tolerated	prob /0.992	C65		0	2 CH	U	-	2 ; UV	CS
c.4856G>T p.Gly1619Val	-	not tolerated	prob /0.972	C65		0	1 CH	U	-	2 ; UV	CS
c.4929C>G p.Cys1643Trp	-	not tolerated	prob /0.992	C65		0	1 CH	U	-	2 ; UV	5
c.5015C>T p.Ala1672Val	-	not tolerated	benign /0.004	C65		1	0	U	-	1 ; UV	dbSNP
c.5050G>A p.Gly1684Ser	-	not tolerated	prob /0.969	C55		0	3 CH	>1 de novo	-	6 ; pathogenic	16, 18, CS
c.5216T>G p.Leu1739Arg	-	not tolerated	benign /0.463	C65		0	1 CH	de novo	-	4 ; pathogenic	1
c.5222G>C p.Arg1741Pro	-	not tolerated	prob /0.972	C65		0	1 CH	de novo	-	5 ; pathogenic	12
c.5225T>A p.Val1742Asp	-	not tolerated	prob /0.962	C65		0	1 CH	de novo	-	5 ; pathogenic	3
c.5234T>C p.Leu1745Pro	-	not tolerated	prob /0.960	C65		0	1 CH	de novo	-	5 ; pathogenic	6
c.5373C>A p.Asp1791Glu	-	not tolerated	prob /0.969	C35		0	1 CH	parent carrier	-	1.5 ; UV	1
c.5390G>T p.Gly1797Val	-	not tolerated	prob /0.998	C65		0	1 CH	de novo	-	5 ; pathogenic	CS
c.5402A>C p.His1801Pro	-	not tolerated	prob /0.935	C65		0	1 CH	de novo	-	5 ; pathogenic	7/12 (same patient)
c.5405G>A p.Gly1802Asp	-	not tolerated	prob /0.990	C65		0	1 CH	de novo	-	5 ; pathogenic	10
c.5434G>C p.Asp1812His	-	not tolerated	prob /0.997	C65		0	1 CH	de novo	-	5 ; pathogenic	CS
c.5435A>G p.Asp1812Gly	-	not tolerated	prob /0.992	C65		0	1 CH	de novo	-	5 ; pathogenic	CS
c.5436C>A p.Asp1812Glu	-	not tolerated	prob /0.978	C35		0	1 CH	de novo	-	4.5 ; pathogenic	7
c.5444T>C p.Leu1815Pro	-	not tolerated	prob /0.984	C65		0	2 CH	de novo	-	5 ; pathogenic	15, CS

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

CHD7 variant[a]	Domain[b]	SIFT[c]	Poly Phen-2[d]	Align-GVGD[e]	Structural model of CHD7[f]	# controls[g]	# index patients[h]	Segregation[i]	Other mutation found in index patient[j]	Total summed score ; classification[k]	References[l]
c.5597A>G p.Asp1866Gly	-	not tolerated	poss /0.856	C0		0	3 CH	de novo	-	3.5 ; UV	1, CS
c.5848G>A p.Ala1950Thr	-	not tolerated	benign /0233	C55		0	1 CH	parent carrier	-	1 ; UV	1
c.5915C>G p.Ala1972Gly	SANT	not tolerated	poss /0.840	C55		0	1 CH	unaffected parent carrier	-	-0.5 ; benign	CS
c.6184C>T p.Arg2062Trp	-	not tolerated	prob /0.990	C65		0	1 CH	U	+	-1 ; benign	CS
c.6194G>A p.Arg2065His	-	not tolerated	prob /0.995	C25		0	2 CH	parent carrier	-	1.5 ; UV	1
c.6221T>C p.Leu2074Pro	-	not tolerated	prob /0.997	C65		0	1 KS/CH	affected sib carrier	-	2 ; UV	CS, 19 (only clinical data were published)
c.6230G>A p.Arg2077Lys	-	not tolerated	poss /0.794	C25		0	1 CLP	parent carrier	-	1 ; UV	6
c.6250A>G p.Ser2084Gly	-	not tolerated	benign /0.032	C55		0	1 CH	U	-	1 ; UV	1
c.6271T>C p.Trp2091Arg	-	not tolerated	prob /0.997	C65		0	1 CH	de novo	-	5 ; pathogenic	CS
c.6287A>G p.His2096Arg	-	not tolerated	prob /0.982	C25		0	2 CH	de novo	-	4.5 ; pathogenic	15, 20
c.6290A>G p.Asp2097Gly	-	not tolerated	prob /0.992	C65		0	1 CH	U	-	2 ; UV	CS
c.6304G>A p.Val2102Ile	-	tolerated/0.18	benign /0.005	C0		0	1 CH	U	-	0 ; benign*	6
c.6308G>A p.Gly2103Asp	-	not tolerated	prob /0.992	C65		0	2 CH	U	-	2 ; UV	1
c.6322G>A p.Gly2108Arg	-	not tolerated	prob /0.999	C65		0	4 CH	>1 de novo	-	6 ; pathogenic	CS, 12/13 (same patient), 21
c.6335C>T p.Thr2112Met	-	not tolerated	prob /0.998	C65		0	1 CLP	parent carrier	-	2 ; UV	6
c.6347T>A p.Ile2116Asn	-	not tolerated	prob /0.993	C65		0	1 KS/CH, 2 CH	>1 de novo	-	6 ; pathogenic	1, 22
c.6352A>G p.Asn2118Asp	-	not tolerated	poss /0.560	C15		0	1 CH	2 family members carrier	-	-1 ; benign	CS
c.6478G>A p.Ala2160Thr	-	tolerated/0.48	benign /0.003	C0		1	3 CH	poss unaffected parent carrier	poss, homo	-7 or -8 ; benign	1, 5, dbSNP

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

CHD7 variant[a]	Domain[b]	SIFT[c]	Poly Phen-2[d]	Align-GVGD[e]	Structural model of CHD7[f]	# controls[g]	# index patients[h]	Segregation[i]	Other mutation found in index patient[j]	Total summed score ; classification[k]	References[l]
c.6673G>A p.Ala2225Thr	-	tolerated/0.33	benign /0	C0		0	1 CH	U	-	0 ; benign*	1
c.6775G>A p.Ala2259Thr	-	not tolerated/0.02	benign /0.001	C0		0	1 CH	parent carrier	-	0 ; benign*	CS
c.6843T>G p.Asp2281Glu	-	not tolerated	benign /0.106	C35		0	1 CH	poss unaffected parent carrier	poss	-1.5 or -2.5 ; benign	5
c.6857G>C p.Gly2286Ala	-	not tolerated	benign /0.095	C55		0	1 CH	de novo	-	4 ; pathogenic	CS
c.6935A>C p.Lys2312Thr	-	not tolerated	poss /0.826	C65		0	1 CH	U	-	1.5 ; UV	CS
c.6955C>A p.Arg2319Ser	-	not tolerated	poss /0.794	C65		0	1 CH	affected parent carrier	-	1.5 ; UV	15
c.6955C>T p.Arg2319Cys	-	not tolerated	prob /0.930	C65		0	6 CH	affected parent and sib carrier	-	2 ; UV	1, 5, 6, 7, 20, 23
c.6989G>C p.Gly2330Ala	-	tolerated/0.09	prob /0.935	C0		6	1 CH	U	-	-1 ; benign	1, dbSNP
c.7097T>G p.Leu2366Arg	-	not tolerated	prob /0.923	C65		0	1 CH	U	-	2 ; UV	CS
c.7243G>T p.Ala2415Ser	-	not tolerated	poss /0.645	C65		2	0	U	-	-0.5 ; benign	dbSNP
c.7390A>G p.Lys2464Glu	-	not tolerated	benign /0.015	C55		0	1 CH	parent carrier	-	1 ; UV	CS
c.7463G>A p.Gly2488Asp	-	not tolerated	poss /0.611	C65		0	1 CH	2 family members carrier	-	-0.5 ; benign	CS
c.7471C>T p.Arg2491Cys	-	not tolerated	benign /0	C65		0	1 CH	U	+	-2 ; benign	CS
c.7485G>T p.Arg2495Ser	-	not tolerated	benign /0.108	C65		0	1 CH	parent carrier	-	1 ; UV	1
c.7579A>C p.Met2527Leu	-	tolerated/0.15	benign /0.064	C0		0	7 CH	parent carrier	-	0 ; benign*	1, CS
c.7958G>A p.Arg2653Gln	BRK2	not tolerated	prob /0.946	C35		0	1 CH	parent carrier	+	-1.5 ; benign	CS
c.8047C>T p.Pro2683Ser	BRK2	not tolerated	prob /0.985	C0		0	1 CH	parent carrier	-	1 ; UV	1
c.8104C>T p.Arg2702Cys	-	not tolerated	prob /0.951	C65		0	1 CH	parent carrier	-	2 ; UV	1
c.8173A>G p.Ile2725Val	-	not tolerated	benign /0.421	C0		1	0	U	-	0 ; benign*	dbSNP

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

CHD7 variant[a]	Domain[b]	SIFT[c]	Poly Phen-2[d]	Align-GVGD[e]	Structural model of CHD7[f]	# controls[g]	# index patients[h]	Segregation[i]	Other mutation found in index patient[j]	Total summed score ; classification[k]	References[l]
c.8197G>A p.Ala2733Thr	-	not tolerated	poss /0.824	C0		0	1 CH	U	-	0.5 ; UV	1
c.8250T>G p.Phe2750Leu	-	not tolerated	poss /0.623	C15		18	0	U	-	-1 ; benign	dbSNP
c.8339C>T p.Ala2780Val	-	not tolerated	benign /0.095	C65		0	1 CH	3 family members carrier	-	-1 ; benign	CS
c.8365G>A p.Ala2789Thr	-	tolerated/0.26	benign /0.082	C0		0	1 HH	U	-	0 ; benign*	2
c.8416C>G p.Leu2806Val	-	not tolerated	poss /0.726	C25		0	5 CH	poss unaffected parent carrier	poss	-1 or -2 ; benign	1, 5, CS
c.8569T>G p.Ser2857Ala	-	tolerated/0.13	benign /0.014	C0		0	1 CH	U	-	0 ; benign*	1
c.8639C>T p.Pro2880Leu	-	not tolerated/0.02	benign /0.015	C15		0	1 HH	U	-	0.5 ; UV	2
c.8791G>A p.Val2931Met	-	not tolerated	benign /0.342	C0		0	1 CH	parent carrier	-	0 ; benign*	1
c.8842A>G p.Lys2948Glu	-	not tolerated/0.05	benign /0.004	C0		0	1 KS	U	-	0 ; benign*	2
c.8950C>T p.Leu2984Phe	-	not tolerated	benign /0.314	C0		0	4 CH	unaffected parent carrier	+	-5 ; benign	1, 7/12 (same patient)

White rows, possible effect on splicing

[a] The GenBank accession number NM_017780.2 was used as reference sequence for the *CHD7* gene. The A of ATG was designated number 1. The intron sequences of the *CHD7* gene can be found in NG_007009.1.

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

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

[d] Polymorphism Phenotyping (PolyPhen-2) output: benign; poss, possibly damaging; prob, probably damaging; naïve Bayes posterior probability that a certain variant is damaging

[e] Align-GVGD output: C0, benign; C15/C25/C35, possibly pathogenic; C45/C55/C65, probably pathogenic

[f] structural model of CHD7 predicts the effect on the stability of the protein; minor, minor effect; detrimental, detrimental effect; undeterm, undetermined; ATP binding, variant located in close proximity to the ATP binding site (probably pathogenic)

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

[h] # 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., 2007); CLP, cleft lip and/or palate

[i] 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., 2007)

[j] -, 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

[k] total summed score and classification according to our classification system (Table 2); *, less reliable prediction (no segregation analysis done, or no detailed phenotypic data available)

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

1 Bartels et al., 2010; 2 Kim et al., 2008; 3 Wincent et al., 2008; 4 Gao et al., 2007; 5 Vuorela et al., 2007; 6 Felix et al., 2006; 7 Jongmans et al., 2006; 8 Delahaye et al. 2007; 9 Wessels et al. 2010; 10 Pauli et al., 2011; 11 Vissers et al., 2004; 12 Bergman et al., 2011a; 13 Bergman et al., 2011b; 14 Dauber et al., 2010; 15 Lalani et al., 2006; 16 Asakura et al., 2008; 17 de Arriba Muñoz et al., 2011; 18 Fujita et al., 2009; 19 Levy and Knudtzon, 1993; 20 Feret et al., 2010; 21 Jongmans et al., 2008; 22 Jongmans et al., 2009; 23 Holak et al., 2008

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	Flanagan et al., 2007
Human CHD1 tandem chromodomains	CD1&2	2B2T	28%	2.45	Flanagan et al., 2005
Yeast Chd1 chromodomain	CD2	2DY8	36%	NMR	Okuda et al., 2007
Human CHD4 chromodomain	CD2	2EE1	32%	NMR	**10.2210/pdb2ee1/pdb**
Zebrafish Rad54 SWI2/SNF2 chromatin-remodeling domain	helicase	1Z3I	30%	3	Thoma et al., 2005
Sulfolobus solfataricus SWI2/SNF2 ATPase domain	helicase	1Z6A	29%	3	Durr et al., 2005
Yeast Chd1 chromatin remodeler	CD1&2, helicase	3MWY	39%	3.7	Hauk et al., 2010

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

CHAPTER 2.3

Exome sequencing in *CHD7* negative CHARGE patients

Nicole Corsten-Janssen, Krista K. van Dijk, Xuanzhu Liu, Jianguo Zhang, Lies Hoefsloot, Cleo C. van Diemen, Conny M.A. van Ravenswaaij-Arts

ABSTRACT

CHD7 mutations are known to be the major cause of CHARGE syndrome. However, in 5-10% of patients with clinically typical CHARGE syndrome, no pathogenic *CHD7* variants are identified. We performed exome sequencing in five clinically typical CHARGE patients in whom no pathogenic *CHD7* variant was previously detected using standard *CHD7* analysis in a diagnostic setting. In four (80%) of these patients, we identified probably pathogenic *CHD7* variants, while the fifth patient's phenotype is most likely explained by her 22q11.2 deletion. The pathogenic *CHD7* variants had been missed in the diagnostic setting due to the use of heteroduplex mutation screening or ignorance of variants in flanking intronic regions. We therefore conclude that *CHD7* is the major causal gene in CHARGE syndrome and that *CHD7* sequencing of all exons including the 20 base pairs flanking intronic regions should be performed or re-analyzed in every patient with clinically typical CHARGE syndrome.

Keywords: CHD7, CHARGE syndrome, exome sequencing

INTRODUCTION

CHARGE syndrome (MIM 214800) is a variable, multiple congenital malformation syndrome. The diagnosis of CHARGE syndrome can be made using clinical diagnostic criteria, which include coloboma, choanal atresia, cranial nerve defects, typical ear anomalies and semicircular canal defects as major features.[1,2]

Pathogenic variants in the *CHD7* gene (MIM 608892) are known to cause CHARGE syndrome by haploinsufficiency.[3] Indeed, most mutations identified in *CHD7* are truncating nonsense and frameshift mutations.[4] However, in 5% to 10% of clinically typical CHARGE patients, no *CHD7* mutation or deletion is identified.[5] The current idea is that these patients might have non-detectable *CHD7* variants (e.g. occurring in intronic regions or regulatory elements) or mutations in other genes that cause an overlapping phenotype. The only other gene that has been described to be involved in CHARGE syndrome is the *SEMA3E* gene (MIM 608166). This gene was shown to be disrupted in two CHARGE patients in 2004, but no mutations of this kind have been described since.[6]

PATIENTS AND METHODS

Patients

We used whole exome sequencing to identify the cause of clinically typical CHARGE syndrome in five unrelated patients declared to be without a *CHD7* mutation or deletion upon routine diagnostics (i.e. Sanger sequencing and MLPA, respectively). Four of these patients fulfilled the criteria of Blake and/or Verloes.[1,2] One patient was included because of her typical external ear anomaly in combination with semicircular canal defects. A summary of the phenotype of all patients is given in Table 1. Diagnostic *CHD7* analysis had been previously performed using heteroduplex analysis for mutation screening in two patients, complete Sanger sequencing in three patients and MLPA in all patients, without mutations being identified.[5,7] Array CGH was performed in four patients and, in patient 5, it revealed a typical 22q11.2 deletion causing 22q11.2 deletion syndrome.[8] We chose to include patient 5 in this study because of her typical CHARGE features which included semicircular canal defects, coloboma and a choanal stenosis. All the patients or their legal representatives gave informed consent for exome sequencing and the collection of clinical data.

Table 1. Clinical characteristics of the patients

Patient	1	2	3	4	5
Sex	F	F	M	M	F
Coloboma	+	-	+	+	+
Choanal anomaly	+	-	CLP	CLP	+
Typical external ear anomaly	?	+	+	+	-
Hearing loss	+	+	+	-	+
Cranial nerve defect	VIII	I	I, VIII	I	-
Abnormal semicircular canals	?	+	+	+	+
Heart defect	+	-	-	+	+
Developmental delay/ intellectual disability	+	+	+	+	+
Genital hypoplasia	+	-	+	-	?
Short stature	-	+	-	+	-
Array results		Normal[1]	Normal[1]	Normal[2]	22q11.2 deletion (3Mb)[3]

+, present; -, absent; CLP, cleft lip and/or palate; F, female; M, male, I, olfactory nerve; VIII, vestibulocochlear nerve.
Genital hypoplasia: present if patient has cryptorchism, micropenis or hypogonadotropic hypogonadism.
Array results:
[1] Agilent 105 K custom HD-DGH microarray; Oxford design (AMADID-nr. 019015)
[2] Affymetrix 250k SNP array
[3] Agilent 180 K custom HD-DGH microarray; Oxford design (AMADID-nr. 023363)

Exome sequencing

Patient DNA was already available from previous blood samples and was isolated using standardized procedures. DNA was enriched using the Agilent SureSelect Human All Exon V3 system. High-throughput sequencing was performed for each captured library on a Hiseq2000 platform. Raw image files were processed by base-calling using Illumina basecalling Software 1.7, with default parameters and the sequences of each individual generated as 90bp pair-end reads. The quality and pollution by adapter was first checked. Data were mapped against the human reference genome (NCBI build37.1). SNPs were called using software SOAPaligner and software SOAPsnp (http://soap.genomics.org.cn/). Indels were called using BWA (http://bio-bwa.sourceforge.net/).

For variant filtering, we assumed that the mutation was present at a very low frequency in public variant databases (≤0.5%, filtered against dbSNP135, 1000 genome database, HapMap database) and at a low frequency in BGI in-house

databases (<10%, including >1,000 samples). We first checked variants in *CHD7* and *SEMA3E*. The data available from diagnostic *CHD7* analysis were re-analyzed in all five patients. Potential pathogenic variants detected by exome sequencing were Sanger sequenced to exclude false positive results.

RESULTS

The mean sequencing depth of the target regions was between 71x and 80x and a minimum of 83% of the target regions of each patient was covered at least 20x. In total, exome sequencing identified six heterozygous variants in the *CHD7* gene in the five patients (see Table 2). Sanger sequencing confirmed five of the variants, while the variant in patient 5 appeared to be a false positive finding. No obvious pathogenic variants were identified in the *SEMA3E* gene.

The missense variant c.4787A>G in exon 21 identified in patient 1 leads to the substitution of an aspartic acid into a glycine at position 1596 of the CHD7 protein. This missense variant has already been described in three CHARGE patients (www.CHD7.org). It is classified as probably pathogenic using the previously published classification system to analyze the pathogenicity of *CHD7* missense variants that combines data from computational algorithms with clinical and segregation data.[9] This missense variant was missed in a diagnostic setting because heteroduplex was used as a screening method and this was apparently not sensitive enough to detect this mutation.

The variant c.5405-17G>A identified in intron 25 of patient 2 was not an obvious candidate after filtering the exome data, although the variant was validated. However, it was identified as a pathogenic variant after the re-analysis of the diagnostically available *CHD7* data. The variant has been reported as occurring *de novo* in at least four other CHARGE patients (www.CHD7.org). It has been shown to create a cryptic splice site through which five codons are inserted into the cDNA in-frame.[10]

The nonsense variant in exon 2 of patient 3 is one of the recurrent arginine to stop mutations of the *CHD7* gene. It has been identified in at least 12 CHARGE patients, with a *de novo* occurrence in several of them (www.CHD7.org). Sanger sequencing confirmed the variant in patient 3. Also in this patient heteroduplex was used for mutation screening in the diagnostic setting and in this way the mutation was missed.

Table 2. CHD7 variants identified by whole exome sequencing (WES)

Patient	CHD7 variant with WES	Sanger sequencing	Segregation	Conclusion
1	c.4787A>G	Confirmed	not maternal*	Probably pathogenic variant
	p.Asp1596Gly			
2	c.5405-17G>A	Confirmed	*de novo*	Pathogenic variant
	p.?			
3	c.1480C>T	Confirmed	unknown	Pathogenic variant
	p.Arg494X			
4	c.6103+8C>T	Confirmed		Benign variant
	p.?			
	c. 5051-15T>A	Confirmed	*de novo*	Probably pathogenic variant
	p.?			
5	c.8097G>A	not confirmed		False positive exome sequencing result
	p.Met2699Ile			

Mutations are numbered according to the current reference sequence (GenBank Accession no. NM_017780.3)
* Father not available.

The variant 6103+8C>T in intron 30 of patient 4 has been identified together with a pathogenic *CHD7* mutation in several CHARGE patients (www.CHD7.org). According to the Exac data from the Exome Aggregation Consortium containing 60,706 unrelated individuals, this variant has a minor allele frequency of 0.07 (dbSNP rs3763592,Version 0.3). It is therefore assumed to be a benign variant. The variant c.5051-15T>A in intron 22 in the same patient has not been previously reported in CHARGE patients or population based databases. Splice site prediction programs predict that this intronic variant creates a new splice acceptor site, but RNA studies have not been performed to prove this functionality. However, *CHD7* analysis of the parents of patient 4 showed that this intronic variant was *de novo*. We therefore conclude that this variant is probably pathogenic.

DISCUSSION

Exome sequencing in our small cohort of five patients with a clinical suspicion of CHARGE syndrome but without a *CHD7* mutation upon routine diagnostics identified pathogenic *CHD7* mutation in 4 out of 5 patients (80%). Moreover, the only patient without a *CHD7* disease-causing variant and clinically typical CHARGE

was diagnosed with a 22q11.2 deletion. This makes it most likely that she has an unusual presentation of the 22q11.2 deletion syndrome (MIM 192430).[8] Our study underscores that *CHD7* is the major causal gene in CHARGE syndrome. It also raises the question of how these *CHD7* mutations were missed in the routine diagnostic analysis of *CHD7*. For patient 1 and 3 the mutation had been missed because *CHD7* analysis was preceded by heteroduplex screening. *CHD7* has a genomic size of 188 kb and consists of 38 exons. Since Sanger sequencing used to be laborious and costly in such large genes, heteroduplex was used as an efficient prescreening method. No Sanger sequencing was performed if the heteroduplex analysis gave only normal signals. However, heteroduplex screening is known to have less than 100% sensitivity.[11] The relevant mutations in patient 1 and 3 were missed because *CHD7* analysis was unfortunately performed during the period of nine months when our diagnostic laboratory used heteroduplex. However, in patient 2 and 4, Sanger sequencing of exons and intron boundaries had been performed. Both intronic variants had been identified in the diagnostic setting, but they were wrongfully classified as non-pathogenic based on the knowledge at that time.

Exome sequencing is a powerful tool to identify pathogenic variants in patients with a well-defined clinical phenotype. The advantage is an unbiased analysis of the data to identify the causal variants. In hindsight, the identification of the pathogenic variants in our four patients would have been more cost-effective if we had reanalyzed their own and their parental *CHD7* genes using traditional Sanger sequencing. However, exome sequencing allows us to search for causal variants in genes presenting with overlapping phenotypes, which is especially useful in atypically presenting patients. As a consequence of the broad phenotypic spectrum of CHARGE syndrome, it has major overlap with other syndromes like Kabuki syndrome (MIM #147920, #300867) and mandibulofacial dysostosis (MIM #610536).[12,13]

In summary, we have confirmed with our small exome sequencing project in five patients that *CHD7* is the major gene causing CHARGE syndrome. In fact, *CHD7* is the only gene that results in clinically typical CHARGE syndrome when mutated. Our study clearly demonstrates that *CHD7* sequencing of all exons including the 20 base pairs flanking intronic regions should be performed in every patient with clinically typical CHARGE syndrome. Targeted reanalysis of *CHD7* is worthwhile when routine diagnostics was performed by heteroduplex screening or flanking intronic regions were not analyzed.

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CHAPTER 3

Phenotype of *CHD7* mutations

CHAPTER 3.1

CHD7 mutations and CHARGE syndrome: the clinical implications of an expanding phenotype

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Adapted from: 'CHD7 mutations and CHARGE syndrome: the clinical implications of an expanding phenotype'. Journal of Medical Genetics 2011; 48(5):334-342. With permission from BMJ Publishing Group.

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 **c**oloboma, congenital **h**eart defects, choanal **a**tresia, **r**etardation of growth and development, **g**enital hypoplasia and **e**ar 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.

Keywords: CHARGE syndrome, CHD7, phenotypic spectrum

INTRODUCTION

The first patients with what later became known as CHARGE syndrome (MIM 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 **c**oloboma, congenital **h**eart defects, choanal **a**tresia, **r**etardation of growth and/or development, **g**enital anomalies, and **e**ar 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.

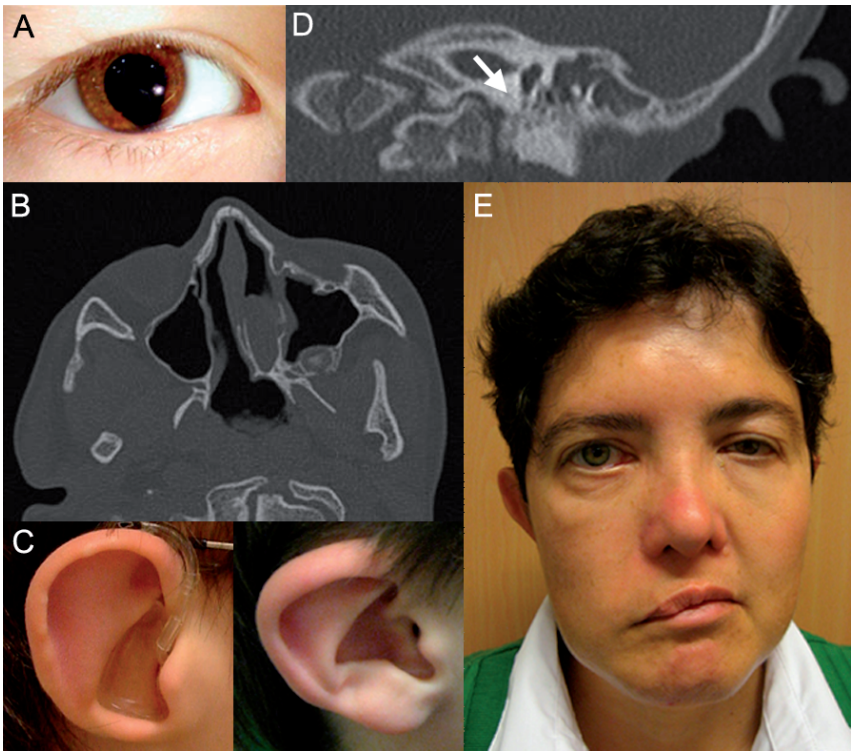
BACKGROUND

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)

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.

Table 1. Clinical criteria for CHARGE syndrome

	Major criteria	Minor criteria	Inclusion rule
Blake[#9]	1. Coloboma, microphthalmia	1. Cardiovascular malformations	Typical CHARGE:
	2. Choanal atresia or stenosis*	2. Tracheo-oesophageal defects	4 major *or*
	3. Characteristic external ear anomaly, middle/inner ear malformations, mixed deafness	3. Genital hypoplasia or delayed pubertal development	3 major + 3 minor
	4. Cranial nerve dysfunction	4. Cleft lip and/or palate	
		5. Developmental delay	
		6. Growth retardation	
		7. Characteristic face	
Verloes[10]	1. Ocular coloboma	1. Heart or oesophagus malformation	Typical CHARGE:
	2. Choanal atresia	2. Malformation of the middle or external ear	3 major *or*
	3. Hypoplastic semicircular canals	3. Rhombencephalic dysfunction including sensorineural deafness	2 major + 2 minor
		4. Hypothalamo-hypophyseal dysfunction (gonadotropin or growth hormone deficiency)	
		5. Mental retardation	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]

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 **C**hromodomain **H**elicase **D**NA 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 (**p**olybromo- and **B**RG1-**a**ssociated **f**actor 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]

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]

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

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 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 C*HD7*-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).

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]

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 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

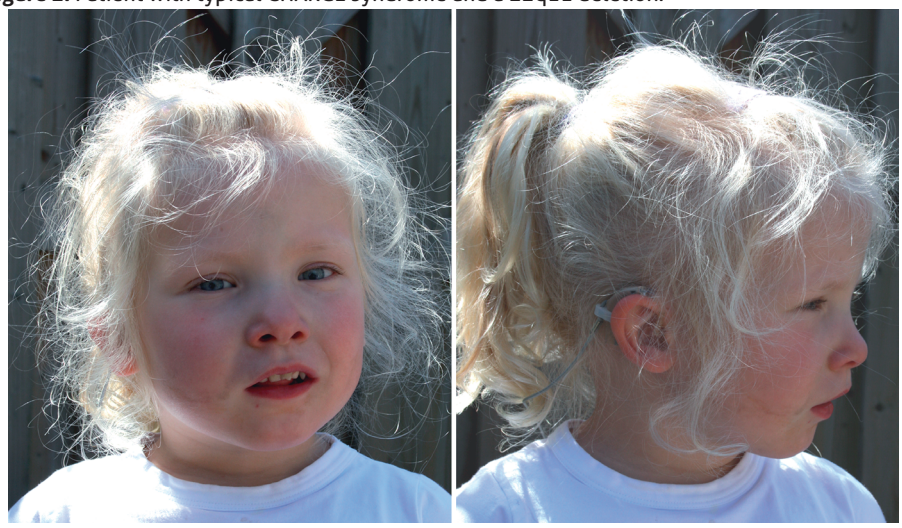
Some authors have undertaken *CHD7* screening in patients with only one CHARGE syndrome feature - e.g., cleft lip and/or palate,[46] congenital heart disease,[47] or scoliosis.[48] These studies did not identify pathogenic *CHD7* mutations. The general impression is that in the absence of other CHARGE 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 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]

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* 3Mb 22q11.2 loss, suggestive for the typical DiGeorge/velocardiofacial syndrome deletion.
Informed consent was obtained for publication of the photographs.

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] 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?

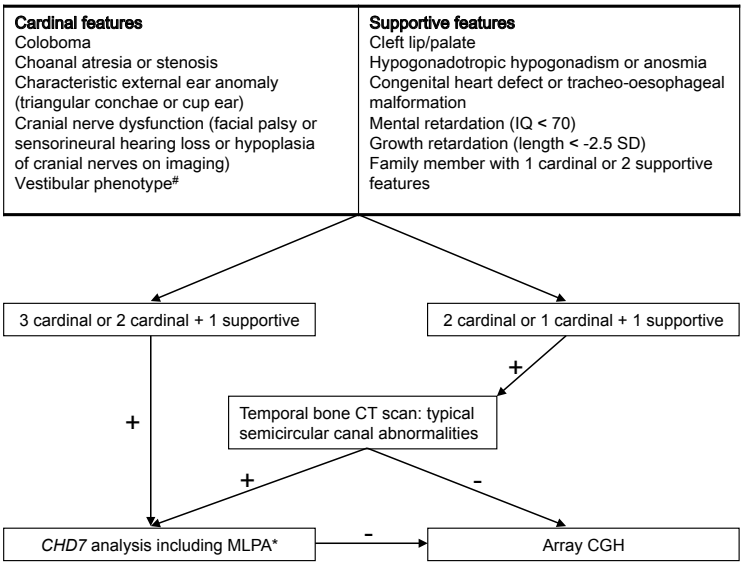
In our opinion, CHARGE syndrome is primarily a clinical diagnosis. If patients fulfil the clinical criteria of Blake or Verloes and chromosomal aberrations and teratogenic exposure effects fully explaining the clinical features have been ruled out, then they have CHARGE syndrome, irrespective of the 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	

Table 4. Clinical surveillance of patients with a *CHD7* mutation (continued)

Evaluation	Tests	Treatment/advice	Be aware of
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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CHAPTER 4

CHD7 and the heart

CHAPTER 4.1

The cardiac phenotype in patients with a *CHD7* mutation

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ABSTRACT

Background: Loss-of-function mutations in *CHD7* cause CHARGE syndrome, a variable combination of multiple congenital malformations including heart defects. Heart defects are reported in 66%-92% of patients with a *CHD7* mutation, but most studies are small and do not provide a detailed classification of the defects. We present the first, detailed, descriptive study on the cardiac phenotype of 299 patients with a *CHD7* mutation and discuss the role of CHD7 in cardiac development.

Methods and Results: We have collected information on congenital heart defects in 299 patients with a pathogenic *CHD7* mutation of whom 220 (74%) had a congenital heart defect. Detailed information on the heart defects was available for 202 of these patients. We classified the heart defects based on embryonic cardiac development and compared the distribution to 1,007 equally classified non-syndromic heart defects of patients registered by EUROCAT, a European Registry of Congenital Anomalies. Heart defects are highly variable in patients with *CHD7* mutations, but atrioventricular septal defects (AVSD) and conotruncal heart defects are overrepresented. Gender did not have an effect on the presence of heart defects, but truncating *CHD7* mutations resulted in a heart defect significantly more often than missense or splice-site mutations (chi-square, $p<0.001$).

Conclusions: CHD7 plays a very important role in cardiac development because we found a wide range of heart defects in 74% of a large cohort of patients with a CHD7 mutation. Conotruncal defects and AVSDs are overrepresented in patients with *CHD7* mutations compared to patients with non-syndromic heart defects.

Keywords: Heart defects, congenital; CHARGE syndrome; *CHD7* gene; genetics; neural crest cells

INTRODUCTION

Congenital heart defects have a high birth prevalence up to 7 per 1,000 live births, and may occur in combination with non-cardiac congenital anomalies.[1] The current hypothesis is that most heart defects without non-cardiac anomalies, referred to as non-syndromic heart defects, are due to a combination of environmental and genetic factors.[2] In contrast, heart defects with non-cardiac congenital malformations that are also referred to as syndromic heart defects are more often associated genetic factors. One of syndromes that cause heart defects and has a known genetic cause is CHARGE syndrome (MIM 214800).

Congenital heart defects occur in 75-80% of the patients clinically diagnosed with CHARGE syndrome.[3] CHARGE syndrome is a highly variable combination of multiple congenital malformations with an incidence between 1 in 15,000 to 17,000 newborns.[4-6] The acronym stands for **C**oloboma, **H**eart disease, Choanal **a**tresia, **R**etardation of growth and/or development, **G**enital hypoplasia and **E**ar abnormalities with or without deafness.[7] Additional major features are cranial nerve defects like anosmia or facial palsy and inner ear defects including abnormalities of the semicircular canals.[8,9] The clinical diagnosis of CHARGE syndrome can be made when the criteria of either Blake et al[8] and/or Verloes[9] are met. In 2004 loss-of-function mutations in the *CHD7* gene (MIM 608892, **C**hromodomain **H**elicase **D**NA-binding protein 7) were identified as the major cause of CHARGE syndrome.[10] CHARGE syndrome is usually a sporadic condition, due to *de novo* mutations, although it is rarely transmitted as an autosomal dominant disease.[5] Over 90% of the patients that fulfill the clinical criteria of CHARGE syndrome have a mutation in the *CHD7* gene, but *CHD7* mutations can be found in atypical patients as well; at least 14-17% of the patients with a *CHD7* mutation do not fulfill the clinical diagnostic criteria, and are referred to as atypical CHARGE syndrome.[5] However, until now every patient with a pathogenic *CHD7* mutation has at least two main clinical features of CHARGE syndrome at careful clinical evaluation.[5] In this study we will focus on the heart defects in patients with a pathogenic *CHD7* mutation, irrespective of their accompanying clinical features as part of their typical or atypical CHARGE syndrome.

Before the identification of *CHD7*, two studies focused specifically on the cardiac phenotype in a group of patients with the clinical diagnosis of CHARGE syndrome.[11,12] Both studies identified conotruncal heart defects as a common heart anomaly in this syndrome. Arch vessel anomalies and atrioventricular septal defects (AVSD) were overrepresented in one of the studies.[11] However, at that time,

the *CHD7* gene had not been discovered and the diagnosis of CHARGE syndrome was hampered by its highly variable clinical presentation. As a consequence, these studies are liable to ascertainment bias towards the more severe end of the clinical spectrum. Thus, studies on prevalence and phenotype should preferably be performed in patients with a proven *CHD7* mutation. Since *CHD7* was identified as a cause of CHARGE syndrome, five other groups have looked at the phenotype in patients with a *CHD7* mutation, revealing a wide range of prevalence of congenital heart defects from 70% to 92%. However, these were small studies containing at most 60 patients, and the studies did not focus on classifying the heart defects.[13-17]

We present the first, detailed, descriptive study on cardiac phenotype in 299 patients with a proven pathogenic *CHD7* mutation, using a classification system based on current developmental and epidemiological insights. We also compare our results with 1,007 non-syndromic heart defects, and discuss the role of *CHD7* in embryonic cardiac development.

POPULATION, MATERIAL AND METHODS

Population and data collection

We set out to collect detailed clinical information on 344 patients with a pathogenic *CHD7* mutation, irrespective of whether they were known to have a congenital heart defect or not. These patients were referred for *CHD7*-analysis on a diagnostic basis to the DNA laboratory in Nijmegen, the Netherlands, between 2004 and 2009, because their local doctors suspected them of CHARGE syndrome. *CHD7*-analysis was performed using methods as described previously.[18] Patients were derived from the Netherlands (34%) and other European countries (54%), but also from Northern America (6%) and other continents (6%). Detailed information on the congenital heart defects was collected via the local doctors or via our Dutch outpatient clinic for CHARGE syndrome. We used a datasheet that included questions on all the possible clinical features of CHARGE syndrome, but with a special focus on the cardiac evaluation and, if present, documentation of the congenital heart defect, age at diagnosis and any heart surgery. We studied reports of cardiac ultrasound, reports of cardiac surgery, autopsy reports and the medical chart if available.

Written informed consent for the collection of medical information was obtained from all patients or their legal representatives. The accredited Medical Ethics Review Committee of the University Medical Center Groningen waived full ethical

evaluation, since, according to Dutch guidelines, no ethical approval is necessary if medical information that was already available is used anonymously and no extra tests have to be performed.

Classification

All heart defects due to *CHD7* mutations were classified by two pediatric cardiologists (L.K. and G.J.D.M.S) using Botto's embryonic development- and epidemiology-based classification system for congenital heart defects.[19] Since patent ductus arteriosus (PDA) and arch vessel anomalies cannot be included in Botto's classification, we added these defects using the overlapping Society of Thoracic Surgeons' (STS) classification modified by Riehle-Collarusso.[20] Both classification systems are made up of three levels: a detailed, a main, and a large level. On the detailed level the heart defects are based on the **I**nternational **P**ediatric and **C**ongenital **C**ardiac **C**odes (IPCCC) and described as specifically as possible. On the main level, some detailed heart defects are grouped together, like the different types of atrioventricular septal defects (AVSD). On the large level, different defects are further combined based on their developmental origin. For example, double outlet right ventricle (DORV), tetralogy of Fallot (TOF) and truncus arteriosus (TA) are grouped together within the large level of conotruncal. We aimed to end up with one large level defect for each patient, whenever possible. When we did end up with two large level defects, each large level defect was counted separately, so the total number of heart defects at the large level exceeds the number of patients.

Patent ductus arteriosus and intra-atrial shunts were considered as a heart defect if they persisted beyond the age of 6 months or if surgery was indicated.

Control group

We compared the distribution of the cardiac phenotypes to that of 1,007 cases with non-syndromic congenital heart defects collected for a regional population-based birth defects registry (EUROCAT Northern Netherlands) from 1997 to 2008. This database holds detailed information on pregnancy outcome and maternal characteristics of more than 80% of all live births, stillbirths and terminations of pregnancy with congenital anomalies that are born in the Northern Netherlands. Data collection for this registry, and specifically for the registration of congenital heart anomalies, has been described in detail by Baardman and colleagues.[21] All cases with non-syndromic congenital heart defect did not have other congenital anomalies or abnormal genetic test (if performed).

Statistical methods

We compared gender and mutation type between the *CHD7*-mutated patients with and without a heart defect using the chi-square test in SPSS PAW Statistics 18. The chi-square test was also used to compare the cardiac phenotypes in patients with a *CHD7* mutation to those in the EUROCAT group with a non-syndromic congenital heart defect. The significance level was set at p=0.05.

RESULTS

We were able to collect information on heart defects in 299 of the 344 live born patients (87%) with a *CHD7* mutation: 165 males (55%) and 134 females (45%). Of these patients, 47 (16%) died postnatally (median age 1 month). Congenital heart defects were present in 220 of the 299 patients (74%): 115 males (52%) and 105 females (48%). The prevalence of heart defects in male and female *CHD7* mutation carriers was thus 70% and 78%, respectively (p=0.09, Table 1). To correct for incomplete data we also calculated the prevalence range, by correcting for the 45 patients out of the 344 patients for whom we had no data (non-responders). If we assume that none or all of these 45 patients had a heart defect, respectively, the prevalence ranges from 64% (=220/344) to 77% (=265/344). The prevalence ranges from 60% (=115/191) to 74% (=141/191) for males, and from 69% (=105/153) to 81% (=124/153) for females.[5]

Table 1. Distribution of heart defects between gender and mutation type

	Gender		Mutation type*	
	Male	Female	Truncating	Non-truncating
Heart defect	115	105	172	48
No heart defect	50	29	44	35
	p = 0.09†		p = 0.000†	
Unknown‡	26	19	30	15

* Truncating mutations include nonsense mutations, frame shift mutations and deletion non-truncating mutations are missense mutations and splice site mutation.

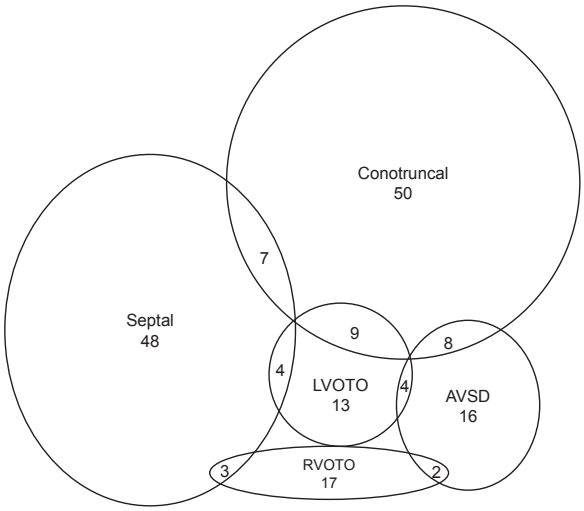
† p-value based on chi-square test.

‡ The male:female distribution in the unknown group (1.4:1) did not deviate significantly from the patients with available information on heart defects (1.2:1). The same is true for the truncation:non-truncation mutation ratios (2.0:1 and 2.6:1, respectively).

The type of *CHD7* mutations in the 299 patients were mostly nonsense (38%) and frame shift mutations (33%), while missense mutations (12%), splice site mutations (16%) and deletions (1%) also occurred. Heart defects were found more frequently in patients with truncating mutations and deletions (80%) than in patients with missense and splice site mutations (58%) ($p<0.001$, Table 1). Of the 45 patients in whom heart defects were not known 30 had truncating mutations and 15 had missense or splice site mutations. Thus the corrected prevalence of heart defects ranges from 70% -82% for the group with a truncating mutation or deletion, and from 49%-64% for the patients with missense and splice site mutations.

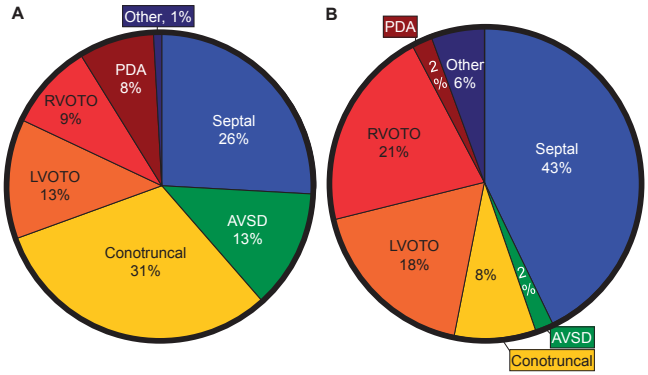
We had sufficient information for classifying the heart defects in 202 of the 220 (92%) patients. The classification showed that the cardiac phenotypes in our cohort were variable, with almost all groups of the classification system being represented. None of the patients had heterotaxy or cardiomyopathy. Conotruncal heart defects, septal defects, AVSD, LVOTO and RVOTO occurred both isolated and in combination with other large level defects (Figure 1). The most common large level heart defects were conotruncal defects, septal defects and AVSD (Figure 2).

Figure 1. Overlapping classification of heart defects at the large level in patients with *CHD7* mutations.



The congenital heart defects of the patients with a *CHD7* mutation that were classified in more than one category are shown at the larger level of the classification system. The groups Other (n = 2) and PDA (patent ductus arteriosus, n = 19) were not included, because these heart defects were only classified in one category.

Figure 2. Distribution of congenital heart defects caused by *CHD7* mutations and of non-syndromic heart defects from the EUROCAT registry.



The distributions of (A) 239 congenital heart defects in a group of 202 patients with a *CHD7* mutation, and (B) 1,007 non-syndromic congenital heart defects collected by EUROCAT, using the classification of Botto et al.[19] The cardiac phenotypes differ significantly in both groups ($p < 0.001$). AVSD, conotruncal defects and PDA are over-represented in patients with *CHD7* mutations compared to patients with non-syndromic heart defects.
AVSD, atrioventricular septal defects; LVOTO, left ventricular outflow tract obstruction; PDA, patent ductus arteriosus; RVOTO, right ventricular outflow tract obstruction.

Among the conotruncal defects, we found different main level heart defects (Supp. Table S1). Many were arch vessel anomalies, either isolated (n = 15) or in combination with other conotruncal and/or other heart defects (n = 25). Examples of arch vessel anomalies were an aberrant subclavian artery (n = 18) or a right aortic arch (n = 19). A TOF was relatively frequent (n = 23), while interrupted aortic arch (IAA, n = 3), DORV (n = 7), transposition of the great arteries (TGA, n = 5) and TA (n = 3) were less frequently seen.

We had information about cardiac surgery for 139 patients: 88 (63%) had undergone cardiac surgery, while in three surgery was not yet necessary and twelve patients had died before the operation could be performed. Thus, in 36 patients (26%) with a heart defect, cardiac surgery was not necessary.

Comparing the cardiac phenotype of *CHD7* mutation to the non-syndromic heart defects at the large level showed that the type of heart defects differed significantly between both groups ($p < 0.001$) (Figure 2). AVSD (13% vs. 2%), conotruncal defects (31% vs. 8%) and PDA (8% vs. 2%) were over-represented in the *CHD7* mutation group (Figure 2). At the main level, significantly more arch

vessel anomalies (40/202 vs. 3/1007, p < 0.001) and TOF (23/202 vs. 49/1007, p < 0,001) were present in patients with a *CHD7* mutation.

DISCUSSION

This study suggests that CHD7 is very important in cardiac development since heart defects have a remarkably high penetrance in patients with pathogenic *CHD7* mutations of 74% (range: 64-77%). The type of heart defects in these patients is variable. Heart defects occur equally in men and women, but are seen significantly more often in patients with a truncating *CHD7* mutation.

The main strength of our study is the size of our patient cohort. Previous phenotypic studies on patients with a *CHD7* mutation by other groups were based on 131 patients in total only, while our results are based on a large group of 299 patients.[13-17] This is also the first study which has specifically focused on heart defects in patients with a *CHD7* mutation and used an embryonic development-based classification system to classify the heart defects in these patients. We classified the heart defects as accurately as possible, asking for original reports of cardiac ultrasound and surgery; however, these were not always available. In approximately 60% of the cases we had to base our classification of the defect on the description given by the physician who requested the *CHD7* mutation analysis, who was not usually the cardiologist. This lack of information might have resulted in a reporting bias and, in particular, the description of heart defects at the detailed level of the classification system might be incomplete. Such a reporting bias probably had less effect at the main and the large levels, where the defects were combined into broader, less-specific groups based on their developmental origin, and most errors at the detailed level would have been filtered out.

In studies like this, we should always be aware of an ascertainment bias due to underreporting of patients without a heart defect. We tried to minimize this by stating in the patient information material that we were mainly, but not solely, interested in cardiac defects and we explicitly stated that in order to estimate an accurate occurrence of heart defects, it was very important for us to be sent information on patients without a heart defect. Nonetheless, we also calculated prevalence ranges assuming that none and all of the 45 non-responding patients had a heart defect. The prevalence of heart defects found (74%, range 64-77%) closely resembles the prevalence of 77% Zentner et al. reported in their review of all 254 patients with *CHD7* mutations that were described in literature earlier, but

within the lower end of the range of reported prevalences (70-92%) in patients with a *CHD7* mutation reported in other original phenotypic studies.[4,13-17,22] This is most likely due to a referral bias towards the more severe end of the clinical spectrum in previous studies, i.e. mildly affected patients being less likely referred for *CHD7* analysis. Although this bias can never be ruled out completely, the unbiased nature of our patient cohort is reflected by the low mutation detection rate in our centre of 41%,[6] indicating a low threshold for performing *CHD7* analysis and thus avoiding a bias to the severe end of the clinical spectrum.

We compared our data on cardiac phenotypes in patients with a *CHD7* mutation to data on non-syndromic heart defects collected by the regional, population-based, birth defects registry, EUROCAT Northern Netherlands (Figure 2). The patients with a non-syndromic heart defect all originated from the Netherlands, while our data from patients with a *CHD7* mutation were collected internationally (see methods). In other, more internationally-based, registries on heart defects the prevalence data on cardiac phenotype show a major overlap with the Dutch EUROCAT data,[1] so we feel our control population is representative. Although none of the patients in our control population had been screened for *CHD7* mutations, they were all known to have a heart defect without anomalies in other organ systems. As mentioned before, thus far no *CHD7* mutations have been detected in patients presenting with only one CHARGE feature, including patients with isolated heart defects.[23] Therefore it is not likely that our control cohort is enriched for *CHD7* mutations. Furthermore, we can extract from the prevalence data on *CHD7* mutations (1:15,000-17,000),[6] and isolated heart defects (1:140)[1] that at the most 1% of our control cohort will have a pathogenic *CHD7* mutation. This will hardly influence the classification of the heart defects in our control cohort. In order to fully exclude the contribution of *CHD7* mutations to isolated (conotruncal) heart defects large series of patients should be sequenced.

A major advantage of our control population is that we were able to select exclusively non-syndromic patients with heart defects and that the heart defects could be classified using the same development-based system. The response rate for the Dutch EUROCAT registry is high, 80%, but nonetheless, there may be a reporting bias. Mild heart defects may be under-reported in the registry, because they become apparent later in life (after the age of 16 years, which is the reporting limit for this registry) or remain undetected. Thus, in the group of non-syndromic heart defects PDA, septal defects, and some arch vessel anomalies might be underrepresented compared to patients with a *CHD7* mutation. In contrast to the general population, patients with a *CHD7* mutation will all undergo extensive cardiac ex-

amination irrespective of their cardiac symptoms. The higher frequency of PDAs, in 8% of the heart defects caused by *CHD7* mutations versus 2% in non-syndromic heart defects of the EUROCAT registry, is thus most likely a reporting artifact of the registry. In contrast, the higher frequency of septal defects in the EUROCAT data (43%) compared to the *CHD7* mutation carriers (24%) is in this respect remarkable, since, just as for PDAs, the prevalence of septal defects in the EUROCAT registry is likely to be underestimated. Also, the difference in prevalence of arch vessel anomalies between the *CHD7* patients (40/202=20%) and the EUROCAT group (3/1007=0.3%) is so large that it cannot be explained by the difference in method of data collection alone.

The cardiac phenotypes in patients with a proven *CHD7* mutation are very similar to those previously reported in CHARGE patients with a heart defect whose *CHD7* status is unknown; conotruncal defects are the most common heart defect, followed by AVSD, arch vessel anomalies, and PDA.[11,12] These results are not surprising, since most clinically typical CHARGE patients have a *CHD7* mutation. Although AVSD and conotruncal defects are overrepresented in CHARGE syndrome, the heart defects among *CHD7* mutation carries are very variable, with all types being represented (Figure 2). For clinical practice this means that one should always be aware of other features of CHARGE syndrome, like coloboma, choanal atresia, cranial nerve dysfunction, balance problems, characteristic ear anomalies (triangular conchae or cup ear) or hypogonatropic hypogonadism, in patients with congenital heart defects especially in patients with AVSD and conotruncal defects.[5,24]

Several types of heart defects are found in animal models with heterozygous *Chd7* mutations: VSDs in adult mice,[25,26] VSDs and defects of the pharyngeal arch arteries in mouse embryos,[27] and abnormal positioning of the truncus arteriosus and cardiac outflow tract in *Xenopus laevis* (African clawed frog).[28] This suggests that CHD7 must be involved in several steps of the cardiac embryogenesis, and especially in the formation of the outflow tract and the atrioventricular cushion. How CHD7 haploinsufficiency exactly causes heart defects is not known and the function of CHD7 in general is only now emerging.

The latest studies show that CHD7 regulates gene expression by enhancer-mediated transcription and ribosomal RNA biogenesis in the nucleolus.[29,30] They also suggest that CHD7 binds to several sites on the DNA with different protein complexes in a tissue- and time-specific manner, regulating various target genes.[29,30] The continuum of influences of CHD7 at different levels and in different cell types could explain the clinical variability seen in different organ systems of individuals

with CHARGE syndrome, including the broad spectrum of heart defects.[31,32] CHD7 probably regulates by chromatin remodeling the expression of cardiac transcription factors.[33,34] The proteins of interest in cardiac development in respect to CHD7 are the ATP-dependent chromatin-remodeling protein complex PBAF and the histone acetyl transferase p300.[33,34] P300 has been shown to co-localize with CHD7 at enhancer elements in mouse embryonic stem cells and PBAF is a protein partner of CHD7 in human neural crest-like cells.[28,29] CHD7 has been shown to be important for neural crest cell migration, and one hypothesis on the pathogenesis of heart defects is that CHARGE syndrome is a neurocristopathy.[28,35]

Cardiac neural crest cells migrate from the neural tube into the caudal pharyngeal arches and a subset migrates into the distal cardiac outflow tract.[36] These cells are known to be important for the development of the pharyngeal arches, the septation of the outflow tract, and closure of parts of the cardiac septum. Ablation of pre-migratory cardiac neural crest cells in animal models results in conotruncal cardiac abnormalities, like persistent truncus arteriosus and malalignment of the outflow tract.[36,37] Whether neural crest cells also contribute to other portions of the heart like the atrioventricular valves is debated.[37,38] Conotruncal defects and AVSD are overrepresented in patients with a *CHD7* mutation, which supports the neural crest hypothesis. However, not all heart defects in patients with *CHD7* mutations can be explained by neural crest cell involvement such as hypoplastic left heart syndrome and ASD. Moreover, rescue of Chd7 expression in neural crest cells of heterozygous mouse embryos did not rescue the defects of the pharyngeal arch arteries, while rescue of Chd7 expression in both neural crest cells and the pharyngeal ectoderm led to a normal phenotype of the pharyngeal arch arteries.[27] Thus, CHD7 has an effect on cardiac development via other cardiac cell types as well. Irrespective of these observations, CHD7 seems to be involved in the signaling pathways that regulate the migration and/or differentiation of cardiac neural crest cells during cardiac development.

Further research is necessary to fully understand how CHD7 effects cardiac development and which other genes are involved in this pathway. What can be concluded from the functional and clinical studies is that the level of CHD7 must be strictly regulated for normal development, including cardiac development. This is supported by our finding that patients with a truncating mutation in *CHD7* more often have a congenital heart defect than patients with a less detrimental, missense or splice site mutation. Nonetheless, in patients with the same mutation, phenotypes differ strikingly, even in monozygotic twins.[13,16,22] In our cohort, patients with the same mutation had different heart defects within the different

groups of the development-based classification. So, the clinical variability is not explained by the type of *CHD7* mutation alone in line with the multi-variable effect of CHD7 mentioned above which changes during development and between tissues.

In conclusion, our prevalence of heart defects in 74% of the patients with *CHD7* mutation show, that the CHD7 protein is very important for cardiac development. Haploinsufficiency, especially due to truncating mutations, results in variable heart defects with a relative over-representation of AVSDs and conotruncal defects, supporting a potential role of CHD7 in the migration and/or differentiation of neural crest cells in the developing heart. However, the variability of the heart defects suggests a pleiotropic effect of *CHD7* mutations, which is not only attributable to a defect of the neural crest cells' lineage.

Unraveling the role of the *CHD7* gene in the developing heart and identifying the signaling pathways influenced by CHD7, could significantly contribute to our knowledge on the mechanisms playing a role in congenital heart disease, which is one of the most frequent congenital anomalies seen in humans. For clinical practice, we advice cardiologist to be aware of other features of CHARGE syndrome in patients with congenital heart defects, especially if the patient has an AVSD or conotruncal defect.

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Classification of congenital heart defects in 202 patients with a CHD7 mutation at Botto's[1] main and large levels.

Large level	N	Main level	N
Conotruncal	50	AVA	15
		DORV	1
		DORV-other	3
		Fallot	16
		Fallot/BAV	1
		Fallot/AVA	2
		IAA	1
		IAA/DORV/absent LAVV	1
		PA-VSD (Fallot)	2
		PDA/AVA	3
		TGA	2
		Truncus	2
		Truncus+AVA	1
Septal	48	any ASD	2
		any ASD 2	20
		any ASD 2/dextrocardia	1
		any VSD	13
		any VSD + any ASD	11
		any VSD/PDA	1
PDA	19	PDA	19
RVOTO	17	Ebstein	2
		HRHS	1
		HRHS/ASD 2	1
		PA	1
		PA-IVS	1
		PVS	9
		TA	1
		TA/PVS/HRHS	1
AVSD	16	AVSD	15
		TA/AVSD	1
LVOTO	13	Abn AV	1
		AS	2
		AS /BAV	1
		BAV	1
		Coarctation	2
		HLHS	2
		SAS	2
		SAS/BAV	1
		VSD/Coarctation	1

Supplementary Table S1. Classification of congenital heart defects in 202 patients with a CHD7 mutation at Botto's[1] main and large levels. (continued)

Large level	N	Main level	N
LVOTO/Conotruncal	9	any VSD + any ASD/BAV/AVA	1
		AS/AVA	1
		AS/BAV + Coarctation/AVA	1
		Coarctation/AVA	1
		HLHS/Coarctation/AVA	1
		HLHS/TGA	2
		SAS/AVA	1
		SAS/Coarctation/VSD/AVA	1
AVSD/Conotruncal	8	AVSD/AVA	4
		AVSD/DORV/TGA	1
		AVSD+TOF	2
		IAA/AVSD/AVA/DORV	1
Septal/Conotruncal	7	any ASD 2/AVA	1
		any VSD + any ASD/AVA	2
		any VSD/AVA	1
		ASD/AVA	1
		VSD/AVA	1
		VSD+ASD/AVA	1
AVSD/LVOTO	4	AVSD/Coarctation	4
LVOTO/Septal	4	any ASD/PDA/BAV	1
		any VSD + any ASD/ BAV	1
		AS/any ASD + any VSD	1
		AS/Coarctation/VSD	1
Septal/RVOTO	3	any ASD 2/PVS	1
		any VSD + any ASD/PVS	1
		any VSD/PVS	1
AVSD/RVOTO	2	AVSD/PVS	2
Complex	1	SV/Complex	1
Abnormal cell Growth	1	TAPVR	1

The classifications at the main and large levels of Botto's system[1] are shown for the 202 patients with a congenital heart defect and *CHD7* mutation.

Abn AV, abnormal atrioventricular valve; AS, aortic stenosis; ASD, atrial septal defect; AVA, arch vessel anomaly; AVSD, atrioventricular septal defect; BAV, bicuspid aortic valve; Coarctation, coarctation of the aorta; DORV, double outlet right ventricle; Ebstein, ebstein anomaly; HLHS, hypoplastic left heart syndrome; HRHS, hypoplastic right heart syndrome; IAA, interrupted aortic arch; IVS, intact ventricular septum; LAVV, left atrioventricular valve; LVOTO, left ventricular outflow tract obstruction; PA, pulmonary atresia; PDA, persistant ductus arteriosus; PS, pulmonary stenosis; PVS, pulmonary valve stenosis; RVOTO, right ventricular outflow tract obstruction; SAS, supravalvular aortic stenosis; SV, single ventricle; TA, tricuspid atresia; TAPVR, total anomalous pulmonary venous return; TGA, transposition great arteries; TOF, tetralogy of Fallot; Truncus, truncus arteriosus; VSD, Ventricular septal defect

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CHAPTER 4.2

Congenital arch vessel anomalies in CHARGE syndrome: a frequent feature with risk for co-morbidity

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Adapted from: 'Congenital arch vessel anomalies in CHARGE syndrome: a frequent feature with risk for co-morbidity'. IJC Heart Vasculature 2016; 12:21-25. With permission from Elsevier.

ABSTRACT

Background: CHARGE syndrome is a complex multiple congenital malformation disorder with variable expression that is caused by mutations in the *CHD7* gene. Variable heart defects occur in 74% of patients with a *CHD7* mutation, with an overrepresentation of atrioventricular septal defects and conotruncal defects - including arch vessel anomalies.

Methods and Results: We report an index patient with an arch vessel anomaly underlying serious feeding problems that resolved after arch vessel surgery. This led us to examine the incidence of arch vessel anomalies in our previously studied cohort of 299 patients with a *CHD7* mutation. Forty-two patients (14%) had an aortic arch anomaly, mostly aberrant subclavian artery or right aortic arch, which usually occurred in combination with other congenital heart defects (81%). The majority of these patients also had feeding problems that may be linked to their arch anomaly, but insufficient information was available to exclude other causes.

Conclusions: Arch vessel anomalies occur in a significant proportion of patients with a *CHD7* mutation, and these anomalies may cause morbidity due to compression of the esophagus or trachea. Since symptoms of vascular compression can mimic those caused by other abnormalities in CHARGE syndrome, it is important to be aware of arch vessel anomalies in this complex patient category. Whether a solitary arch vessel anomaly is an indicator for CHARGE syndrome still needs to be studied, but doctors should look out for other CHARGE syndrome features in patients with arch vessel anomalies.

Keywords: Arch vessel anomalies; CHARGE syndrome; *CHD7* gene; Feeding problems; Congenital heart defects; Aberrant subclavian artery

INTRODUCTION

CHARGE syndrome (MIM 214800, **C**oloboma, **H**eart disease, Choanal **a**tresia, **R**etardation of growth and/or development, **G**enital hypoplasia and **E**ar abnormalities with or without deafness) is a multiple congenital malformation disorder with variable expression and an incidence of 5.8-6.7 per 100,000 newborns.[1] CHARGE syndrome is usually a sporadic condition that is caused, in particular, by *de novo* loss-of function mutations in the *CHD7* gene (MIM 608892).[2]

Congenital heart defects occur in 74% of patients who have CHARGE syndrome due to a *CHD7* mutation, and in 80% of patients with a truncating *CHD7* mutation.[3] Our previous study showed that while the types of heart defects found in CHARGE syndrome patients are variable, atrioventricular septal defects and conotruncal defects are overrepresented compared to non-syndromic heart defects.[3] Congenital arch vessel anomalies such as aberrant right subclavian artery (ARSA) were highly overrepresented present within our group of patients with CHARGE syndrome.[3]

The aortic arch and its vessels are formed after the fourth week of embryogenesis by remodeling and re-arrangement of the aortic sac, the branchial arch arteries and the dorsal root aorta's. A normal developing embryo initially has one aortic sac which communicates with the heart via the truncus arteriosus and is connected to two dorsal root aorta via paired branchial arch arteries. The eventual left sided aortic arch derives from the aortic sac, left 4th branchial arch artery and left dorsal root aorta. The first origin, the brachiocephalic trunk arises from the aortic sac. The right and left common carotid arteries develop from the 3rd branchial arch arteries. The root and first part of the right subclavian artery is formed by the right 4th branchial arch artery and right dorsal root aorta. The rest of the right subclacian artery and the complete left subclavian artery derive from an intersegmental artery that originates directly from the dorsal root aorta. The molecular control of this complex process is not well understood, but defective remodeling results in congenital arch vessel anomalies.[4-6]

A common congenital arch vessel anomaly is an aberrant subclavian artery in which the right or left subclavian artery has an abnormal anatomical position. An aberrant right subclavian artery, which is also called arteria lusoria, passes posterior of the esophagus and left aortic arch. It occurs when during embryological development the right fourth branchial arch artery and proximal portion of the right dorsal root aorta disappears, while the distal right dorsal root aorta persists.[6] Aberrant subclavian arteries have been found in 1-2% of pediatric patients who

had echocardiograms and in cardiac autopsy specimens.[7,8] Another frequent arch vessel abnormality is a right-sided aortic arch (RAA) which is caused by the persistence of the right dorsal root aorta and disappearance of the left fourth branchial arch artery and left dorsal root aorta.[6] A RAA is usually associated with a congenital heart malformation.[8,9]

Arch vessel anomalies are usually asymptomatic, but problems may occur when a complete or incomplete vascular ring causes compression of the esophagus and the trachea. A double aortic arch in which both left- and right-sided aortic arches surround the trachea and esophagus is the most common cause of vascular compression in children.[10] Presenting symptoms of vascular compression vary, but include recurrent respiratory infections, stridor, wheezing, cough, dyspnea, respiratory distress, dysphagia, feeding difficulties and vomiting.[5,10]

In this study we describe CHARGE patients with congenital arch vessel anomalies and focus on the health problems that might be caused by arch vessel anomalies in these patients.

PATIENTS AND METHODS

Case report

We report a clinically diagnosed CHARGE patient with dysphagia due to an arch vessel anomaly. Clinical information was obtained from the extensive medical correspondence concerning this patient. The patient's parents have given consent for the publication of this data.

Cohort of patients with an arch anomaly and a CHD7 mutation

We previously studied heart defects in 299 patients with a proven *CHD7* mutation, of whom 220 had a congenital heart defect.[3] This cohort consisted of patients tested for a *CHD7* mutation because of a clinical suspicion of CHARGE syndrome. The *CHD7* analysis was performed on a diagnostic basis at the DNA laboratory in Nijmegen, the Netherlands, between 2004 and 2009. Patients lived in the Netherlands (34%) and other European countries (54%), but also on other continents (12%). The accredited Medical Ethics Review Committee of the University Medical Center Groningen waived full ethical evaluation because, according to Dutch guidelines, no ethical approval is necessary if medical information that was already available is used anonymously and no extra tests have to be performed.

We selected patients from this previous study who had a vascular ring of any type, a RAA, an interrupted aortic arch, an aberrant left or right subclavian artery, or an aberrant origin of an aortic arch vessel. We studied cardiac phenotype and extra-cardiovascular symptoms in these patients. The patient described in the case report was not part of this cohort.

Control cohort to compare extra-cardiovascular features

The data collected about our study cohort were compared descriptively to a previously published group of 280 CHARGE patients with a known *CHD7* mutation.[2] Because there is some overlap between this group and our present study group, statistical comparisons were not possible. However, excluding these overlapping patients described here might bias the control group.

RESULTS

Case report

We report new findings on a twenty-year-old male with CHARGE syndrome. He was born after an uneventful full-term pregnancy and with a birth weight of 8 pounds (about 3500 gram). He was evaluated directly after birth because of congenital anomalies and respiratory distress. He was diagnosed with laryngomalacia and had a tracheostoma until he was 8.5 years old. A diagnosis of CHARGE syndrome (which was then still an association) was made based on the combination of following anomalies: colobomata of the optic nerve and fundus, choanal stenosis, pulmonary valve dysplasia, genital hypoplasia with unilateral cryptorchism, small kidneys with subcortical cysts, a grade IV vesicoureteral reflux, velopharyngeal incompetence (due to abnormal 9th and 10th cranial nerves), right sided facial nerve palsy and external ear anomalies with absent response to BAER. Further evaluation during the years showed profound sensorineural deafness with absent auditory nerves, absent semicircular canals, dysplastic cochlea, anosmia, hypogonatropic hypogonadism and significant short stature with growth hormone deficiency. He had a normal conventional karyotype, but *CHD7* analysis had never been done. He does fulfill the current diagnostic criteria for CHARGE syndrome.[11,12]

The boy experienced feeding problems from birth, for which he received tube feeding until the age of 9 years. Even after decannulation and removal of the feeding tube, his feeding problems persisted; he aspirated water and could only eat soft foods. He had several swallowing studies done through the years that showed a constriction of the esophagus. From the age of 10 years his esophagus

was dilated several times, but his feeding problems did not improve. He had several periods of choking, which warranted further evaluation. At the age of 18 years, he had a gastroscopy, which indicated a vessel compressing the esophagus. An angiogram confirmed an aberrant right subclavian artery as the cause. After surgical re-implantation of the aberrant subclavian artery, the boy was finally able to eat normally, and no new feeding problems or periods of choking have occurred since that time.

Arch vessel anomalies in a cohort of patients with a *CHD7* mutation

Of the 299 patients with a *CHD7* mutation, 42 had a congenital arch vessel anomaly (14%). This group consists of 23 males and 19 females (see Table 1). Most patients had a truncating *CHD7* mutation (33/42, 79%). Fourteen patients were deceased (33%), ten of the twelve patients for whom the age of death was known died in the first year of life (see Table 1).

Right sided aortic arch (20 patients) and aberrant subclavian arteries (19 patients) were most frequently identified (see Table 1). A vascular ring was identified in five patients. An abnormal origin of an arch vessel was diagnosed in four patients, two concerning the subclavian artery (patient 1 and 37) and two the carotid arteries (patient 17 and 20). In patient 1, who had an interrupted aortic arch type B and a malalignment ventricular septal defect, the subclavian artery derived from the descending aorta. In patient 37, who had a right-sided aortic arch and a bicuspid aortic valve, the left subclavian artery derived from the pulmonary artery. Patient 17 had a persistent ductus arteriosus (PDA) and ARSA in combination with a right internal carotid artery that was inserted higher than usual. Patient 20 had a PDA and ARSA with a truncus bicaroticus, which means both carotid arteries originated from one common origin of the aortic arch.

Most patients had other heart defects in addition to their arch vessel anomaly (34/42, 81%), and one patient had a congenital conduction disorder. Interestingly, seven patients (17%) had an arch vessel anomaly as an isolated cardiovascular feature (see Table 1). The accompanying heart defects were variable, but often included septal defects (atrial as well as ventricular), PDA and tetralogy of Fallot or double outlet right ventricle.

Table 1. Overview of 42 patients with a *CHD7* mutation and an arch vessel anomaly

		Mutation type	Arch vessel anomaly	Other heart defect
1*	f	Fs	Aberrant origin SA, IAA	VSD
2	m	Fs	ASA	PS
3*	f	Fs	ASA, IAA	AVSD, DORV
4	f	Fs	ASA	ASD
5	f	Fs	ASA	SAS
6	f	Mis	ASA	VSD
7	m	Non	ASA	Truncus
8	m	Non	ASA	AVSD
9	m	Non	ASA	ASD, BAV, VSD
10*	f	Non	ASA	ASD, coarctation, VSD
11	m	Non	ASA	Fallot
12*	f	Non	ASA, IAA	Absent left AV valve, DORV
13*	m	Splice	ASA	Fallot, TAPVR
14	f	Splice	ASA	PDA
15*	f	Splice	ASA	HLHS, coarctation
16	m	Transl	ASA	ASD, PDA
17*	m	Non	ASA, aberrant origin RCA	PDA
18	m	Mis	ASA, RAA	Peripheral PS
19	m	Non	ASA, RAA	Coarctation
20	f	Fs	ASA, Truncus bicaroticus	PDA
21	m	Fs	RAA	Fallot
22	f	Fs	RAA	
23	f	Fs	RAA	PDA
24*	m	Fs	RAA	Fallot
25	f	Fs	RAA	
26	f	Non	RAA	Coarctation, VSD
27	m	Non	RAA	Coarctation, SAS, VSD
28	f	Non	RAA	Fallot
29*	f	Non	RAA	
30*	m	Non	RAA	ASD, VSD
31	f	Non	RAA	Fallot
32*	m	Non	RAA	
33	m	Non	RAA	ASD, PDA
34*	m	Splice	RAA	DORV, VSD
35*	m	Splice	RAA	AVSD
36	m	Splice	RAA	PDA
37*	m	Fs	RAA, aberrant origin LSA	BAV
38	m	Fs	Vasc. ring	ASD, VSD

Table 1. Overview of 42 patients with a *CHD7* mutation and an arch vessel anomaly (continued)

		Mutation type	Arch vessel anomaly	Other heart defect
39	m	Fs	Vasc. ring	
40	m	Non	Vasc. ring	
41	f	Splice	Vasc. ring	
42	f	Non	Vasc. ring, RAA	

Sex: f, female; m, Male
Mutation type: Fs, frameshift; Mis, Missense; Non, nonsense; Splice, Splice site or intronic variant; Transl, translocation t(2;8)(q11.2;q11.2).
Arch vessel anomalies: ASA, aberrant subclavian artery; LSA, left subclavian artery; RAA, right aortic arch; RCA, right coronary artery; SA, subclavian artery; Vasc. ring, vascular ring.
Other heart defects: ASD, atrial septal defect; AVSD, atrioventricular septal defect; AV valve, atrioventricular valve; BAV, bicuspid aortic valve; coarctation, coarctation aorta; DORV, double outlet right ventricle; Fallot, tetralogy of Fallot; HLHS, hypoplastic left heart syndrome; IAA, interrupted aortic arch; PDA, persistent ductus arteriosus; PS, pulmonary stenosis; SAS, subvalvular aortic stenosis; TAPVR, total anomalous pulmonary venous return; Truncus, truncus arteriosus; VSD, ventricular septal defect.
* deceased

The most common extracardiovascular features were external ear anomaly (36/36), hearing loss (34/34) and semicircular canal abnormalities (23/24), which were present in almost all patients for whom the information was known (see Table 2). Developmental delay, genital hypoplasia (e.g. micropenis or hypogonatropic hypogonadism) and cranial nerve dysfunction were present in the majority of patients (>80%). These extracardiovascular features did not clearly differ between our study cohort and the control cohort (see Table 2).

Information on feeding or swallowing history was known for 26 of 37 patients who were alive at the age of 1 month. Only one out of these 26 patients was recorded not to have feeding or swallowing problems. Thus, these problems were present in 96% of patients (range 25/37-36/37=68-97%). Remarkably, at least twenty patients (77%, range 20/37-36/37=54-97%) had feeding problems that necessitated tube feeding. Information on feeding was known for 110 patients in our control cohort, and tube feeding was necessary in 90 patients (82%, range 90/280-260/280=32-93%). We have no information on recurrent respiratory infections, stridor, wheezing, cough or dyspnea in both our study and control group.

Table 2. Other features of CHARGE syndrome in patients with arch vessel anomalies

Patient		C	A	DD	GR	G	E	HL	SCC	CLP	F	CN
1*	f	y	y	?	?	?	y	?	y	?	?	?
2	m	n	n	y	n	y	y	y	y	n	y	y
3*	f	y	y	?	?	?	y	y	y	n	n	?
4	f	n	?	?	?	?	y	y	?	y	?	?
5	f	y	y	y	y	?	?	y	?	?	y	?
6	f	n	n	?	y	?	?	?	n	n	y	?
7	m	n	n	y	?	y	y	?	y	?	y‡	y
8	m	y	n	y	y	?	y	y	y	?	y	?
9	m	y	?	?	?	y	y	y	y	y	y‡	y
10*	f	?	?	?	?	?	y	y	?	y	?	?
11	m	?	?	y	?	y	?	y	?	?	?	?
12*	f	n	y	?	?	?	?	?	?	?	?	?
13*	m	y	n	?	n	?	y	?	?	y	?	?
14	f	y	n	y	y	y	y	y	?	n	?	y
15*	f	y	n	?	?	?	y	?	?	y	?	?
16	m	n	y	?	y	y	y	y	?	y	y	y
17*	m	y	n	?	y	y	y	y	?	?	y†,‡	y
18	m	y	?	?	?	y	y	?	?	?	?	y
19	m	n	y	?	n	y	y	y	y	n	y	?
20	f	y	n	y	n	?	y	y	y	?	?‡	?
21	m	y	n	y	n	y	y	y	y	n	?	y
22	f	n	?	y	?	?	y	y	y	y	y	n
23	f	y	?	?	?	?	y	y	?	?	?	y
24*	m	n	y	?	n	y	y	y	y	n	?	?
25	f	?	y	y	?	?	?	y	?	?	?	?
26	f	y	n	n	y	n	y	y	y	n	y	y
27	m	y	y	?	n	n	y	y	y	n	y	y
28	f	y	n	n	y	?	y	y	y	n	y	y
29*	f	y	y	y	y	n	y	y	y	n	y	y
30*	m	y	y	?	?	?	y	y	?	?	y†	?
31	f	y	?	y	y	?	y	y	?	?	y‡	y
32*	m	y	n	y	?	?	y	y	y	y	y	n
33	m	n	n	y	y	y	y	y	?	n	y	?
34*	m	y	n	?	y	y	y	y	y	?	y‡	?
35*	m	n	n	y	y	y	y	y	?	n	?	y
36	m	y	n	?	y	y	y	y	y	y	?	n
37*	m	y	y	?	?	y	y	?	y	n	?	y
38	m	n	n	y	y	n	y	y	?	y	y	y
39	m	y	y	n	n	y	y	y	y	n	y	?
40	m	y	n	?	n	y	y	y	y	n	y	y

Table 2. Other features of CHARGE syndrome in patients with arch vessel anomalies (continued)

Patient		C	A	DD	GR	G	E	HL	SCC	CLP	F	CN
41	f	n	n	y	y	?	?	y	y	y	?‡	?
42	f	n	y	y	n	?	y	y	y	n	y	y
Total		25/39	14/34	18/21	16/26	18/22	36/36	34/34	23/24	11/28	20/24	19/22
%		64	41	86	62	82	100	100	96	39	83	86
Range%		60-67	33-52	43-93	38-76	43-90	86-100	81-100	55-98	26-60	48-90	45-93
Control %		81	55	99	37	81	97	?	94	48	82	99
Control Range%		68-84	35-71	53-99	13-79	42-90	80-98	?	39-98	28-70	32-93	62-100

m, male; f, female; y, feature present; n, feature absent; ?, unknown/no information.
C, Coloboma or microphthalmia; A, Choanal atresia or stenosis; DD, Developmental delay: GR, Growth retardation; G, Genital hypoplasia, e.g. micropenis, hypogonadotropic hypogonadism; E, External ear anomaly; HL, Hearing loss; SCC, Semicircular canal anomaly, CLP, Cleft lip and or palate; F, Feeding problems, needing tube feeding; CN, Cranial nerve defect.
Total, Patients in whom feature was present/all patients of whom information was known; %, Percentage of patients of whom information was known who had this feature; Range%, Shows the minimum-maximum frequency of a feature in this cohort as calculated by (positive/total) x 100% - (positive+unknown/total) x 100%; Control, Numbers based on a previously studied cohort of 280 patients with a pathogenic *CHD7* mutation.[2]
* deceased
† no information on tube feeding, not included in total number of patients
‡ Swallowing problems are mentioned.

DISCUSSION

In our study cohort, arch vessel anomalies were present in 14% (42/299) of patients with a *CHD7* mutation and in 19% (42/220) of patients with a *CHD7* mutation and a cardiovascular defect. We might have missed patients with an arch vessel anomaly in our retrospective study because it can be missed with echocardiography, and because we know the collected data are not complete. We also did not have enough information to classify heart defects in 18 patients (8%), and in approximately 60% we had to base our classification on the information from the medical doctor who requested the *CHD7* analysis.

Several previous studies on smaller populations (between 47 and 83 patients) also documented arch vessel anomalies in 4 to 23% of the patients with CHARGE syndrome, or in 5 to 36% of the patients with CHARGE syndrome and a heart defect.[13-16] However the data from our study and the previous studies cannot easily be compared for a number of reasons. First, not every study used the same definition for arch vessel anomalies, while the type of heart defects that are categorized as arch vessel anomaly are not clear in others. For example, we did not include

hypoplastic aortic arch as an arch vessel anomaly based on the classification system we used to classify heart defects,[17,18] while a previous study did.[16] Second, we included patients with arch vessel anomalies and other cardiac anomalies in our percentages while, in at least one other study, patients with an arch vessel anomaly and another heart defect were partly categorized in a different group.[16] Finally, the populations differ because patients in all previous studies had a clinically based diagnosis of CHARGE syndrome, while we included only patients with a definite molecular diagnosis. Nonetheless, both our study and all previous studies show that arch vessel anomalies do occur more frequently in CHARGE syndrome than in the general population.

We primarily identified patients with aberrant subclavian arteries and right-sided aortic arch in our cohort, but rarer arch vessel anomalies can also occur in patients with CHARGE syndrome. For example, we identified an abnormal origin of an arch vessel in four of our patients (see Table 1). An aberrant origin has also been described previously in CHARGE patients for the left brachiocephalic trunk and left subclavian artery out of the pulmonary artery, respectively.[19,20] In our study cohort, the arch vessel anomalies usually occurred in combination with other heart defects. However, it is important to note that arch vessel anomalies such as right aortic arch and aberrant subclavian artery were solitary in 17% of our patient cohort.

Based on these clinical observations, CHD7 probably has an effect on the embryonic development of the branchial arch arteries. This hypothesis is supported by animal studies in which knockdown of CHD7 has been shown to have an effect on pharyngeal arch development.[21,22] We did not find an indication that truncating mutations in *CHD7* are more likely to be the cause of arch vessel anomalies, as they were present in comparable percentages in our study and control cohort (79% vs. 71%), while they are known to be present significantly more often in CHARGE patients with a congenital heart defect.[3]

CHARGE syndrome is a complex multiple congenital malformation disorder. Children with CHARGE syndrome face significant problems. Feeding problems, chronic aspiration and swallowing dysfunction are often present and can result in recurrent respiratory infections.[23,24] Identifying the cause of feeding problems in CHARGE syndrome is complex, because they can be associated with structural problems of the oral cavity, the nasal cavity, the pharynx or larynx; cranial nerve defects; congenital heart defects; or a combination of factors. Since respiratory aspiration is a risk factor for early death in CHARGE syndrome, it is important to

carefully evaluate feeding problems.[25] A vascular ring, caused by an arch vessel anomaly, may present as feeding problems and respiratory problems. Our study indicates that arch vessel anomalies are often present in patients with molecularly diagnosed CHARGE syndrome, but we could not identify predictive factors for the existence of an arch vessel anomaly, e.g. *CHD7* mutation type or other CHARGE-related congenital malformations (see Table 2). Furthermore feeding problems for which tube feeding was needed doesn't occur more often in patients with arch vessel anomalies (83% range 48-90%) compared to the control population of patients with a *CHD7* mutation (82%, range 32-93%, see Table 2). However, the medical history described in our case report clearly illustrates that vascular compression due to an arch vessel anomaly should be taken into account in patients with CHARGE syndrome who also have respiratory and/or feeding problems, especially when choking occurs. The exact prevalence of symptomatic vascular compression of the trachea and/or esophagus in CHARGE syndrome needs to be established.

Since 74% of the patients with molecularly proven CHARGE syndrome have a heart defect, an echocardiography is usually performed in CHARGE patients.[3] However, a normal transthoracic echocardiography does not exclude an arch vessel anomaly since its sensitivity for detecting arch vessel anomalies is low.[26] To indicate the presence of a vascular ring, a regular chest X-ray for tracheal compression, and barium contrast esophagography for esophageal compression, respectively, have a higher sensitivity.[9,26] For identifying the exact morphology of an arch vessel anomaly, non invasive imaging techniques like magnetic resonance imaging and computed tomography are warranted, and they can be used with the same efficiency as invasive angiographic techniques, which has been the gold standard for decades.[9,27] The identification of abnormal aortic arch arteries can also be important for asymptomatic CHARGE syndrome patients who need interventional or surgical procedures because routine procedures may be complicated in patients with arch vessel anomalies, e.g., when associated with anomalies of the laryngeal nerve.

Given the high prevalence of arch vessel anomalies in CHARGE syndrome, it remains interesting to study how often patients with arch vessel anomalies have a *CHD7* mutation. Our recent study in 46 patients with syndromic conotruncal heart defects or AVSD, including eight with an arch vessel anomaly, was unable to identify pathogenic *CHD7* mutations.[28] In a previous study that focused on the prevalence of bicarotid trunk in patients who underwent cardiac catheterization, genetic syndromes were also assessed; CHARGE syndrome was present in three of

the 310 patients (1%) with a bicarotid trunk.[29] A study of 257 patients with a tetralogy of Fallot with pulmonary stenosis showed that the incidence of chromosomal or genetic abnormalities, including CHARGE syndrome, increased significantly in patients who had an aberrant subclavian artery with either a left or right aortic arch.[30] While we don't yet have enough support to advise *CHD7* analysis in all patients with arch vessel anomalies, current studies suggest arch vessel anomalies might be an indicator of CHARGE syndrome. We therefore do advise health care professionals to look carefully for other features of CHARGE syndrome (e.g. external ear anomalies, balance problems, deafness and coloboma) in patients with arch vessel anomalieIn conclusion, arch vessel anomalies are present in a significant portion of patients with a *CHD7* mutation. They may cause problems due to compression of the esophagus and/or trachea. Therefore, doctors caring for patients with CHARGE syndrome should be aware of this underlying cause of swallowing and respiratory problems. Future studies are warranted to identify more precisely the frequency of symptomatic arch vessel anomalies in CHARGE syndrome. More evidence is needed to support that an arch vessel anomaly is an indicator of CHARGE syndrome, but doctors should be aware of other features of this complex entity in patients with an arch vessel anomaly.

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CHAPTER 4.3

CHD7 mutations are not a major cause of atrioventricular septal and conotruncal heart defects

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Adapted from: 'CHD7 mutations are not a major cause of atrioventricular septal and conotruncal heart defects'. Am J Med Genet Part A 014;164A(12):3003–3009. With permission from Wiley.

ABSTRACT

Since 2004, *CHD7* mutations have been a known cause of CHARGE (Coloboma, Heart defects, Atresia of choane, Retardation of growth and development, Genital hypoplasia, Ear anomalies) syndrome, but the full clinical spectrum of *CHD7* mutations is only now gradually emerging. *CHD7* mutations have been identified in patients who do not fulfill the clinical criteria for CHARGE syndrome and in patients with overlapping syndromes. Variable congenital heart defects occur in the majority of patients with *CHD7* mutations, with an overrepresentation of atrioventricular septal defects and conotruncal heart defects. This prompted us to study *CHD7* in 46 patients with these heart defects and one other feature of CHARGE syndrome. We identified two *CHD7* variants that were inherited from a healthy parent (c.3778+17C>T, c.7294G>A), but no pathogenic *CHD7* mutations. We conclude that *CHD7* mutations are not a major cause of the atrioventricular septal defects and conotruncal heart defects, not even if one extra phenotypic feature of CHARGE syndrome is present. Therefore, *CHD7* analysis should not be performed routinely in this group of patients. However, we do recommend adding *CHD7* to massive parallel sequencing gene panels for diagnostic work in patients with syndromic heart defects.

Keywords: atrioventricular septal defects; CHARGE syndrome; CHD7; conotruncal; Heart defect, congenital

INTRODUCTION

CHARGE syndrome (MIM 214800, Coloboma, Heart defects, Atresia of choanae, Retardation of growth and development, Genital hypoplasia and Ear anomalies) is a variable multiple congenital malformation syndrome with a prevalence of 5.8-6.7 per 100,000 live-born children.[1] A patient can be clinically diagnosed with CHARGE syndrome when the diagnostic criteria of either Blake et al.[2] or Verloes[3] are fulfilled. Major criteria are coloboma, choanal atresia, characteristic external ear anomalies, cranial nerve dysfunction, and hypoplasia of the semicircular canals. Other features can be present as well, including congenital heart defects, which are seen in the majority of patients with CHARGE syndrome.[4]

In 2004 heterozygous *CHD7* mutations (MIM 608892) were identified as the major cause of CHARGE syndrome, and these mutations are found in over 90% of the clinically typical CHARGE patients.[5,6] The full clinical spectrum of *CHD7* mutations only gradually emerging, especially the mild end of the spectrum. At least 14-17% of patients with a *CHD7* mutation have a mild presentation of CHARGE syndrome and do not fulfill the clinical diagnostic criteria.[7] In addition, *CHD7* mutations have also been identified in patients with overlapping clinical syndromes, like Kallmann syndrome (MIM 612370), a combination of hypogonadotropic hypogonadism and anosmia.[8,9]

CHD7 mutations might thus be found in atypical CHARGE patients presenting with only a few features of CHARGE syndrome. It is important to identify a *CHD7* mutation in these patients, because this has significant consequences for their clinical follow-up. A *CHD7* mutation increases the risk of co-morbidity for which timely recognition can improve outcome. Furthermore, a molecular diagnosis is important for counseling parents on their recurrence risk. Guidelines are available to help doctors identify which patients should be advised to perform *CHD7* analysis,[7,10] but patients with a mild clinical presentation might still be missed.

Heart defects are the most common congenital malformation, with an incidence of 0.8% in the general population. The cause is unknown in the majority of patients. A congenital heart defect is present in 74% of patients with a *CHD7* mutation. The types of heart defects in these patients are variable, but atrioventricular septal defects (AVSD) and conotruncal heart defects, including aortic arch anomalies, are overrepresented.[4]

Based on the observations that *CHD7* mutations can result in mild, atypical CHARGE syndrome, and on the high prevalence of heart defects in patients with a *CHD7* mutation, one can speculate that such mutations might also be present in patients with an isolated cardiac defect. However, no pathogenic *CHD7* mutations were identified in 67 patients with unselected congenital heart defects.[11] We hypothesized that *CHD7* mutations are more likely to be identified in patients selected for the type of heart defects that are overrepresented in CHARGE syndrome, i.e. AVSD or conotruncal heart defect, and who have at least one other feature of CHARGE syndrome. To test our hypothesis we studied *CHD7* in 46 patients fulfilling these criteria.

PATIENTS AND METHODS

Patients

The patients were selected from databases of patients with a congenital heart defect held at the Departments of Genetics (University Medical Center Groningen), the Center for Congenital Heart Diseases (University Medical Center Groningen), and the Children's Heart Center (Radboud University Nijmegen Medical Center). Inclusion criteria were an AVSD or conotruncal heart defect and at least one of the following CHARGE syndrome features: gait anomalies indicating balance problems, abnormal semicircular canals, Mondini malformation, external ear malformation, coloboma or microphthalmia, cranial nerve dysfunction including severe feeding difficulties necessitating tube feeding, hearing loss, choanal anomalies, genital hypoplasia, tracheoesophageal anomalies, developmental delay, cleft lip and/or palate, growth retardation, immunological dysfunction, or hypocalcaemia. None of the patients fulfilled the clinical criteria for CHARGE syndrome and none had had *CHD7* analysis performed. No other cause for their congenital malformations had been identified at the time of inclusion.

All patients and/or their legal representatives gave informed consent for the analysis of *CHD7*, the collection of medical information, and clinical re-evaluation if a pathogenic *CHD7* mutation was identified. This study was prospectively reviewed and approved by the ethical review boards of the University Medical Center Groningen and Radboud University Nijmegen Medical Center.

CHD7 analysis

DNA was already available for diagnostic DNA analysis or extracted from peripheral blood lymphocytes or saliva using standard procedures. All individual exons of the

CHD7 gene were amplified by polymerase chain reaction, and direct sequencing was performed as described previously.[6] Multiplex ligation-dependent probe amplification (MLPA) was performed if no *CHD7* mutation was found upon sequence analysis (method as described previously).[12] When an unclassified variant was identified, the parents of the patient were analyzed for this variant, whenever available, and the classification system described by Bergman et al. was used for classifying missense variants.[13]

RESULTS

Of the 80 patients selected from the databases who fulfilled the inclusion criteria, 71 patients were asked by letter or by their genetic counselor to participate in this study. The other nine were not approached, because they did not live in the Netherlands, their address was not available, or the parents of a deceased child had not had any contact with the cardiologist or geneticist recently. Of the 71 patients, 15 patients did not respond and ten patients did not wish to participate.

The 46 patients included consisted of 17 females and 29 males with a median age of 10 years (age range: 1 week-69 years). Data on congenital heart defects and other congenital malformations are presented in Table 1. We also collected information on any previous genetic analysis performed in these patients (Table 2).

CHD7 analysis in the 46 patients revealed two unclassified variants in patients 30 and 33 (see Tables). The missense variant c.7294G>A was identified in a 12

Table 1. Clinical features in the 46 patients with congenital heart defects.

Number	Sex	Age in years	Heart defect	Other clinical features
1	M	0	CC-TGA, IAA	Choanal stenosis, cryptorchism, cup ears, single umbilical artery, kidney anomaly, facial dysmorphism
2	M	0	AVSD, underdev. AA	Growth retardation, cryptorchism, small penis, hypospadia, posteriorly rotated ears, extra cervical vertebrae and ribs, facial dysmorphism
3	M	0	AVSD, CoA, DORV	Abnormal external ears, anal atresia, low implanted thumbs, fusion vertebrae S2-S3
4	F	0	AVSD	Cleft palate, low set and posteriorly rotated ears, retrognathia

Table 1. Clinical features in the 46 patients with congenital heart defects. (continued)

Number	Sex	Age in years	Heart defect	Other clinical features
5	F	1	Absent LAVV, AVSD, DORV, PA	Growth retardation, large clitoris, posteriorly rotated ears, prominent helices, thrombopenia, anal fistula, microcephaly, facial dysmorphism, flexed wrists, sacral dimple
6	M	2	TOF	Cryptorchism
7	F	2	Vasc. ring with RAA	Esophageal atresia, tracheomalacia, hypoplastic thumb
8	F	5	AVSD	Short stature, hypotonia, feeding difficulties, upslanting palpebral fissures
9	M	5	TGA	Mild gross motor developmental delay, feeding difficulties, focal seizures
10	F	6	DILV/DORV, TGA, VSD, CoA	Feeding difficulties, aplasia of the thymus
11	M	6	TOF with RAA	Feeding difficulties, thymic aplasia with T-cel dysfunction, hypoparathyreodism, hypocalcaemia, club feet, vertebral and rib anomalies, bilateral inguinal hernia
12	M	7	TOF, RAA	IQ 65, abnormal position of the ears, PDDNOS, ankyloglossia, facial dysmorphism
13	F	7	LI, DORV, AVSD, ASD, IPS, PVS	Psychomotor retardation, feeding difficulties, periventricular leucomalacia, spastic paraplegia
14	F	7	TOF	Abnormal motor development
15	F	7	TGA	Small clitoris, minimal plexus cyst
16	M	7	TOF	Cryptorchism, esophageal atresia with tracheoesophageal fistula, large kidneys
17	M	8	IAA type B, VSD	Speech delay, hypocalcaemia, hypomagnesaemia
18	M	8	AVSD	Growth retardation, IQ 78, sensorineural hearing loss, recurrent airway infections, osteomyelitis
19	M	9	TOF	Small stature
20	M	9	TOF, HPS	Motor delay, IQ 75, feeding difficulties, pes planovalgus, encopresis
21	M	9	AVSD	Motor delay, IQ 80, facial dysmorphism, syndactyly 2^{nd}-3^{rd} toes, pectus excatum
22	F	10	RAA, aberrant LSA	Cleft palate, facial dysmorphism, epilepsia
23	M	10	TOF	Psychomotor retardation, microcytic anemia, ADHD, PDDNOS
24	M	10	TOF	Learning difficulties, PDDNOS
25	M	10	TOF, PA	Esophageal atresia with tracheoesophageal fistula, coxitis fugax
26	F	10	TOF	Long ears, suspected for 22q11.2 deletion
27	M	11	aberrant RSA	Esophageal atresia
28	M	11	TA, IAA	Psychomotor retardation, infantile encephalopathy, nefrocalcinosis, toilet training problems
29	F	12	AVSD	Unilateral microtia, feeding difficulties, unilateral longitudinal reduction defect arm, hip dysplasia, abnormal vertebrae

Table 1. Clinical features in the 46 patients with congenital heart defects. (continued)

Number	Sex	Age in years	Heart defect	Other clinical features
30*	F	12	aberrant RSA	Motor delay, IQ 70-80, thumb hypoplasia, constipation, myopathy
31	F	13	PA with VSD	Learning problems, IQ 72, conductive hearing loss, feeding difficulties, faillure to thrive
32	M	13	TOF	Cryptorchism, anal atresia
33*	M	13	PA, TOF-like	Psychomotor retardation, phimosis, toilet training problems
34	M	14	TOF	Hypogammaglobulinemia with absent B-cells
35	F	14	TOF, AS	Learning difficulties
36	M	15	TOF	Growth retardation, gross motor delay, sensorineural hearing loss, feeding difficulties, esophageal atresia with tracheoesophageal fistula, tracheomalacia, VUR, abnormal sacral vertebrae
37	F	15	TOF	Short stature, developmental delay, nasal speech, facial dysmorphism
38	M	17	CC-TGA, VSD, PA	Intellectual disability, protuding ears, PDDNOS, kyphoscoliosis
39	M	17	TOF	Coloboma, unilateral microtia, conductive hearing loss, cervical vertebral anomalies, bilateral pre-auricular tag, inguinal hernia, mild frontal microgyria
40	M	20	CC-TGA, DOLV, PVS, IPS, HPS	Cleft lip, 3th grade AV-block, low activity of protein C, inguinal hernia
41	M	22	TOF	Right-sided Cleft lip and palate, left cleft lip
42	F	33	TOF	Short stature, psychomotor retardation, feeding difficulties, balance problems
43	M	33	TOF	Mixed hearing loss, otosclerosis
44	F	34	TOF	Unilateral microphthalmia, developmental delay, IQ 75,
45	M	54	RAA	Coloboma, developmental delay, IQ 80, ear pit, walks on toes
46	M	69	TOF	Mixed hearing loss, vertigo

Number: patient ID number. * Patients with a *CHD7* variant.
Sex: F, female; M, male
Heart defects: AS, aortic stenosis; ASD, atrial septal defect; AVSD, atrioventricular septal defect; AVV, atrioventricular valve; CC-TGA, congenitally corrected transposition of the great arteries, CoA, Coartctation of the aorta; DILV, Double inlet left ventricle; DOLV, double outlet left ventricle; DORV, double outlet right ventricle; HPS, hypoplastic pulmonal system; IAA, interrupted aortic arch; IPS, infundibular pulmonary stenosis; LAVV, left atrioventricular valve; LI, left isomerism; LSA, left subclavian artery; PA, pulmonary atresia; PVS, pulmonary valve stenosis; RAA, right-sided aortic arch; RSA, right subclavian artery; TA, truncus arteriosus; TGA, transposition of the great arteries, TOF, tetralogy of Fallot; Underdev. AA, underdeveloped aortic arch; Vasc. Ring, vascular ring; VSD, ventricular septal defect
Other clinical features: feeding difficulties are only mentioned when tube feeding was needed; ADHD, attention deficit hyperactivity disorder; PDDNOS, pervasive developmental defect not otherwise specified; VUR, vesico-ureteral reflux.

Table 2. Genetic analysis in 46 patients with congenital heart defects.

Number	Sex	Age in years	Karyotype	FISH	Array	DNA
1	M	0			Agilent 105K	*NOTCH1*
2	M	0	46,XX	22q11	Agilent 105K	*NOTCH1*
3	M	0	46,XY		Agilent 105K	*FANCA, SALL1*
4	F	0	46,XX	22q11	BAC 1Mb	
5[1]	F	1	46,XX	22q11, 12p	BAC 1Mb	
6	M	2			Agilent 105K	
7	F	2	46,XX[4]		Agilent 105K[5]	
8	F	5	46,XX	22q11	Subtelomer	
9	M	5				
10	F	6	46,XX	22q11, 10p13p14		
11	M	6	46,XY	22q11, 10p13p14	Agilent 180K[6]	*JAG1*
12	M	7	46,XY	22q11	Agilent 180K	
13	F	7				
14	F	7				
15	F	7				
16	M	7	46,XY	22q11		
17	M	8	46,XY	22q11		
18	M	8	46,XY	22q11		
19	M	9				
20	M	9	46,XY	22q11		
21	M	9			Agilent 180K[7]	*DHCR7*
22	F	10	46,XX	22q12	Agilent 180K	
23	M	10	46,XY	22q11	BAC 1Mb	
24	M	10				
25	M	10	46,XY	22q11		
26	F	10	46,XX	22q11		
27	M	11				
28	M	11	46,XY	22q11		
29	F	12				
30[2]	F	12	46,XX		250K SNP array	
31	F	13	46,XX	22q11	Subtelomer, BAC 1Mb	
32	M	13				
33[3]	M	13	46,XY		BAC 300kb	*FMR1*
34	M	14				
35	F	14				
36	M	15	46,XY			

Table 2. Genetic analysis in 46 patients with congenital heart defects. (continued)

Number	Sex	Age in years	Karyotype	FISH	Array	DNA
37	F	15	46,XX	22q11, 22q13, 10p13p14	Subtelomer, BAC 1Mb	
38	M	17			Agilent 180K	*CFC1, NODAL, NOTCH1, ZIC3*
39	M	17	46,XY	22q11	Subtelomer	
40	M	20				
41	M	22				
42	F	33	46,XX	Subtelomer	250K SNP array	*RAI1*
43	M	33				
44	F	34			Agilent 180 K[8]	*NOTCH1*
45	M	54	46,XY	22q11, 14cen/ 22cen	Agilent 105K	*FMR1*
46	M	69				

This table summarizes the results of genetic analyses previously performed in a diagnostic setting. Number: patient ID number. Patients with a *CHD7* variant are in bold. [1] no MLPA performed of *CHD7* because insufficient DNA available, [2] *CHD7* variant c.7294G>A p.Val2432Met maternally inherited, [3] *CHD7* variant c.3778+17C>T p.? maternally inherited.

Sex: F, female; M, male

Karyotype: [4] also normal mytomycine test

FISH: fluorescent in-situ hybridization

Array: type of whole genome array is mentioned, if performed. 250K SNP array, Affymetrix GeneChip 250K (NspI) SNP array platform; Agilent 105K, Agilent 105 K custom HD-DGH microarray; Agilent 180K, Agilent 180 K custom HD-DGH microarray; BAC 1Mb, average 1 Mb resolution; BAC 300kb, average 300 kb resolution; Subtelomer, subtelomer array or MLPA in which the terminal 5 Mb of all chromosomes were analyzed.

[5] duplication 17q25.1, 840 kb, maternal. [6] dupXp22.33, 970kb. [7] deletion 15q13.1q13.2, 135kb, maternal. [8] duplication 12q13,12, 359 kb, de novo. The copy number variations mentioned were not classified as a cause for the congenital heart defect.

DNA: other sequence analyses performed in diagnostics prior to *CHD7* analysis without identifying causal variants

year-old girl (patient 30) with an aberrant right subclavian artery, thumb hypoplasia, an unclassified myopathy, delayed motor development, constipation, and an IQ between 70 and 80. This missense variant causes a change of the conserved valine into methionine at position 2432 of the CHD7 protein. The variant was also found in her mother, who did not have a congenital heart defect nor any other features of CHARGE syndrome on clinical examination. The variant was classified as probably benign using the classification system of *CHD7* missense variants.[13] We identified the intronic variant c.3778+17C>T in a 13-year-old boy (patient 33) with a pulmonary atresia with ventricular septal defect, psychomotor retardation,

and toilet training problems; it appeared to be inherited from his healthy mother. We classified this variant as probably benign.

DISCUSSION

We identified a *CHD7* variant in 2/46 patients. The c.7294G>A (p.Val2432Met) was classified as probably benign: it had not previously been identified in CHARGE patients (www.CHD7.org). The prediction programs Align GVGD and Polyphen predicted it to be benign and it was seen once in the Exome Sequencing project (ESP) database in >11,000 alleles (based on ESP6500 VCF files as of 13/07/2012; Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA; http://evs.gs.washington.edu/EVS/) and more importantly, the variant was found in the patient's mother, who had no CHARGE syndrome features nor a congenital heart defect.

The intronic variant c.3778+17C>T was also classified as probably benign, since it was identified in the patient's healthy mother, the nucleotide is not conserved, and splice prediction programs predict no major effect on the splice-site and branchpoint.

Taken together, although congenital heart defects occur in 74% of the patients with a *CHD7* mutation, with an overrepresentation of AVSDs and conotruncal heart defects, we found no pathogenic *CHD7* mutations in a group of 46 patients selected with these types of heart defect and at least one other feature of CHARGE syndrome.[4] We know from previous studies that the phenotype of patients with a *CHD7* mutation can be mild. For example, a patient has been described with a heart defect and characteristic external ear abnormality as the only features of her *CHD7* missense mutation.[7] Most previously described mildly affected patients with a *CHD7* mutation, who did not fulfill the diagnostic criteria of Blake et al. or Verloes, had at least one of the major criteria, being coloboma, choanal atresia, characteristic ear abnormalities, cranial nerve dysfunction or semicircular canal defects,[2,3,7] while in our current cohort only 5 patients (patients 1, 18, 36, 39 and 45) had one major criterium, so the patients in our cohort had less specific features of CHARGE syndrome. The results of the *CHD7* analysis in our present cohort show that *CHD7* mutations are not a major cause of the congenital heart defects in patients with at least one other feature of CHARGE syndrome, even when the patients were selected for CHARGE syndrome-specific types of congenital heart defects. Post hoc statistical analysis of our results show with high probability

that *CHD7* mutations are present in less than 6.3% of our selected population. A previous study already excluded *CHD7* mutations as a major cause of unselected congenital heart defects.[11] Thus, *CHD7* analysis does not need to be performed routinely in patients with non-syndromic congenital heart defects.

This recommendation is in line with the previously published guideline for *CHD7* analysis, in which analysis is suggested based on the presence of a minimum number of cardinal features (coloboma, choanal atresia or stenosis, characteristic external ear abnormalities, cranial nerve dysfunction and vestibular phenotype) and supportive features (cleft lip and/or palate, hypogonadotropic hypogonadism or anosmia, congenital heart defect or tracheoesophageal anomaly, mental retardation, growth retardation, or a family member with CHARGE syndrome features). *CHD7* analysis is certainly advised in patients with three cardinal features, or with two cardinal and one supportive feature. A CT scan of the semicircular canals is advised in patients with two cardinal features, or one cardinal and one supportive feature.[7] If we apply this guideline to our cohort, every patient had at least one supportive feature (their congenital heart defect), but *CHD7* screening would be directly recommended in only one patient (patient 1). A *CHD7* mutation or deletion was not found in this patient. Our results strongly support that *CHD7* analysis should only be performed in accordance with the guideline for *CHD7* analysis.[7] If we had used more stringent inclusion criteria, e.g. an AVSD or conotruncal heart defect in combination with one of the cardinal CHARGE features according to the *CHD7* guideline, the chance of finding a mutation might have been higher.

All the patients in our cohort had a syndromic congenital heart defect, which means that they had other problems in addition to their congenital heart defect. Other diagnoses had been suggested in some patients, like oculoauriculovertebral spectrum (including Goldenhar syndrome, MIM 164210), VACTERL association (MIM 192350), and diabetic embryopathy, but none had been clearly proven. All these diagnoses have overlapping features with CHARGE syndrome and are included in the more extensive differential diagnosis of CHARGE syndrome. Chromosomal abnormalities can also have overlapping features with CHARGE syndrome. Patients with a known chromosomal aberration that caused their heart defect were excluded from the present study. A whole genome array had been performed in about half of our cohort, and a 22q11.2 deletion, that causes a syndrome that significantly overlaps with CHARGE syndrome, was excluded in most patients.[14] After our study, a complex chromosomal rearrangement of the short arm of chromosome 20 (46,XX,der(20)trp(20)(p11.21p12.1)dup(20)(p12.1p12.2) dup(p12.3p12.3)) was identified in patient 26 with a whole genome array (Agilent

180 K custom HD-DGH microarray; AMADID-no. 27730) as a cause of her congenital heart defect. Small deletions and duplications probably cause around 15% of the congenital heart diseases in patients.[15] So if we had screened 46 patients with a normal whole genome array result, the chance of finding a *CHD7* mutation might have been higher.

Recently, exome sequencing of 362 parent-offspring trios with a child with a severe congenital heart defect revealed a truncating *CHD7* mutation in one patient with a tetralogy of Fallot and pulmonary atresia, who also had other features of CHARGE syndrome (cleft lip and palate, inguinal hernia, micropenis, sensorineural hearing loss, abnormal neurological development) including one cardinal feature.[16] This patient fulfilled the inclusion criteria of our study, but might also have been eligible for *CHD7* analysis based on the guideline.[7] Interestingly, in the same study, mutations were identified in seven other patients, in different genes that are all involved in the production, removal or reading of methylation of histone 3 lysine at position 4 (H3K4).[16] H3K4 methylation is a mark of activation that is found at promotors or enhancers of important developmental genes. CHD7 is known to bind to H3K4 methylated sites in different cell types during development.[17] Zaidi et al.[16] suggested a complex heterogenetic origin for congenital heart defects, in which genes that modulate H3K4 methylation play an important role. In our small, carefully selected cohort, we could not demonstrate that *CHD7* mutations alone are an important factor in causing syndromic congenital heart defects. New, massive parallel sequencing techniques will offer the opportunity to analyze *CHD7* together with other genes involved in cardiac development, especially the genes involved in H3K4 methylation, in larger cohorts of patients with congenital heart defects. With these techniques *CHD7* mutations can be identified in mildly or atypically affected patients and patients with poor phenotypic characterization. Finding a *CHD7* mutation gives important information for clinical follow-up and reproductive options. We therefore advise adding *CHD7* to gene panels used to sequence patients with syndromic congenital heart defects.

We conclude that *CHD7* mutations are not a major cause of AVSD and conotruncal defects within the mild end of the phenotypic spectrum of CHARGE syndrome, but previous studies have shown that *CHD7* mutations are a minor cause of syndromic congenital heart defect, and the majority of patients with a *CHD7* mutation do have a heart defect. A *CHD7* mutation thus belongs in the extensive differential diagnosis of patients with congenital heart defects. Furthermore the clinical diagnosis of a *CHD7* related disorder is not always straight forward and identification of a *CHD7* mutation has clinical relevance. We therefore recommend including the

CHD7 gene in screening panels using massive parallel sequencing for patients with syndromic congenital heart malformations.

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CHAPTER 4.4

Congenital heart disease and CHARGE syndrome: Molecular genetics, principles of diagnosis and treatment.

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ABSTRACT

CHARGE syndrome is a multiple congenital malformation syndrome occurring in 6-7 per 100,000 live births and presenting with a variable combination of coloboma, choanal atresia, cranial nerve defects, specific ear anomalies and semicircular canal defects as its major features. In most patients a *de novo* loss-of-function mutation in the *CHD7* gene can be identified. CHARGE syndrome can be diagnosed using clinical criteria, but 17% of the patients in whom a *CHD7* mutation has been identified do not fulfill these criteria. So identifying a *CHD7* mutation in a suspected patient confirms the diagnosis and assures appropriate clinical follow-up and genetic counseling.

Heart defects occur in approximately 75% of patients with a pathogenic *CHD7* mutation. The types of heart defects are variable, but atrioventricular septal defects and conotruncal defects are relatively overrepresented.

CHD7 is a member of the chromodomain helicase DNA-binding protein family that regulates transcription by chromatin remodeling. CHD7 has been shown to have a cell-type-specific and stage-dependent function in enhancer-mediated transcription. In the heart, CHD7 is emerging as an important epigenetic factor, but further research is needed to fully understand the function of CHD7 and the broad spectrum of clinical features that is caused by haploinsuffciency of CHD7.

Keywords: CHD7, CHARGE syndrome, heart defects

INTRODUCTION

CHARGE syndrome (MIM 214800) is a multiple congenital malformation syndrome that is highly variable and has an estimated birth prevalence of 6-7 per 100,000.[1] The acronym CHARGE was introduced in 1981 and summarizes some of the cardinal features: ocular coloboma, congenital heart defects, choanal atresia, retardation of growth and development, genital hypoplasia, and ear anomalies with or without deafness.[2] The first patients with what we now call CHARGE syndrome were described in 1961,[3,4] but it was not until 1979 that the association between coloboma, congenital heart defects and choanal atresia was recognized by an ophthalmologist as well as a pediatrician.[5,6] In 2004, loss-of-function mutations in the *CHD7* (Chromodomain Helicase DNA-binding protein 7) gene (MIM 608892) were identified as the major cause of CHARGE syndrome.[7]

The *CHD7* gene is located at chromosome 8q12, has a genomic size of 188 kb and consists of 38 exons, of which the first is non-coding.[7] The encoded protein consists of 2,997 amino acids and belongs to the third subfamiliy of a group of CHD proteins that play a role in regulating the of transcription of genes by chromatin remodeling.[7,8] For this function all CHD members have two N-terminal tandem chromo (chromatin organization modifier) domains, and a centrally located SNF2-like ATPase domain, which uses the energy from ATP hydrolysis to alter the histone-DNA contracts with the nucleosome. In addition, the third subfamily, which includes CHD7, has a C-terminal paired BRK (Brahma and Kismet) domain and a SANT-like (Switching-defective protein 3, Adaptor 2, Nuclear receptor corepressor, Transcription factor IIIB-like) domain.[8] CHD7 probably plays a role in controlling gene expression by ATP-dependent chromatin remodeling in embryonic stem cells and in other cell types.[9,10]

CHARGE syndrome is usually a sporadic condition caused by *de novo* mutations, although rarely it can be transmitted as an autosomal dominant disease.[11] The full clinical spectrum of CHARGE syndrome has gradually emerged since the *CHD7* gene was recognized as its major cause in 2004.[11]

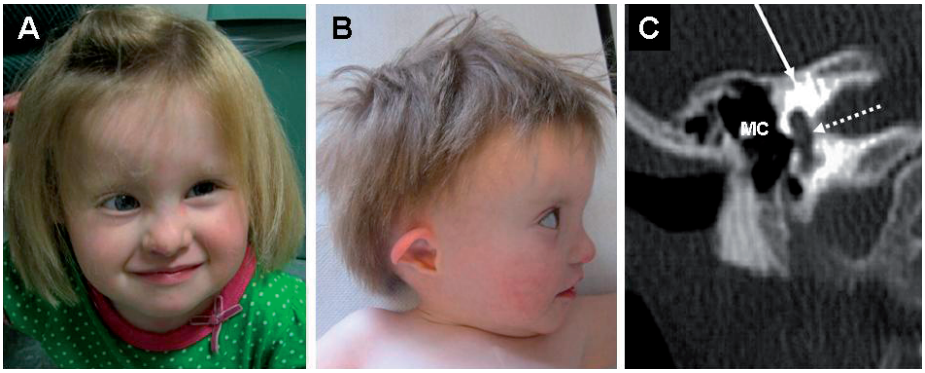
DIAGNOSIS

Clinical diagnosis

Before the discovery of the *CHD7* gene, CHARGE syndrome could only be diagnosed using clinical criteria: the major ones are coloboma, choanal atresia, cranial

nerve dysfunction, ear anomalies, and semicircular canal anomalies (Figure 1).[12,13] In both sets of clinical criteria that are currently used, the clinical diagnosis of CHARGE syndrome could only be made if a patient had at least coloboma or choanal atresia (Table 1). However, since the discovery of the *CHD7* gene, patients with a *CHD7* mutation have been identified who do not fulfill the clinical criteria and have a milder clinical phenotype.[11] So *CHD7* analysis now makes a major contribution to the diagnosis of CHARGE syndrome, although there is some discussion on the naming of patients with only a few of its clinical features. In contrast, no *CHD7* mutations can be identified in 5-10% of the patients who do fulfill the clinical diagnostic criteria.[11,14,15] These patients are still registered as having CHARGE

Figure 1. Clinical features of CHARGE syndrome, a typical patient report



Female patient with CHARGE syndrome due to a nonsense mutation in the *CHD7* gene (c.7879C>T; p.Arg2627*), at the age of 4.5 years (**A**, frontal view) and 3.5 years (**B**, lateral view). She was born with a complex heart defect (double outlet right ventricle, ASD, VSD, pulmonalis stenosis, right descending aorta), a diaphragmatic hernia, bilateral intra-ocular coloboma, a mild right-sided facial palsy (**A**) and dysmorphic ears (**B**). She did not have a choanal atresia or cleft lip/palate. Her right ear is deaf and she has a bilateral absence of the semicircular canals (**C**, MC=middle ear cavity, dashed arrow is basal curve of the cochlea, arrow indicates absence of semicircular canals). The hernia and cardiac defect were surgically treated (Blalock-Taussig shunt, followed by complete correction at the age of 19 months). She had percutaneous gastric tube feeding until the age of 2 years. Her speech development was normal at the age of 4.5 years. Because of growth deficiency, she is treated with growth hormone with good response (age 9 years, 1.16m, -3.5 standard deviation score (SDS), before treatment <-5 SDS). At the age of 9 years she is visiting a main stream school. She has some problems with calculating, but her IQ is within the normal range.
(published with informed parental consent)

Table 1. Clinical criteria for CHARGE syndrome

	Blake	Verloes
Major criteria	1. Ocular coloboma, microphthalmia 2. Choanal atresia or stenosis* 3. Characteristic external ear anomaly, middle/inner ear malformations, mixed deafness 4. Cranial nerve dysfunction	1. Ocular coloboma 2. Choanal atresia 3. Hypoplastic semicircular canals
Minor criteria	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	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
Inclusion rule	Typical CHARGE: 4 major criteria *or* 3 major + 3 minor	Typical CHARGE: 3 major criteria *or* 2 major + 2 minor Partial CHARGE: 2 major + 1 minor criteria Atypical CHARGE: 2 major + 0 minor criteria *or* 1 major + 3 minor

There are currently two clinical systems in use to diagnose CHARGE syndrome: Blake's, updated by a consortium in 2006 and 2009[13] and Verloes'.[12]
* Choanal atresia can be substituted by cleft palate, since these anomalies rarely occur in combination.

syndrome on clinical grounds as long as other diagnoses have been excluded, like chromosomal defects and clinically overlapping syndromes.

Molecular diagnosis

Molecular genetic analysis of *CHD7* comprises the sequencing of all the gene's coding exons and intron-exon boundaries. If no mutation is identified, a partial or whole gene deletion should be excluded by multiplex ligation-dependent probe amplification (MLPA).[16] The analysis of *CHD7* helps clinicians to make the diagnosis of CHARGE syndrome and anticipate problems and assures appropriate clinical surveillance and accurate genetic counseling. There is a guideline to help clinicians decide when to perform *CHD7* analysis (Table 2); it is based on the

Table 2. Guidelines for *CHD7* analysis

Cardinal features	Supportive features
• Coloboma • Choanal atresia or stenosis • Characteristic external ear anomaly (triangular conchae or cup ear) • Cranial nerve dysfunction (facial palsy, sensorineural hearing loss or hypoplasia of cranial nerves on imaging) • Vestibular phenotype*	• Cleft lip/palate • Hypogonadotropic hypogonadism or anosmia • Congenital heart defect or tracheo-oesophageal malformation • Mental retardation (IQ < 70) • Growth retardation (length < -2.5 SD) • Family member with 1 cardinal or 2 supportive features
Perform temporal bone CT scan when patient has:	
2 cardinal features *or* 1 cardinal + 1 supportive feature	
Perform *CHD7* analysis including MLPA when patient has:	
3 cardinal *or* 2 cardinal + 1 supportive feature *or* 2 cardinal + typical SCC abnormalities on temporal bone CT scan *or* 1 cardinal + 1 supportive feature + typical SCC abnormalities on temporal bone CT scan	
Perform genome-wide array analysis when patient has:	
CHD7 analysis reveals no mutation or deletion *or* 2 cardinal + no typical SCC abnormalities on temporal bone CT scan *or* 1 cardinal + 1 supportive feature + no typical SCC abnormalities on temporal bone CT scan	

* A convincing history of vestibular problems (e.g. five-point crawl) or abnormal vestibular test or semicircular canal hypoplasia.
SCC = semicircular canal, adapted from reference [11].

clinical features seen in 280 patients with a proven *CHD7* mutation.[11] In addition to the features that are part of the clinical diagnostic criteria (Table 1), the *CHD7* guideline includes features seen in family members as supporting information. The guideline also indicates when imaging of the semi-circular canals is an appropriate next diagnostic step.

CHD7 analysis in a clinical setting reveals mutations in 32-41% of the patients suspected of having CHARGE syndrome.[1,17] Whereas in a research setting, the detection rates range from 33-100% depending on the patient inclusion criteria and the techniques used for *CHD7* analysis (reviewed in [1]). In patients fulfilling the clinical diagnostic criteria, the mutation detection rate is over 90%.

Over 500 different pathogenic *CHD7* mutations have been identified (www.CHD7.org), of which 97% are intragenic mutations. The mutations are spread throughout the coding region of the *CHD7* gene. Most are truncating frameshifts (44%) or

nonsense alterations (34%), while splice site and missense mutations occur in 11% and 8%, respectively. Complete and partial deletions of the *CHD7* gene and translocations including *CHD7* rarely occur. Mutations are often unique for a patient or a family, but recurrent mutations do occur, especially arginine to stop codon alterations are overrepresented at codon 494 and 2627.[1,17]

The interpretation of the effect of missense variations is often difficult, especially in rare syndromes like CHARGE syndrome where most variations are unique and no functional assay is available. The correct interpretation is essential for adequate genetic counseling and a classification system was therefore proposed to predict the effect of missense variations. The prediction is based on the combination of results from computational algorithms, a structural model, segregation data, and phenotypic data.[18] For the correct interpretation of missense variations, it is essential to collect the segregation and phenotypic information in patients.

Other causes of CHARGE syndrome

CHD7 mutations are the major cause of CHARGE syndrome, but its cause is unclear in 5-10% of the clinically typical CHARGE patients, and in an even higher percentage of the patients who are suspected of CHARGE syndrome but who do not have the full clinical presentation. Non-detectable re-arrangements of *CHD7* might partly explain the CHARGE syndrome features in these patients, but there might also be other genes involved. Besides the *SEMA3E* gene that was found to be disrupted in two patients with the clinical diagnosis of CHARGE syndrome, no other genes have been identified so far in relation to CHARGE syndrome.[1,19] Different chromosomal imbalances which cause phenocopies of CHARGE syndrome have been described (reviewed in reference Janssen et al.)[1] and a recurring phenotypic overlap has been reported for 22q11.2 deletions.[20,21] So when patients suspected of CHARGE syndrome do not harbor a *CHD7* mutation, a genome-wide array analysis should be performed to exclude chromosomal alterations. Besides 22q11.2 deletion syndrome, other syndromes may also mimic CHARGE syndrome, like branchio-oto-renal syndrome, Kabuki syndrome, and mandibulofacial dysostosis caused by *EFTUD2* mutations.[13,22]

BROAD PHENOTYPIC SPECTRUM OF CHD7 MUTATIONS

Clinical features of patients with a CHD7 mutation

A study of clinical features in 280 patients with a proven *CHD7* mutation showed that three features are almost always present: external ear anomalies, cranial

nerve dysfunction, and semicircular canal defects. Therefore, when a patient is suspected of having CHARGE syndrome, a clinician needs to pay special attention to these features – indicated by cup shaped ears with triangular conchae, facial palsy or sensorineural deafness, and delayed motor milestones, respectively.[11] Other features that occur in more than 75% of the patients are coloboma, genital hypoplasia, congenital heart defects, and intellectual disability. However, it is important to realize that around 20% of the patients with a *CHD7* mutation have a normal level of intelligence (Figure 1).[11,23]

Since the identification of the *CHD7* gene, several studies have focused on the mild end of the phenotypic spectrum and shown that up to 17% of the patients do not fulfill the clinical criteria.[11,14] The clinical variability is nicely demonstrated in the 18 familial cases reported thus far (reviewed in reference Janssen et al. and Bergman et al.).[1,11] Monozygotic twins may have different features of CHARGE syndrome and especially patients from two-generation families have a mild presentation of the disorder.

CHD7 mutations in other syndromes

CHD7 mutations have also been identified in patients diagnosed with two other syndromes: Kallmann syndrome and 22q11.2 deletion syndrome.

Kallmann syndrome is the combination of hypogonadotropic hypogonadism and anosmia. Both features also occur in around 80% of the patients with CHARGE syndrome.[24] Other features that occur in both syndromes are cleft lip and/or palate, and hearing loss. Kallmann syndrome is heterogeneous with mutations identified in the genes *CHD7, FGF8*, *FGFR1*, *KAL1*, *PROKR2* and *PROK2;* together these explain 30-45% of the patients.[25] Mutational analysis of *CHD7* has been performed in patients with hypogonadotropic hypogonadism with or without anosmia in four studies and mutations were identified in 13 out of 319 patients in total.[26-29] The chance of finding a *CHD7* mutation seems highest if at least anosmia or one other feature of CHARGE syndrome is present. Heart defects are not a common feature of Kallmann syndrome, but a bicuspid aortic valve was present in 1 of the 13 patients with a *CHD7* mutation.

In contrast, 22q11.2 deletion syndrome does share heart defects as a common feature with CHARGE syndrome, in addition to cleft palate, deafness, and immunodeficiency.[20,21] In most patients with 22q11.2 deletion syndrome, a common 3 Mb deletion including the *TBX1* gene has been identified. *CHD7* analysis in 20 patients clinically suspected of 22q11.2 deletion syndrome but without a deletion

or mutation of *TBX1* revealed truncating mutations in 5 of them.[30] Therefore, *CHD7* analysis should also be performed in patients suspected of 22q11.2 deletion syndrome without a TBX1 mutation or deletion.

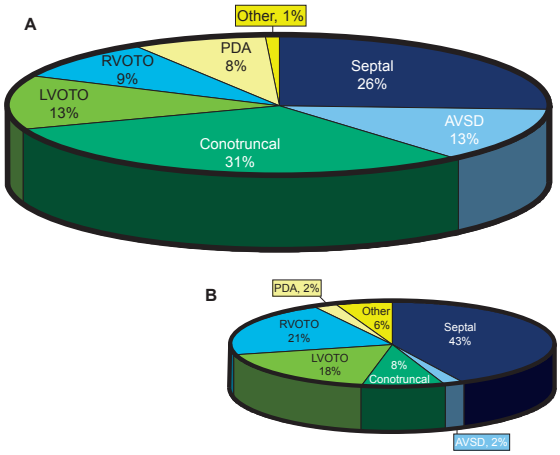
Genotype-phenotype relations

There is no clear genotype-phenotype relation for *CHD7* mutations but, in general, missense mutations are associated with a milder phenotype. Especially cleft lip/palate, choanal atresia, and congenital heart defects are seen less often in patients with a missense mutation.

HEART DEFECTS IN CHARGE SYNDROME

Before the identification of mutations in the *CHD7* gene as the major cause of CHARGE syndrome, two studies had focused on congenital heart defects in CHARGE syndrome in 83 and 59 patients, respectively.[31,32] Both studies identified that conotruncal heart defects often occur in CHARGE syndrome. More recently, a study was performed specifically on congenital heart defects in 299 patients with a proven *CHD7* mutation.[30] Congenital heart defects were present in 74% of these patients. The type of heart defect was variable but, compared to a group of patients with non-syndromic congenital heart defects, especially atrioventricular septal defects and conotruncal defects were overrepresented (Figure 2). In the group of conotruncal heart defects, there was a remarkable overrepresentation of arch vessel anomalies like an aberrant subclavian artery. Heart defects occurred equally in males and females, but were significantly more frequent in patients with truncating mutations than splice- or missense mutations.

Figure 2. Type of heart defects in patients with a *CHD7* mutation (A) and in patients with a non-syndromic heart defect (B).



The distribution of congenital heart defects in a group of 202 patients with a *CHD7* mutation (**A**), and in 1007 patients with non-syndromic congenital heart defects (**B**), adapted from reference [30]. The type of heart defects differ significantly between these two groups; especially conotruncal heart defects and AVSD are overrepresented in patients with *CHD7* mutations.
AVSD = atrioventricular septal defects, LVOTO = left ventricular outflow tract obstruction, PDA = patent ductus arteriosus, RVOTO = right ventricular outflow tract obstruction.

When looking at congenital heart defects, it is worth remembering the overlap already mentioned between CHARGE syndrome and 22q11.2 deletion syndrome, since both syndromes show an overrepresentation of conotruncal heart defects and, in mouse, *Tbx1* and *Chd7* are the only genes known to cause anomalies of the fourth pharyngeal arch arteries that can result in interrupted aortic arch, for instance.[20,33,34]

Based on the high frequency of congenital heart defects and the observation that *CHD7* mutations may be found in patients with only a few features of CHARGE syndrome, it was thought that *CHD7* mutations might also be found in patients with isolated heart defects. However, *CHD7* analysis in 67 patients with isolated congenital heart defects did not reveal any pathogenic mutations.[35] Also in 46 patients selected for atrioventricular or conotruncal heart defects and one other feature within the spectrum of the CHARGE syndrome, no pathogenic CHD7 variants could be identified.[36] In a recent study of 362 parent-offspring trios with a child with a severe congenital heart defect that were exome sequenced, a truncating *CHD7* mutation in a patient with a tetralogy of Fallot and pulmonary atresia

was identified.[37] This patient also had other features of CHARGE syndrome, namely a cleft lip and palate, an inguinal hernia, a micropenis, a sensorineural hearing loss, and abnormal neurodevelopment. However, the patient did not fulfill the clinical criteria of CHARGE syndrome. Based on these observations, one should be aware of other features of CHARGE syndrome, like hearing loss, external ear anomalies, cranial nerve dysfunction, and semicircular canal defects, and look for them in patients with a congenital heart defect, especially if they have an atrioventricular septal defect or conotruncal heart defects, including arch vessel anomalies. In addition, if patients are suspected of having 22q11.2 deletion syndrome but no 22q11.2 deletions are found, they should be examined carefully for CHARGE features.[20,30] Furthermore, *CHD7* should be included in gene panels that screen patients with syndromic or other congenital heart defects.

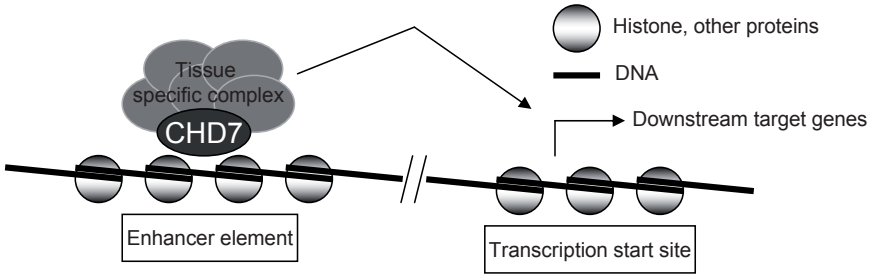
CHD7 FUNCTION IN THE HEART

CHD7 expression has been studied in human, mouse and chicken embryos, amongst others. Mouse embryo *in situ* hybridization at embryonic day 10.5 showed expression of Chd7 in the cardiac outflow tract and truncus arteriosus in one study, while in another study Chd7 expression did not appear to be above background in the myocardium and conotruncal region of the heart at embryonic day 12.5.[15,38] In a heterozygous Chd7-deficient mouse (due to a gene-trapped lacZ reporter embryonic stem cell line), expression was seen in the heart at several embryonic stages.[39,40] In chicken embryos, no expression in the heart was seen at any stage in one study, while another study reported expression in the branchial arches that will form the cardiac outflow tract, at Hamburger and Hamilton stage 20.[41,43] *In situ* hybridization of normal human fetal and embryonic tissue showed expression of CHD7 at the mesenchyme of the pharyngeal arches at days 26 and 33, but no expression in the heart was observed at any stage.[43]

Heart defects have been studied in different animal models with heterozygous *Chd7* mutations, including mice and the African clawed frog (*Xenopus laevis*). In some mice embryos, edema, ventricular septal defects, and pharyngeal arch defects were noticed.[34,38,44] In *X. laevis,* heart defects, including abnormal positioning of the truncus arteriosus and cardiac outflow tract were identified.[9] These findings, together with the high prevalence of heart defects in patients with a *CHD7* mutation, suggest that CHD7 is important in embryonic cardiac development. Although the function of CHD7 is gradually becoming clear, it is still not known exactly how CHD7 haploinsufficiency causes heart defects.

CHD7 has been shown to regulate gene expression by enhancer-mediated transcription and ribosomal RNA biogenesis in the nucleolus.[10,45] CHD7 also binds to different sites on the DNA with various protein complexes in a time- and tissue-specific manner, thereby regulating various target genes (Figure 3).[10,45] The clinical variability seen in different organ systems of individuals with CHARGE syndrome, including the variety of heart defects, is probably caused by the continuum of influence of CHD7 in different cell types.[46,47] CHD7 probably regulates the expression of important cardiac development genes by chromatin remodeling.[48,49] A recent study in mouse embryos showed Chd7 interacts with intracellular BMP-activated SMADs (SMAD1, 5, and 8) in order to specifically regulate transcription of important cardiac development genes like *Nkx2.5*.[40] Other proteins that are known to be important in cardiac development and that have been associated with CHD7 are the ATP-dependent chromatin-remodeling protein complex PBAF (Polybromo- and BRGI-associated factor) and the histone acetyl transferase p300.[9,10,48,4] Furthermore, CHD7 has been shown to be important for the formation of multipotent migratory neural crest cells and neural crest cells are important for cardiac development.[9] It has also been shown that, for normal great vessel development in mice, Chd7 is required in the ectoderm of the pharyngeal arch during embryogenesis.[34] So the heart defects seen in CHARGE syndrome cannot be explained by the effect of CHD7 on neural crest cells alone.

Figure 3. Function of CHD7.



Based on current studies, CHD7 has been shown to bind to different sites on the DNA with various protein complexes in a time- and tissue-specific manner, regulating various target genes. Adapted from Layman et al.[46]

Further research is necessary to fully understand how CHD7 affects cardiac development and which other genes are involved in this pathway. Since monozygotic twins can have different cardiac phenotypes, environmental factors cannot be ignored in this respect.

CLINICAL SURVEILLANCE AND GENETIC COUNSELING

CHARGE syndrome is a complex disorder involving different organ systems and therefore follow-up is recommended in a multidisciplinary expert setting. There are guidelines for the clinical surveillance of patients.[11,13] In all patients with CHARGE syndrome, the heart and kidneys should be screened, since mild congenital anomalies may remain undetected, but can have clinical consequences. Cranial nerve evaluation is important, since dysfunction is involved in respiratory aspiration, post-operative complications, and sudden death. Evaluation of hypogonadotropic hypogonadism should also be done at an appropriate age, in order to induce puberty development at a natural age and to prevent osteoporosis. Evaluation of hearing loss and visual impairment should be done early on, so that devices can be used and communication can be adapted to the child's possibilities. An individualized educational program is also important to fully stimulate the child's intellectual potential and to manage behavioral problems.

Heart defects in patients with CHARGE syndrome should be treated just as in other patients with heart defects. However, one should be aware that anesthetic complications can arise if an operation is necessary and procedures that need anesthetics should therefore be combined whenever possible.[11,13]

Identifying a *CHD7* mutation provides tools for genetic counseling. CHARGE syndrome is a sporadic condition, but familial recurrence has been described due to germline or somatic mosaicism and in mildly affected parents. Parents of a patient should therefore be screened for the *CHD7* mutation found in their child. If a mutation is found *de novo* in a patient, the recurrence risk for another child is 2-3% due to germline mosaicism. CHARGE patients, if they are fertile, have a 50% risk of having a child with CHARGE syndrome. Prenatal diagnosis (molecular analysis and/or ultrasound) should be discussed with the parents of affected children and patients. However, the degree of severity of CHARGE syndrome cannot be predicted by the mutation found, and only a limited prediction can be given from prenatal ultrasound.

CONCLUDING REMARKS

CHARGE syndrome is an extremely variable, multiple congenital malformation disorder that is caused by mutations in the chromodomain gene *CHD7* in the majority of patients. The diagnosis of CHARGE syndrome can be made using clinical criteria, but *CHD7* mutational analysis can be helpful to confirm the diagnosis in suspected cases. A diagnosis offers tools for more accurate clinical surveillance and genetic counseling.

Heart defects are present in the majority of patients with a *CHD7* mutation, although the types of heart defect are variable, with an overrepresentation of conotruncal heart defects and atrioventricular septal defects.

The function of CHD7 and its interaction with other genes is slowly emerging, indicating a role in embryonic stage-specific and tissue-specific expression of genes. In the heart, CHD7 has also been shown to be an important epigenetic factor regulating cardiac development genes, but further research is needed to fully understand why patients with CHARGE syndrome show so much clinical variability.

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CHAPTER 5

Overlap with other syndromes

CHAPTER 5.1

More clinical overlap between 22q11.2 deletion syndrome and CHARGE syndrome than often anticipated

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Adapted from: 'More clinical overlap between 22q11.2 deletion syndrome and CHARGE syndrome than often anticipated'. Molecular Syndromology 2013; 4(5):235-245.With permission from Karger.

ABSTRACT

Background: CHARGE and 22q11.2 deletion syndromes are variable, congenital malformation syndromes that show considerable phenotypic overlap. We further explore this clinical overlap and propose recommendations for the genetic diagnosis of both syndromes.

Methods: We describe two patients clinically diagnosed with CHARGE syndrome, who were found to carry a 22q11.2 deletion and searched the literature for more cases. In addition, we screened our cohort of *CHD7* mutation carriers (n=802) for typical 22q11.2 deletion features and studied *CHD7* in 20 patients with phenotypically 22q11.2 deletion syndrome, but without haploinsufficiency of *TBX1*.

Results: In total, we identified five patients with a clinical diagnosis of CHARGE syndrome and a proven 22q11.2 deletion. Typical 22q11.2 deletion features were found in thirty patients (30/802, 3.7%) of our *CHD7* mutation-positive cohort. We found truncating *CHD7* mutations in five out of the twenty patients with phenotypically 22q11.2 deletion syndrome.

Conclusions: Differentiating between CHARGE and 22q11.2 deletion syndromes can be challenging. CHD7 and TBX1 probably share a molecular pathway or have common target genes in affected organs. We strongly recommend performing *CHD7* analysis in patients with a 22q11.2 deletion phenotype without *TBX1* haploinsufficiency, and conversely, performing a genome-wide array in CHARGE syndrome patients without a *CHD7* mutation.

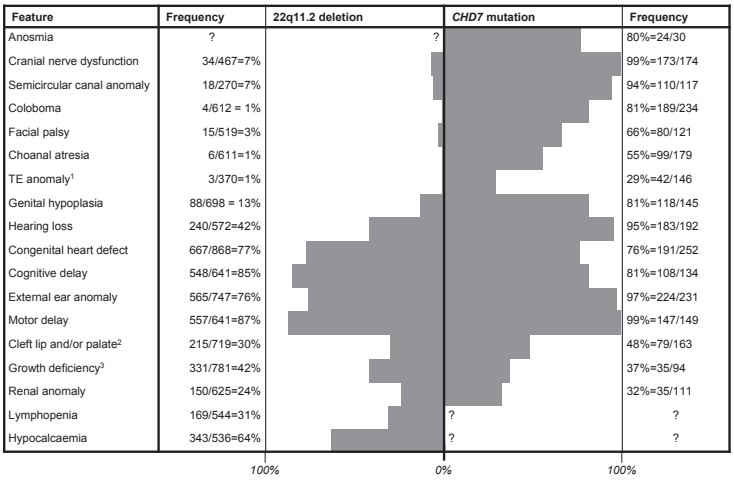
Keywords: CHARGE syndrome, 22q11 deletion syndrome, *CHD7*, *TBX1*

INTRODUCTION

CHARGE syndrome (MIM 214800, Coloboma, Heart defects, Atresia of choanae, Retardation of growth and development, Genital hypoplasia and Ear abnormalities) is a highly variable, multiple congenital malformation syndrome that shows considerable clinical overlap with other syndromes like Kallmann syndrome,[1,2] VACTERL association (Vertebral anomalies, Anal atresia, Cardiac defects, Tracheo-oesophageal fistula, Oesophageal atresia, Renal anomalies and Limb defects),[3,4] Goldenhar syndrome (Oculo-Auriculo-Vertebral spectrum),[4,5] and SOX2 anophthalmia syndrome.[6] The most striking similarity of clinical features, however, is seen with 22q11.2 deletion syndrome, as illustrated by Jyonouchi et al.[7] Here, we further explore the clinical similarities between CHARGE syndrome and 22q11.2 deletion syndrome.

CHARGE syndrome has an estimated birth incidence of 5.8-6.7 per 100,000 live births.[8] A patient is currently diagnosed with CHARGE syndrome if the clinical diagnostic criteria of Blake et al. or of Verloes are fulfilled.[9,10] The major clinical features include choanal atresia, coloboma of the eye, hypoplastic semicircular canals, external ear anomalies and cranial nerve dysfunction (as summarised in Figure 1).[11] CHARGE syndrome is inherited in an autosomal dominant fashion, but most cases are sporadic due to *de novo* mutations in the *CHD7* gene.[11,12] *CHD7* codes for a chromodomain helicase DNA-binding protein that has a cell type-specific and embryonic stage-dependent function in regulating the expression of other developmental genes.[13-15] Heterozygous *CHD7* mutations are found in more than 90% of the patients who fulfil the clinical criteria of CHARGE syndrome, but can also be detected in patients with an atypical phenotype.[11,16]

Figure 1. Frequency of the most common clinical features seen in patients with a 22q11.2 deletion and patients with a *CHD7* mutation



Feature: TE anomaly, tracheo-oesophageal anomaly; [1] in 22q11.2 deletion group only tracheo-oesophageal fistula; [2] Including 28 patients with hypospadia in 22q11.2 deletion group; [3] 148 patients in 22q11.2 deletion group had a submucosal cleft; [4] Height below <2.5 SD in *CHD7* cohort or below 5th percentile in 22q11.2 deletion cohort.
Frequency: number of patients with feature/number of patients investigated
Data based on 943 patients from Children's Hospital of Philadelphia Database for 22q11.2 deletion group.
Data based on 280 patients indentified at the RUNMC with a *CHD7* mutation, as described in Bergman et al.[11]

Chromosome 22q11.2 microdeletions have an estimated birth incidence between 10 and 26 per 100,000 live births and cause a highly variable clinical phenotype including velocardiofacial (VCF) syndrome (MIM 192430), which is the combination of velopharyngeal incompetence and other palate abnormalities, congenital heart defects, and dysmorphic facial features. DiGeorge syndrome (MIM 188400) is another associated phenotype that includes features of congenital heart defects of the outflow tract, hypocalcaemia, and immunodeficiency.[17-19] Since VCF syndrome and DiGeorge syndrome describe variable clinical expressions of the same entity, the term 22q11.2 deletion syndrome is now more commonly used (features summarised in Figure 1).[7,17] This syndrome is inherited in an autosomal dominant manner from a parent in 10% of new cases, but mostly occurs *de novo*.[17] The majority of patients with velocardiofacial syndrome and DiGeorge syndrome have a 3.0 Mb (90%) or 1.5 Mb (8%) hemizygous deletion of chromosome 22q11.2 that can be identified using FISH, MLPA or genome-wide array analysis.[19,20] Mutations in the *TBX1* gene, located in the commonly deleted region, cause a similar

phenotype and thus *TBX1* haploinsufficiency appears to significantly contribute to the features of 22q11.2 deletion syndrome.[21]

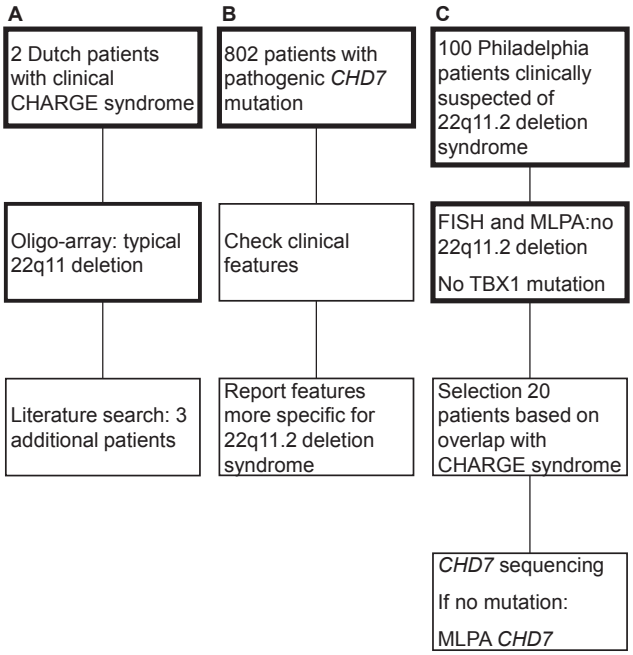
The overlap between CHARGE syndrome and 22q11.2 deletion syndrome has long been recognised.[22-26] The overlapping clinical features include congenital conotruncal heart defects, cleft palate, ear abnormalities, hearing loss, growth deficiency, developmental delay, renal abnormalities, hypocalcaemia and immune deficiency.[7,11] Clinical features like coloboma, choanal atresia, facial nerve palsy, tracheo-oesophageal fistula, hypoplastic semicircular canals, micropenis or hypogonadotropic hypogonadism generally occur more often in patients with CHARGE syndrome than in those with 22q11.2 deletion syndrome, although not exclusively (see Figure 1). Because of the clinical resemblance between the two syndromes, in our recent review, we recommended that *CHD7* is a good candidate gene to analyse in patients with clinical features of 22q11.2 deletion syndrome, but who do not have a deletion or mutation of *TBX1*. We further recommended that a whole-genome array should be performed in patients suspected of CHARGE syndrome but without a *CHD7* mutation or deletion.[11]

The overlap between CHARGE syndrome and 22q11.2 deletion syndrome and the variable expression of both syndromes can hamper clinical diagnosis, but also provides interesting clues to the aetiology and pathogenesis of both syndromes. We provide further details of the overlap between the two syndromes by describing case reports of patients diagnosed with CHARGE syndrome, but carrying a 22q11.2 deletion, by reporting the typical 22q11.2 deletion features present in a *CHD7*-positive cohort, and by describing the results of *CHD7* sequencing in a cohort of patients with features of 22q11.2 deletion syndrome but without a deletion or mutation of *TBX1*. The molecular pathways underlying this clinical resemblance and the implications for genetic diagnostic work are discussed.

METHODS

A schematic overview of our study design is shown in Figure 2.

Figure 2. Overview of our study design



This flow diagram illustrates the three different parts of our study. The data in thickened boxes was available at the start of the study.
A: Clinical CHARGE patients with 22q11.2 deletions
B: Patients with a *CHD7* mutation and features of 22q11.2 deletion syndrome
C: *CHD7* analysis in patients clinically presenting as 22q11.2 deletion syndrome

Patients

We describe the clinical findings of two Dutch patients who were diagnosed with CHARGE syndrome according to the clinical criteria of Blake and/or Verloes, but who appeared to have a 22q11.2 deletion. We further summarise the available clinical data of three patients described in the literature with clinically typical CHARGE syndrome and a 22q11.2 deletion.

We screened our database of 802 patients with a pathogenic *CHD7* mutation for clinical features more specific for 22q11.2 deletion syndrome, like hypocalcaemia, thymus anomalies and immunological problems, or who were reported to have a DiGeorge or 22q11.2 deletion phenotype.

In addition, we analysed *CHD7* in a cohort of 20 patients from the Department of Paediatrics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, USA. These patients were selected from a cohort of 100 cases who were clinically suspected of 22q11.2 deletion syndrome, but in whom FISH did not detect a deletion of chromosome 22q11.2 and MLPA did not find any atypical deletions of the 22q11.22 region. In the patients selected for *CHD7* analysis, point mutations in the *TBX1* gene had also been excluded. The 20 patients were selected because their clinical features overlapped with features seen in CHARGE syndrome, including the presence of congenital heart defects, coloboma, immune defects, ear anomalies, renal malformations and hearing loss.

CHD7 analysis

Blood samples from all patients were drawn after informed consent. 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 earlier.[16] If no mutations were identified, *CHD7* was screened for whole exon deletions and duplications 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).[27]

Analysis of 22q11.2 deletions and *TBX1* sequencing

Patients gave informed consent and blood samples were taken. 22q11.2 deletions were detected or excluded in the Dutch patients by array comparative genomic hybridisation (CGH) using an Agilent 180 K oligonucleotide array (custom design no. 023363, Agilent Technologies Inc., Santa Clara, CA, USA) and/or fluorescence in situ hybridisation (FISH) using probe RP11-481H20 and RP11-590C5. The array-CGH procedures were carried out according to the manufacturer's protocols. Normal male or female reference DNA was used as a control and DNA analytics version 4.0.81 (Agilent) was used to analyse the results.

The 20 Philadelphia cases were studied by FISH using the commercially available probe N25 and then analysed by an MLPA assay specific for the chromosome 22q11.22 region (MRC Holland; SALSA P250 kit) to identify deletions whose proximal endpoints are distal to the standard FISH probes and would not have been identified in the original cohort.[28] Point mutations in the *TBX1* gene were excluded by capillary sequencing following PCR amplification as previously reported.[29]

RESULTS

Clinical CHARGE patients with 22q11.2 deletions

Table 1 lists all the patients with a CHARGE syndrome phenotype known to carry a 22q11.2 deletion, including the two described below.

Table 1. Clinical features in patients with clinical CHARGE syndrome and a 22q11.2 deletion

		Patients				
		Emanuel[22]	*Digilio*[23]	*Devriend*[24]	*Our Pt1*	*Our Pt2*
Features	***Coloboma***	**u**	**+**	**+**	**+**	**-**
	Heart defect	u	+	+	+	+
	Choanal atresia*	**u**	**+**	**+**	**+**	**+**
	Growth retardation	u	u	+	u	u
	Developm. delay	u	u	+	+	+
	Genital hypoplasia	**u**	**+**	**-**	**-**	**-**
	Ear anomaly	u	+	+	-	+
	SCC hypoplasia	**u**	**u**	**u**	**+**	**-**
	Cleft lip and/or palate	u	-	-	-	-
	Hearing loss	u	u	+	+	-
	*Feeding difficulties***	u	u	u	+	+
	Facial palsy	**u**	**u**	**u**	**-**	**-**
	TE-anomaly	**u**	**-**	**u**	**-**	**-**
	Hypocalcaemia	u	+	-	-	-
	Immunological abn.	u	+	u	-	-
	Thymus abn.	u	-	-	u	-
	Other	CHARGE association, no further information	unilateral absent radius, hypoplastic ulna	pre-auricular tags, pit in left cheek	Bronchotra-cheomalacia, GERD	pharyngo-malacia

Features more commonly seen in CHARGE syndrome than in 22q11.2 deletion syndrome are highlighted in bold.

+ present; – not present; u unexamined/unknown

* atresia or stenosis of choanae

** only if necessitating tube feeding

Abn., abnormalities; Developm., developmental; GERD, gastro-oesophageal reflux disease; SCC, semicircular canal; TE-anomaly, Tracheo-oesophageal anomaly.

Patient 1

This girl was briefly described by Bergman et al.[11] She was born by Caesarean section because of foetal distress at 33+3 weeks of gestation. The pregnancy was complicated by pregnancy-related diabetes and polyhydramnios. Her birth weight was 2830 grams (97.7th percentile), and her APGAR scores were 9 and 9 after 1 and 5 minutes, respectively. During the first four weeks of life she had feeding problems, necessitating nasogastric tube feeding. She was diagnosed with bronchotracheomalacia and gastro-oesophageal reflux disease. Several congenital anomalies were noticed: coloboma of the left iris and both retinas, unilateral choanal stenosis, mild pulmonic stenosis, and bilateral mixed hearing loss. A CT scan of the mastoid showed abnormal semicircular canals as well as an abnormal vestibulum and an abnormal basal convolution of the cochlea. The girl had a developmental motor delay; she started walking at the age of 2 years and 3 months. At her last examination, at the age of 3 years, she had a normal language comprehension (quotient score of 91, comprehension scales of the Dutch Reynell Developmental Language Scales). Physical examination showed simple ears, an anteriorly placed anus and a hockey-stick line crease on both palms. The diagnosis CHARGE syndrome was made based on the clinical diagnostic criteria of both Blake and Verloes.[9,10] Analysis of *CHD7* did not show a mutation or deletion. Subsequently, array CGH showed a *de novo* 2.6 Mb loss of 22q11.2 (proximal breakpoint 17,210,818-17,270,293; distal breakpoint 19,891,492-19,870,318). The deletion was confirmed by FISH analysis.

Patient 2

This female infant was born prematurely at 33+4 weeks, with a birth weight of 1565 grams (10th – 20th percentile). The pregnancy was complicated by polyhydramnios. Postnatally, she experienced respiratory distress and was suspected of having a partial choanal atresia. She had a congenital heart defect consisting of ASD, VSD and PDA. Morphologic evaluation at the age of 2 days revealed microcephaly (28 cm; -5.6 SD below mean), short palpebral fissures, dysmorphic ears with broad superior crus of the antihelices, overfolded helices and a bulbous nasal tip. CHARGE syndrome or a chromosomal abnormality were suspected and array CGH, fundoscopy, renal ultrasound, and brain imaging with special attention for the semicircular canals were suggested. Array CGH showed a 2.9-3.0 Mb deletion of 22q11.21 (proximal breakpoint 17,210,818-17,270,293; distal breakpoint 20,142,009-20,247,225) that was confirmed by FISH analysis. The other investigations had normal results, except for subtle abnormalities of the brain MRI scan, with slightly delayed myelinisation and mildly enlarged ventricles.

At the age of 2 months the VSD was surgically corrected. From age 4-22 months she required a tracheostomy because of the combination of very narrow choanae and pharyngomalacia. At the age of 10 months, a gastrostoma with feeding tube were placed. At her last examination at the age of 2 years and 4 months, her height was 86 cm (-1 SD). Her head circumference was not measured, but she was normocephalic before (at the age of 22 months her head circumference was 47.7 cm (-0.7 SD)). She was still being fed through her feeding tube and had just started to use some spoken words, in addition to sign language, after the removal of her tracheotomy.

CHD7 mutations in phenotypic 22q11.2 deletion syndrome

Patients with a CHD7 mutation and features of 22q11.2 deletion syndrome

Table 2 summarises the 30 patients out of our international database of 802 patients in whom typical features of 22q11.2 deletion syndrome were described.[8] We also included three additional patients from the literature in whom the precise *CHD7* mutation was not mentioned.[30] All 33 patients have features that are more commonly seen in deletion 22q11.2 syndrome than in CHARGE syndrome. A 22q11.2 deletion was excluded in 25 of the 33 patients (76%). In one patient, a paternally inherited 2.5 Mb 22q11.23 deletion, located distal to the 22q11.2 deletion syndrome region, was identified in addition to the *CHD7* mutation.[31] At least 16 of the 33 patients died in infancy.

CHD7 analysis in patients clinically presenting as 22q11.2 deletion syndrome

We identified 5 pathogenic *CHD7* mutations in our group of 20 patients that were clinically suspected of 22q11.2 deletion syndrome.

Table 3 summarises the mutations and known clinical features of the five patients clinically presenting as deletion 22q11.2 syndrome, but in whom a *CHD7* mutation was identified. Remarkably, all five patients carried a truncating mutation in

Table 2. Patients with a *CHD7* mutation who show clinical features of 22q11.2 deletion syndrome

		Features																
ID	Type of mutation	*Coloboma*	*Heart defect*	*Choanal atresia**	*Growth retardation*	*Developm. delay*	*Genital hypoplasia*	*Ear anomaly*	*SCC hypoplasia*	*Cleft lip and/or palate*	*Hearing loss*	*Feeding difficulties***	*Facial palsy*	*TE-anomaly*	Hypocalcaemia	Immunological abn.	Thymus abn.	*Other*
0[60]	del	+	+	-	-	u	+	+	-	-	u	u	+	u	+	+	+	
0[49]	del wg	-	+	-	u	+	u	+	u	-	u	u	u	u	u	u	u	22q11 phenotype, long slender fingers
P99	fs	+	+	-	u	+	u	+	u	u	+	+	u	+	u	+	+	
P129[60]	fs	+	+	+	+	+	+	+	+	-	+	u	+	u	+	+	u	PTH def
P137	fs	+	+	-	+	+	u	+	+	u	+	+	u	-	u	u	+	bronchomalacia, torticollis, GERD, absent thumbs
P147	fs	+	+	-	+	u	+	+	u	u	+	u	u	-	u	u	+	fissure upper lobe right long, hypothyroidism
P197[31]	fs	+	+	-	u	u	+	+	+	u	u	u	+	u	+	+	+	laryngomalacia, erythroderma, total alopecia
P238	fs	u	u	u	u	u	u	u	u	u	u	u	u	u	u	+	u	
P245[57]	fs	+	+	u	u	+	u	+	u	u	u	u	u	u	+	u	+	
P293[65]	fs	+	+	u	u	u	u	u	u	u	+	u	u	+	+	+	+	tracheomalacia, PTH def, ectopia of one kidney
P304	fs	+	u	+	u	u	+	+	-	u	+	u	u	u	+	+	u	
P767	fs	-	+	u	-	+	+	+	+	-	+	+	+	-	+	u	u	PTH def, OSAS
P800[16]	fs	+	+	-	-	+	+	+	+	-	+	+	+	+	u	-	+	obstructive apneus
P866	fs	u	u	u	u	u	+	+	u	u	u	u	u	u	+	u	u	
P875	fs	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	laryngeomalcia, hypothyreoidy, vermis dysplasia
P987[63]	fs	+	+	u	u	u	+	+	u	u	+	u	u	u	+	u	u	PTH def, limb abnormality
P29[57]	non	+	+	u	u	u	+	+	u	+	u	u	u	u	u	u	+	Abnormal limbs
P40	non	+	+	+	+	u	u	+	u	u	u	u	u	u	u	+	u	
P37[58]	non	-	+	+	u	u	+	+	+	u	+	u	-	-	+	u	+	PTH def
P44[59]	non	+	+	+	u	u	-	+	u	u	u	u	+	u	+	+	+	PTH def, laryngomalacia, abnormal limbs
P122	non	+	+	+	u	u	+	u	u	u	u	u	u	+	+	+	u	
P189[58]	non	u	+	-	u	u	+	+	u	u	-	u	u	+	+	+	u	hypothyroidism, partial agenesis corpus callosum, horseshoe kidney

Table 2. Patients with a *CHD7* mutation who show clinical features of 22q11.2 deletion syndrome (continued)

		Features																
ID	Type of mutation	*Coloboma*	*Heart defect*	*Choanal atresia**	*Growth retardation*	*Developm. delay*	*Genital hypoplasia*	*Ear anomaly*	*SCC hypoplasia*	*Cleft lip and/or palate*	*Hearing loss*	*Feeding difficulties***	*Facial palsy*	*TE-anomaly*	Hypocalcaemia	Immunological abn.	Thymus abn.	*Other*
P256[61,62]	non	u	+	u	u	u	+	u	u	+	u	u	u	u	u	u	+	terminated pregnancy, left isomerism, hypoplastic optic nerves
P279[13,16,52]	non	-	+	+	+	+	+	+	+	-	+	+	u	-	u	-	+	cortical brain atrophy
P780[13,16]	non	-	+	+	+	+	+	+	+	-	+	-	-	+	+	-	-	NEC, hydrocephalus, GERD
P834[16]	non	+	+	u	+	+	+	+	+	+	+	u	+	u	u	u	+	laryngomalacia, PTH def, corpus callosum hypoplasia, horseshoe kidney
P898	non	+	+	u	u	u	u	+	u	+	+	u	u	u	u	+	u	hydrocephaly
P1003[64]	non	-	+	+	u	u	-	+	+	u	u	u	u	u	+	+	+	glottic web, laryngomalacia, vertical talus
0[57]	non	u	+	u	u	u	+	+	u	u	u	u	u	u	+	u	+	pre-auricular tags
P616	spl	+	+	-	+	u	+	+	+	u	u	u	+	-	+	u	+	scalp cutis aplasia, PTH def
0[30]	U	+	+	u	u	u	u	+	u	-	+	u	u	+	u	+	+	
0[30]	U	+	+	+	+	+	u	u	u	-	+	u	+	u	u	+	u	seizures
0[30]	U	+	+	u	u	u	u	u	u	-	+	u	-	u	u	+	u	bulbar palsy

Features more commonly seen in 22q11.2 deletion syndrome than in CHARGE syndrome are highlighted in bold.
ID: patient ID in online database of *CHD7* mutations www.chd7.org. 0, not mentioned in the online database. Superscript numbers of references:
Type of mutations: del, deletion exon 4; del wg, whole gene; fs, frameshift; non, nonsense; spl, splice site; U, mutations unknown (all patients described in reference [30]).
Features:
* atresia or stenosis of choanae
** only if necessitating tube feeding
Abn., abnormalities; Developm., developmental; GERD, gastro-oesophageal reflux disease; NEC, necrotising enterocolitis; OSAS, obstructive sleep apnea syndrome; PTH def, low parathyroid hormone or hypoparathyroidism; SCC, semicircular canal; TE-anomaly, Tracheo-oesophageal anomaly.

the *CHD7* gene. In addition, we identified two silent *CHD7* mutations (c.4014C>T, p.Gly1338Gly and c.6216C>G, p.Pro2072Pro) in two other patients. Both silent mutations had been identified in the 1000 Genomes project (DbSNP rs199828744, rs188679907).

DISCUSSION

We highlight the clinical overlap between 22q11.2 deletion syndrome and CHARGE syndrome. Although some of the clinical features are present far more often in one of the syndromes (Figure 1), none are seen exclusively in either 22q11.2 deletion or CHARGE syndrome. For example, the presence of choanal atresia or semicircular canal hypoplasia does not exclude a 22q11.2 deletion, and severe T-cell dysfunction also occurs in CHARGE syndrome.

Remarkably, almost exclusively we found truncating *CHD7* mutations in both groups of patients suspected of having *TBX1* haploinsufficiency (Tables 2 and 3). This is in line with our previous observation that *CHD7* missense mutations result in a milder phenotype. Features also seen in 22q11.2 syndrome, like congenital heart defects and cleft palate, occur more often in patients with a truncating mutation than in those with a missense mutation.[32] Thus, the clinical overlap between these syndromes is predominantly seen in the more severely affected patients with a 22q11.2 deletion or *CHD7* mutation.

Table 3. Features of five patients with clinical 22q11.2 deletion syndrome but without *TBX1* haploinsufficiency in whom *CHD7* mutations were found

Patient ID	CHD7 c.	CHD7 p.	Clinical features Typical CHARGE features are indicated in bold
CH95-172	493_496delinsGG	Pro165fs	suspected of having 22q11.2 deletion syndrome, no further information available
CH95-218	2440C>T	Gln814X	ADHD, speech therapy, sensorineural/ conductive hearing loss, scoliosis, myopia, retinal coloboma, dysmorphic features
CH94-143	3024T>G	Tyr1008X	thymic hypoplasia, CLP, hypocalcemia, normal cardiac ultrasound, auricular dysplasia
CH96-184	4357C>T	Gln1453X	low PTH, low Ca, low T cells, small thymus, dysmorphia, micrognathia, low-set malformed ears, choanal atresia, velopharyngeal incompetence, laryngotracheomalacia, ASD
CH99-214F	4424del	Glu1475fs	suspected of having 22q11.2 deletion syndrome, no further information available

ADHD, attention deficit hyperactivity disorder; ASD, atrial septal defect; Ca, calcium; CLP, cleft lip and palate; PTH, parathyroid hormone.

How can we explain the remarkable clinical overlap between these two syndromes? It is possible that both syndromes could be present simultaneously, but this must be extremely rare since no cases have been reported yet. However, it cannot be excluded since not all mutations in *CHD7* and *TBX1* are detectable by current techniques. It is well known that no *CHD7* mutation can be detected in 5-10% of the patients with a clinical diagnosis of CHARGE, suggesting either undetectable *CHD7* mutations (e.g. in the promoter region) or the existence of a second gene that can cause CHARGE syndrome when mutated. Nonetheless, both syndromes should be included in a common differential diagnosis, as discussed in our recent reviews.[11,17,18]

The most likely explanation for the phenotypic overlap between both syndromes is that the causative genes, *CHD7* and *TBX1*, function in the same embryonic pathway or in pathways with a common target. The *CHD7* gene is expressed ubiquitously during human embryonic development, with a high expression in the foetal inner ear, eye, central nervous system and in the neural crest of the pharyngeal arches.[33] The CHD7 protein belongs to the Chromodomain Helicase DNA-binding (CHD) family,[34] and is thought to regulate gene transcription by ATP-dependent chromatin modification during embryogenesis.[15] CHD7 cooperates with, amongst others, PBAF (polybromo- and BRG1-associated factor containing complex) in controlling neural crest gene expression and cell migration.[35]

The *TBX1* gene codes for the T-box transcription factor TBX1 that regulates the expression of downstream growth and transcription factors that are involved in the development of the heart, thymus, parathyroid and palate. TBX1 physically interferes with SMAD1, influencing its binding to SMAD4 and thus signal transduction.[36] Interestingly, CHD7 was found to co-localise with SMAD1 and other transcription factors at enhancer elements near genes that are repressed.[37] Thus, both TBX1 and CHD7 regulate gene transcription and might well regulate the transcription of the same genes.

Mice with heterozygous *Chd7* mutations show semicircular canal defects, septal heart defects, cleft palate, choanal atresia, hyposmia, olfactory bulb anomalies, testes hypoplasia, hearing loss and low body weight.[38-43] Other CHARGE features, e.g. coloboma, external ear anomalies, and tracheo-oesophageal defects, have not been reported in Chd7-deficient mice. This discrepancy in phenotype between man and mice might be caused by species-specific differences in CHD7 requirements or differences in genetic background.[12]

Mice with haploinsufficiency for Tbx1 show the full range of malformations that can be present in the 22q11.2 deletion syndrome.[21] Tissue-specific conditional mutagenesis of Tbx1 has shown its role in the secondary heart field,[44] pharyngeal mesoderm,[45] pharyngeal endoderm,[46] pharyngeal epithelia,[47] and otic epithelium.[48]

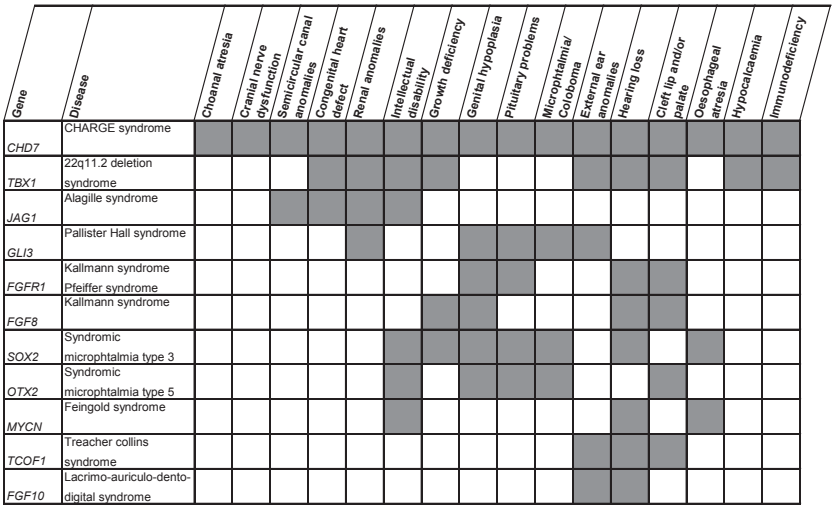
Surprisingly, mice with a double heterozygous mutation of *Chd7* and *Tbx1* show a severe cardiovascular phenotype and severely reduced postnatal viability compared to mice with a heterozygous mutation of *Chd7* or *Tbx1*.[49] The synergistic haploinsufficiency of both genes resulted in an enhanced effect on the fourth pharyngeal arch morphogenesis, abnormal thymus development, and malformations of the semicircular canals. These observations in mice, together with our observations in the patients presented in this paper, suggest that both genes act in the same developmental pathway. Randall et al. hypothesised that Chd7 might modulate *Tbx1* expression, but was unable to prove that the expression of either gene changed in mouse embryos mutated at the other locus.[49] Hurd et al. showed that *Tbx1* expression was expanded more ventrally in the developing inner ear of a *Chd7* null mouse conditional mutant ($Chd7^{Gt/Gt}$) compared to the wild-type mouse embryo. This effect was not seen in heterozygously mutated mice ($Chd7^{Gt/+}$), suggesting that there is a dose-dependent inhibiting effect of Chd7 on Tbx1 in the inner ear, which might be essential for inner ear neurogenesis.[42]

As an alternative theory, a shared convergent pathway via FGF8 has been suggested, but has not been proven.[49] It was shown that reduced CHD7 dosage in the olfactory placode, pituitary and hypothalamus in mice reduced the expression of the FGF8 receptor FGFR1.[50] FGF8 and its receptor FGFR1 are interesting linking factors, since both are also involved in the pathogenesis of other organs frequently affected in CHARGE syndrome, like the combination of hypogonadotropic hypogondism and anosmia.[51-53] The fact that this combination is seldom seen in patients with 22q11.2 deletion could be explained by the more ubiquitous expression of *CHD7* compared to *TBX1*.

The tight relationship between 22q11.2 deletion syndrome and CHARGE syndrome is not an isolated observation. Both syndromes share common features with other syndromes that may reveal further clues for interaction of their causative genes and underlying embryonic pathways. For instance, *SOX2* mutations result in a phenotype characterised by anophthalmia, tracheo-oesophageal abnormalities, pituitary defects and genital abnormalities. Like CHD7, SOX2 is assumed to play a role in neural stem cells and Engelen et al. showed that CHD7 is a SOX2 transcriptional cofactor, with their common target genes being JAG1, GLI3 and MYCN

(NOTCH and SHH signalling pathways).[6] SHH signalling regulates the expression of TBX1 in the pharyngeal arch probably through transcription factors of the FOX family.[54] TBX1 has also been described as an upstream regulator of the NOTCH-signalling effector HES1 in the pharyngeal arch and a downstream target of JAG2 in tooth morphogenesis.[55,56] CHD7 and TBX1 have been described to interact with proteins known from other overlapping syndromes (Figure 3). Thus, CHD7 and TBX1 may also interact indirectly through different pathways, like the NOTCH and SHH signalling pathways.

Figure 3. Multiple congenital anomaly syndromes having clinical overlap with CHARGE syndrome and 22q11.2 microdeletion syndrome



In Figure 3 we show the overlapping clinical features of CHARGE and 22q11.2 deletion syndrome with known genetic syndromes. All the genes mentioned in this figure or their proteins have been associated with either CHD7 or TBX1. The expression of FGFR1, OTX2 and TBX1 depends on CHD7 in some tissues, TBX1 and FGF8 are in epistasis in ectodermal development, binding of the protein treacle, which is encoded by TCOF1, partly depends on the presence of CHD7, and SOX2 and CHD7 are co-factors that regulate the expression of JAG1, MYCN, GLI3, amongst others.[6,14,49,50,66] Figure 3 shows that the linked molecular pathways are reflected by the shared clinical features of the syndromes.

In conclusion, the clinical diagnosis of two highly variable syndromes, CHARGE syndrome and 22q11.2 deletion syndrome, can prove challenging. The syndromes should therefore be included in a common differential diagnosis and we strongly recommend performing *CHD7* analysis in any patients with a 22q11.2 deletion

phenotype but without TBX1 haploinsufficiency, and performing a genome-wide array for 22q11.2 deletions in clinical CHARGE patients without a *CHD7* mutation. We have shown there is strong clinical evidence that both molecular pathways are linked, although the precise nature of this link needs further exploration.

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CHAPTER 5.2

Definition of 5q11.2 microdeletion syndrome reveals overlap with CHARGE syndrome and 22q11 deletion syndrome phenotypes

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ABSTRACT

Microdeletions of the 5q11.2 region are rare; in literature only two patients with a deletion in this region have been reported so far. In this study, we describe four additional patients and further define this new 5q11.2 microdeletion syndrome. A comparison of the features observed in all six patients with overlapping 5q11.2 deletions showed a phenotypic spectrum that overlaps with CHARGE syndrome and 22q11.2 deletion syndrome including choanal atresia, developmental delay, heart defects, external ear abnormalities, and short stature. No colobomas or abnormalities of semicircular canals and olfactory nerves were reported. Two male patients had genital abnormalities. We estimated a 2.0Mb (53.0–55.0 Mb) Shortest Region of Overlap (SRO) for the main clinical characteristics of the syndrome. This region contains nine genes and two non-coding microRNAs. In this region DHX29 serves as the candidate gene as it encodes an ATP dependent RNA-helicase that is involved in the initiation of RNA translation. Screening a small cohort of 14 patients who presented the main features, however, did not reveal any pathogenic abnormalities of DHX29.

Keywords: 5q11.2 microdeletion syndrome; DHX29; CHARGE syndrome; 22q11.2 deletion syndrome; intellectual disability; choanal atresia

INTRODUCTION

During the last decade, the introduction of genome-wide array technologies has led to the identification of numerous novel microdeletion and microduplication syndromes, which previously escaped detection by routine cytogenetic and molecular cytogenetic techniques.[1,2]

The phenotypic characteristics of some microdeletion syndromes were shown to be caused by haploinsufficiency of single genes, such as EHMT1 in Kleefstra syndrome,[3] RAI1 in Smith Magenis Syndrome,[4] and TCF4 in Pitt-Hopkins syndrome.[5] Moreover, the application of genome-wide array technologies and the study of overlapping microdeletions has led to the identification of several novel genes associated with developmental disorders or abnormal brain development such as CHD7 in CHARGE syndrome[6] and FOXG1 in the congenital variant of Rett syndrome.[7]

In the current study, we aimed to define the 5q11.2 microdeletion syndrome and to identify the genetic cause of its core phenotype, by collecting individuals with overlapping deletions comprising the 5q11.2 region.

In literature, so far only two individuals with microdeletions in the 5q11.2 region were described. The first had a short stature, learning and behavioral difficulties, tetralogy of Fallot, a bifid uvula, and velopharyngeal insufficiency.[8] The other patient had multiple congenital abnormalities (MCA) comprising tracheal agenesis, cartilage rings in the esophagus, an aberrant right bronchus, anal atresia, cryptorchid testes, and mild dysmorphic features.[9] This patient died already at neonatal age.

We recently diagnosed two patients with overlapping 5q11.2 deletions. Interestingly, in both cases, CHARGE syndrome was among the differential diagnoses because the patients presented with several features commonly observed in CHARGE syndrome as choanal atresia/stenosis, developmental delay, growth retardation, and external ear abnormalities. Some of these features are also common in patients with the 22q11.2 deletion syndrome. So, apparently, heterozygous loss of the 5q11.2 region is associated with a certain spectrum of MCA. This led us to the hypothesis that this MCA phenotype might be explained by haploinsufficiency of one gene or contiguous genes located at 5q11.2.

To further investigate genotype–phenotype correlations we collected additional cases with overlapping 5q11.2 deletions. In total, we review the clinical and molecular characteristics of four novel cases and two previously published cases and aim to estimate the critical region for this MCA syndrome.

PATIENTS AND METHODS

Patients

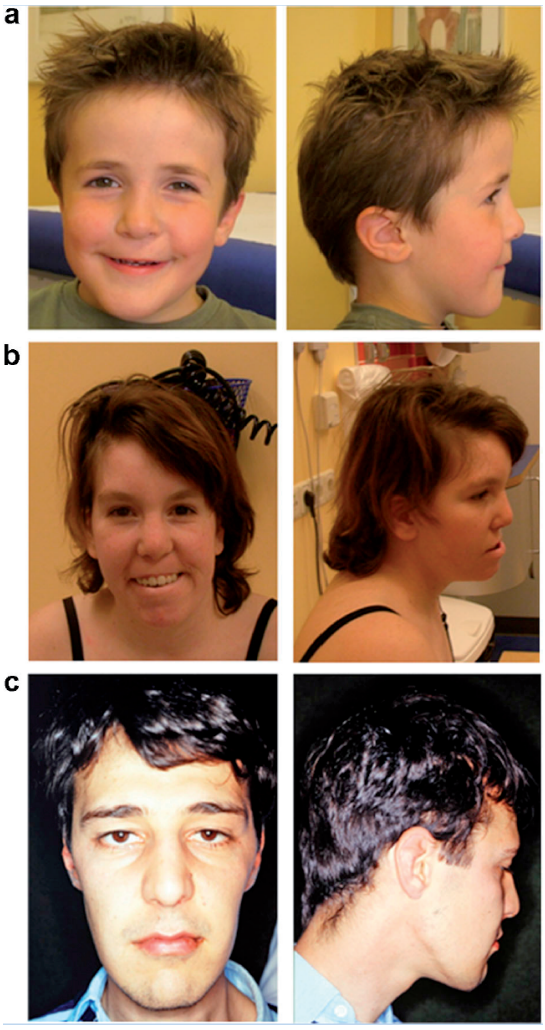
The clinical and molecular data of Patients 1 and 2 were obtained by our clinical genetic diagnostic center. The other two cases (Patient 3 and Patient 4) were collected through our international network. All patient's legal representatives provided informed consent for the use of their data and photographs.

Patient 1

Patient 1 was born after an uncomplicated pregnancy of 39þ4 weeks, with a birth weight of 4,120 g (+1.5 SD). The patient was the third child of healthy parents. The first child, a boy, suddenly died at the age of 14 months, with unknown cause. He had an apparently normal development. The second child was a boy with tricho–dento-osseous dysplasia.

The Apgar score of the index patient was 9/10.When he was 4 days old, he was hospitalized for 3 weeks because of a RS-virus infection, feeding problems, and weight loss. During this period, he received nasogastric tube feeding and it was noticed that the choanae were very narrow. The first year he showed a delayed development, there was no eye contact until the age of nine months. Feeding problems and growth retardation continued. He suffered from colds and recurrent ear infections, for which he received ear tubes. At the last examination, at the age of 7 years he had a height of 117cm (-2.3 SD) and a head circumference of 52 cm (0 SD). Beside his short stature, he showed some dysmorphic features; down slanting palpebral fissures, strabismus, posteriorly rotated ears, and brachycephaly (Figure 1a). He had hirsutism, in particular at the position of his spinal column, a mild pectus excavatum, and an asymmetric thorax due to hypoplasia of thorax muscles. He had an intellectual disability and a delayed speech development. His total IQ was 50. He showed behavioral problems and was diagnosed with autism.

Figure 1. Facial profiles of patients



a. Patient 1 at an age of 7 years showing down slanting of the eyes, brachycephaly and posteriorly rotated ears.
b. Patient 2 at an age of 22 years showing a flat midface and asymmetry of the face.
c. Patient 4 at an age of 24 years with a sloping forehead, flattened tip of the nose and coarse, underdeveloped external ears.

Patient 2

Patient 2 was born after a pregnancy of 37 weeks, with a birth weight of 2,500 g (-1 SD). She is the second child of parents with normal intelligence, her father, however, died of a frontal/ethmoidal sinus carcinoma. She had neonatal feeding problems and growth retardation. She had unilateral choanal atresia (right side), velopharyngeal insufficiency, and a nasal speech which required several operations. She also had cochlear hearing loss (55 dB right and 47 dB left). At the age of 11 years, she was hospitalized with encephalomyelitis with unknown cause. She was treated with growth hormone from her 4th until 16th year of life. Upon her last referral at the age of 22 years, she had a height of 144 cm (-4 SD) and a head circumference of 51.5 cm (-2.3 SD). She had flat and asymmetric face, bulbous nose, clinodactyly of both fifth fingers and hirsutism (Figure 1b). Her total IQ was 51. She also showed behavioral problems, including aggressive outbursts and stereotypic movements, at this age. An EEG showed diffuse abnormalities, but no seizures were observed. Because of the characteristics similar to CHARGE syndrome, CHD7 was screened for mutations and intragenic deletions/duplications but showed no abnormalities. At the age of 22 years, she stayed in an assisted-living environment and was able to do simple work.

Patient 3

Patient 3 was born after a pregnancy of 32 weeks with a birth weight of 1,247 g (-1.6 SD). He was found to have bilateral choanal atresia which required immediate and repeated surgical dilation over the first 4 years of life. He required nasogastric feeding for the first few months and then had a PEG tube until the age of 5 years but thereafter, no feeding difficulties were present. He had intestinal malrotation and constipation. He also had hypospadias, penile chordae, left side cryptorchidism, and a right-sided inguinal hernia. At the age of 10 years, he had no significant growth retardation, his height was 132.4 cm (-1.0 SD), weight 32.45 kg (+0.2 SD), and head circumference 54.9 cm (+0.3 SD). He showed frontal bossing and had dysplastic ears. At this age he was attending a mainstream school with learning support. He was diagnosed with a mild autistic spectrum disorder. He had no cardiac problems, and no visual or hearing problems. He has had a few fractures with trauma. He was generally in good health. A diagnosis of atypical CHARGE was made and he has CHD7 analysis performed, but this did not reveal any pathogenic variants.

Patient 4

Patient 4 was born by spontaneous vaginal delivery after a 40-week pregnancy complicated by threatened abortion in the fourth month of gestation. His birth

weight was 4,300 g. In the third month of life, an episode of ear infection with high fever is recalled. He walked at the age of 11 months. He had absence seizures since 2 years and started drug treatment. He had severe intellectual disability, speech problems, and behavioral problems. At the age of 24 years, his height was 173.6 cm (-0.5 SD). A brain MRI at the age of 43 years demonstrated cerebral atrophy, dilation of the occipital horns of the lateral ventricles, and hyper intense spots in the periventricular white matter. This patient had several facial dysmorphisms, including a sloping forehead, flattened tip of the nose, and coarse, underdeveloped external ears. He also had myopic maculopathy (Figure 1c).

METHODS

Array Analysis

The microdeletion screening in the patients included in this study was performed on a number of different array platforms. Patients 1 and 2 were analyzed on the AffymetrixGeneChip 250k (NspI) SNP array platform (Affymetrix, Inc., Santa Clara, CA). Patient 3 was analyzed on the Nimblegen ISCA array (Roche, Basel, Switzerland) and Patient 4 was analyzed on the Human Genome CGH Microarray 60K (Agilent Technologies, Palo Alto, CA). Hybridizations were performed according to the manufacturer's protocols. For the interpretation of the array results, the Human Genome Browser, February 2009 freeze (Hg19/GRCh37) was used in all cases (http://genome.ucsc.edu).

RESULTS

Clinical Delineation

The patients included in this study show a spectrum of features that are common in CHARGE syndrome and/or 22q11.2 microdeletion syndrome. The clinical features of the four patients in this study and the two previously published cases[8,9] are summarized in Table 1. In the patient published by de Jong et al.,[9] the developmental status and further growth parameters are unknown since this patient died at neonatal age.

Table 1. Patient characteristics

	Patient 1	Patient 2	Patient 3	Patient 4	Prescott et al.	De Jong et al.
Genomic location of deletion from 5pter (in Mb) in Hg19/GRCh37 (Feb.2009)	49.8-58.4	50.5-56.3	50.4-55.4	53.0-56.9	50.3-56.8 (maximum deleted region)	51.0-55.0 (maximum deleted region)
Array platform used	Affymetrix 500k	Affymetrix 250k	Nimblegen ISCA Array	Agilent 60k	BAC Array (details unknown)	Agilent 105K
Clinical features						
Coloboma*	-	-	-	-	-	-
Choanal atresia/hypoplasia*	+	+	+	-	NR	NR
Hypoplastic semicircular canals*	-	-	-	-	NR	NR
External ear abnormalities	+	-	+	+	NR	+
Hearing loss	-	+	-	-	NR	NA
Genital defect	-	-	+	-	-	+
Developmental delay	+	+	-	+	+	NA
Cardiovascular malformations	-	-	-	-	+	+
Growth retardation	+	+	-	-	+	NA
Velopharyngeal defects	-	+	-	-	+	-
Tracheoesophageal abnormalities	-	-	-	-	-	+
Facial dysmorfism	Down-slanting palpebral fissures, posteriorly rotated ears, brachycephaly	Flat face, asymmetric face, bulbous nose	Frontal bossing, dysplastic ears	Sloping forehead, flattened nasal tip, coarse, underdeveloped external ears	Asymmetrical face, prominent ears, thin lips	NR
Behavioural problems	+	+	-	+	+	NA
Feeding problems	+	+	-	-	NR	NA
Hirsutism	+	+	-	-	NR	NR
Seizures	-	+	-	+	+	NR

NA: not applicable, NR: not reported, + present, - absent

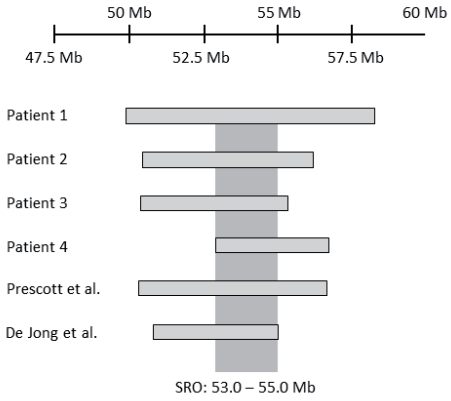
* Major signs of CHARGE syndrome (Verloes criteria)

At least three of the six patients in this study have choanal atresia or stenosis. Three out of five patients showed growth retardation. Four out of five patients had developmental delay, varying from language delay to severe intellectual disability. Five patients showed behavioral problems two were diagnosed with autism spectrum disorder and one showed aggressive behavior and stereotypic movements. Two patients had seizures. Velopharyngeal insufficiency was seen in two patients, one patient had tracheal agenesis. Main facial dysmorphisms of patients in this study included external ear abnormalities (4/6 patients), frontal bossing (2/6 patients), and a flat midface (3/6 patients). Two male patients had genital abnormalities. Additionally, hirsutism was seen in two patients. None of the patients had colobomas, and no abnormalities of the semicircular canals and olfactory nerves were reported.

Array Results

The combined array results are shown in Table I and schematically depicted in Figure 2. The deletion sizes ranged from 3.9 to 8.6 Mb. The most proximal position was at 49.8Mb and the most distal at 58.4 Mb. The SRO appeared to be 2.0Mb in size, located at 53.0–55.0Mb and covering nine OMIM annotated genes, two OMIM annotated noncoding microRNAs, and 13 genes or sequences with unknown data (Table 2 and Figure 2). All deletions were checked in the parents and considered to be de novo, although the father in Patient 2 could not be tested because he was deceased.

Figure 2. Overlapping deletions and SRO



Schematic representation of deleted regions in the presented patients and previously reported patients, and shortest region of overlap (SRO). All deletions were mapped according to the UCSC human genome browser build 19 (2009). The SRO is 2.0 Mb (53.0-55.0) and shown as a grey bar.

Table 2. Genes and microRNAs in shortest region of overlap (OMIM-Annotated)

Gene	Name	OMIM	Gene / Protein Function
NDUFS4	NADH-Ubiquinone Oxidoreductase Fe-S Protein 4	602694	Subunit of mitochondrial complex I. Homozygous or compound heterozygous mutations can lead to Leigh syndrome.
HSPB3	Heat-Shock 27-KD protein 3	604624	A missense mutation in the gene encoding small heat shock protein B3 was discovered in 2 siblings with an assymmetric axonal motor neuropathy. This supports the theory that the small heat shock protein gene family coordinately plays a role in motor neuron viability.
ESM1	Endothelial Cell-Specific Molecule 1	601521	Mainly expressed in endothelial cells in lung and kidney tissues. Expression is regulated by cytokines, suggesting that it may play a role in endothelium-dependent pathologic disorders.
GZMK GZMA	Granzyme K and Granzyme A	600784 140050	Members of a group of related serine proteases that may function as a common component necessary for lysis of target cells by cytotoxic T lymphocytes and natural killer cells.
MIR449A MIR449B	MicroRNA 449A MicroRNA 449B	613131 613132	Non-coding
MCIDAS	MCIDAS/IDAS	614086	Geminin-binding partner that functions in cell cycle progression and may regulate proliferation and differentiation during development.
CCNO	Cyclin O	607752	In vitro translated UDG2 showed significant uracil-DNA glycosylase activity.

DISCUSSION

In this study, we further defined a novel 5q11.2 microdeletion syndrome, based on genotype–phenotype studies in six patients with overlapping deletions in the 5q11.2 region. We estimated a 2.0Mb minimal SRO (53.0–55.0 Mb) which was associated with choanal atresia, heart defects, developmental delay, velum insufficiency, short stature, external ear abnormalities, genital defects, behavioral problems, and hirsutism. Although facial dysmorphisms were reported in all patients, there is no consistent recognizable facial phenotype. The complete phenotype of these patients overlaps significantly with both CHARGE syndrome and 22q11 deletion syndrome. According to the updated Verloes criteria,[10] at least one patient meets the criteria for atypical CHARGE syndrome. In clinical setting, this diagnosis was considered in three of the patients in this study.

CHARGE syndrome and 22q11.2 deletion syndrome share many features. Cleft palate, cardiac malformations, hearing loss, growth retardation, developmental de-

lay, ear abnormalities, and renal abnormalities are relatively common in patients diagnosed with one of these two syndromes.[11,12] Due to the presence of choanal atresia in three of our patients, we particularly considered this microdeletion syndrome as "CHARGE-like syndrome." What seems to distinguish this syndrome from CHARGE syndrome is absence of coloboma of the eye and abnormalities in the semicircular canals, since these features are not present in any of the patients with the 5q11.2 deletions as far as we know.

About 58% of the clinically suspected CHARGE-patients have mutations in CHD7.[13] This gene encodes Chromodomain Helicase DNA-binding protein 7. CHD7 is an ATP-dependent DNA-helicase and a member of the MI-2/CHD protein family that regulates gene transcription by chromatin remodeling.[14] CHD7 is involved in the transcriptional activation of nuclear genes in a time dependent and cell-specific manner,[15,16] and also in the regulation of ribosomal RNA.[17]

Interestingly, amongst the nine genes and two noncoding micro-RNAs in the SRO, one gene, DHX29 (MIM 612720), encodes an ATP-dependent RNA-helicase that is involved in the initiation of RNA translation.[18] RNA helicases are highly conserved enzymes that play a role in RNA transcription, splicing, and translation.[19] DHX is one of the different families of human RNA helicases that are known.[19]

DHX29 is a DExH-box protein that is required for the translation of mRNAs with structured 5'UTRs.[18,20] DHX29 is important for translation initiation and cell proliferation.[21] Down-regulation of DHX29 causes reduced assembly of 80S ribosomal complexes and therefore, inhibits mRNA translation.[21] Silencing of DHX29 is also associated with the inhibition of cancer cell proliferation.[21] DHX29 is therefore, considered as the most important candidate gene in this novel 5q11.2 microdeletion syndrome. Haploinsufficiency for this gene might be the main contributor to the origin of the core symptoms of this syndrome, including short stature, developmental delay, velum insufficiency, heart defects, choanal atresia, and dysmorphisms.

Noteworthy, a search in the Decipher database (http://decipher.sanger.ac.uk) revealed an additional case with a deletion at 5q11.2 (Decipher 250281) that is presented with choanal atresia in addition to various other features. However, due to the large deletion of 16.4Mb (54.3–70.7 Mb) that was present in this case, we did not include this case in the analysis of the SRO. The presence of choanal atresia in this patient though, might suggest that a genetic locus for this feature

could be found in the region between 54.3 and 55.0Mb which also comprises the gene DHX29.

We subsequently studied a cohort of 14 patients with a CHARGE- or 22q11.2-like phenotype by screening for mutations in DHX29. They were selected based on the core phenotype of 5q11.2 deletions: growth retardation, velopharyngeal insufficiency, and or/choanal atresia/stenosis. All these patients were clinically suspected for CHARGE and/or 22q11.2 deletion syndrome, but had no mutations in CHD7 or deletions in the 22q11 region. No mutations in DHX29 could be identified in this cohort. Thus, the potential role of DHX29 in this 5q11.2 microdeletion phenotype is neither confirmed nor excluded, since the sample size of the patient cohort was relatively small.

Meanwhile, we cannot exclude one or more other genes in this region that could possibly contribute in the pathophysiology of the characteristic phenotype. Identification of further cases either through copy number variation detection or possibly by next generation sequencing, might reveal additional arguments for the candidate gene DHX29 proposed in this study.

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CHAPTER 6

Summary, general discussion and future perspectives

6.1 SUMMARY

Chapter 1 is a general introduction to CHARGE syndrome and *CHD7* variants in which the objectives and scope of this thesis are presented. The main aim of this thesis was to contribute to the knowledge about CHARGE syndrome caused by *CHD7* variants, with a special focus on heart defects, and to learn more about syndromes that clinically overlap with CHARGE.

Chapter 2 focuses on the *CHD7* gene. In **chapter 2.1** we give an overview of 528 pathogenic *CHD7* variants in 802 patients. The pathogenic variants were equally distributed along the coding region of the *CHD7* gene. The most common types of pathogenic variants were nonsense and frameshift variants. Most variants were unique, but 94 were recurrent, including 27 arginine to stop mutations. A pathogenic variant usually occurs *de novo*, but familial cases with variable phenotypes have been described with germline or somatic mosaicism. We built a locus-specific database listing all variants that is available online at www.CHD7.org. Furthermore, based on the number of pathogenic *CHD7* variants detected in live-born children in the Netherlands from 2006 to 2010 and the overall birth prevalence in these years, we have now estimated the birth incidence of CHARGE syndrome between 5.9 and 6.7 per 100,000 live born children per year.

In **chapter 2.2** we introduced a classification system for predicting the pathogenicity of missense variants using two computational algorithms and a structural model in combination with segregation and phenotypic data. Using this classification system, we classified 145 *CHD7* missense variants. Pathogenic *CHD7* variants are mainly located in the middle coding region of the *CHD7* gene. Furthermore, pathogenic missense variants are, in general, associated with a milder phenotype when compared to pathogenic truncating *CHD7* variants.

In **chapter 2.3**, using exome sequencing, we identified potential pathogenic *CHD7* variants that were previously missed in a diagnostic setting in four out of five CHARGE patients.

In **chapter 3** we studied the phenotypic spectrum of 280 patients with a pathogenic *CHD7* variant. Four features were almost always present in patients with a pathogenic *CHD7* variant: external ear anomalies, cranial nerve dysfunction, semicircular canal hypoplasia and delayed motor milestones. We explored the

mild end of the phenotypic spectrum of pathogenic *CHD7* variants by studying familial cases, mildly affected patients with a proven pathogenic *CHD7* variant, and the results of *CHD7* analysis in cohorts with only one CHARGE feature or with partly overlapping syndromes. Based on these results, we established guidelines for *CHD7* analysis, including advice on when evaluation of semicircular canals is warranted. We also gave our recommendations on the clinical surveillance of CHARGE patients.

In **chapter 4** we focused on heart defects due to *CHD7* variants. In **chapter 4.1** we studied the cardiac phenotype of 299 patients with a pathogenic *CHD7* variant. Heart defects occurred in 74% of these patients. The types of defects were variable, but atrioventricular septal defect (AVSD) and conotruncal heart defects were overrepresented in CHARGE syndrome compared to a EUROCAT-based group of patients with non-syndromic heart defects.

We focused specifically on arch vessel anomalies in **chapter 4.2** because they may be the cause of treatable feeding and breathing problems in CHARGE syndrome. We identified arch vessel anomalies in at least 14% of the patients, but our information was insufficient to link feeding or breathing problems to arch vessel anomalies.

In **chapter 4.3** we performed *CHD7* analysis in 46 patients with a conotruncal heart defect and/or AVSD and one other feature of CHARGE syndrome, but did not identify pathogenic *CHD7* variants. This indicates that *CHD7* analysis should not routinely be performed in patients with conotruncal heart defect or AVSD.

In **chapter 4.4** we summarized the clinical aspects of heart defects in CHARGE syndrome, as described in the previous chapters, and we reviewed the function of CHD7 in cardiovascular development, which showed that CHD7 is important in regulating other genes involved in cardiovascular development.

In **chapter 5** we focused on two clinically overlapping microdeletion syndromes. In **chapter 5.1** we studied the clinical overlap of CHARGE syndrome with 22q11.2 deletion syndrome, based on their molecular diagnosis. The phenotypes of both syndromes overlap especially for the features congenital heart defect, cleft palate, developmental delay, renal abnormalities and hearing loss. Even features that seem specific to CHARGE syndrome, e.g. coloboma, choanal atresia and semicircular canal anomalies, occur in a small percentage of 22q11.2 deletion syndrome patients. Inversely, features regarded specific for 22q11.2 deletion syndrome, e.g.

thymus hypoplasia, occur in patients with pathogenic *CHD7* variants. Based on these observations, we show that 22q11.2 deletions can be identified in clinical CHARGE patients and pathogenic *CHD7* variants in patients with phenotypically 22q11.2 deletion syndrome. This has consequences for the genetic work-ups of both these groups of patients.

In **chapter 5.2** we describe the clinical phenotype of six patients with a 5q11.2 deletion. The 5q11.2 microdeletion syndrome clinically overlaps with CHARGE syndrome and 22q11.2 deletion syndrome due to the presence of choanal atresia and stenosis, external ear defect, growth retardation, autism, velopharyngeal insufficiency and heart defects. The most interesting candidate gene in the smallest region of overlap is *DHX29*, which codes for a protein important for mRNA translation. However, we do not have enough evidence to prove haploinsufficiency of this gene causes the phenotype of 5q11.2 microdeletion syndrome.

6.2 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

This thesis has contributed to our knowledge of the phenotype of CHARGE syndrome caused by pathogenic *CHD7* variants, with a special focus on heart defects, and taught us more about overlapping phenotypes. The key results of this thesis are:

- A comprehensive overview of *CHD7* variants, an algorithm to interpret missense variants and an online database, www.CHD7.org, currently comprising over 1000 entries.

- Guidelines based on extensive phenotyping on when to perform *CHD7* analysis and recommendations for clinical surveillance of patients with a pathogenic *CHD7* variant.

- A 74% prevalence of cardiac anomalies in patients with a pathogenic *CHD7* variant, with a higher prevalence in patients with a truncating variant. Some types of heart defect were more prevalent, but pathogenic *CHD7* variants are not a major cause of these heart defects when isolated.

- Demonstration that CHARGE syndrome clinically overlaps with other syndromes, especially 22q11.2 deletion syndrome. 22q11.2 deletions can be identified

in clinical CHARGE patients, while pathogenic *CHD7* variants are identified in patients with 22q11.2 deletion phenotype.

In the following sections, I discuss these findings against the background of what is known and what still needs to be unravelled about CHD7 function; the identification and interpretation of *CHD7* variants and their clinical effects, most specifically on the heart; and the relation of *CHD7*-related phenotypes to other overlapping syndromes.

6.2.1 *CHD7* function

Since the discovery of the *CHD7* gene as a cause of CHARGE syndrome more than 10 years ago, several groups have focused on the function of CHD7. Published studies have shown that haploinsufficiency of CHD7 changes the transcription of tissue-specific target genes that are normally regulated by CHD7 or complexes in which CHD7 is involved.[1-4] The effect of CHD7 haploinsufficiency is tissue- and developmental-stage-dependent based on different binding sites, protein complexes and target genes (see chapter 2.1).[2,3] Functional studies have proven that CHD7 changes the position of nucleosomes at the DNA in the cell nucleus by acting as an ATP-dependent nucleosome remodeling factor.[5]

Knowledge about the function of the *CHD7* gene helps in understanding how one gene can cause such broad phenotypic variability. Current thinking is that the reduced level of CHD7 fluctuates mildly in tissues during development and that some organs are more sensitive to these fluctuations than others.[3,6] A major challenge remains the identification of the downstream target genes whose expression is affected if CHD7 goes below a specific threshold. These target genes are then responsible for the phenotype of CHARGE syndrome.[7] The hypothesis, and hope, is that by identifying CHD7's target genes, possible targets for therapy can be determined. In animal models some therapies have already shown an effect on the whole or a specific phenotype caused by *CHD7* defects. This has been seen, for example, in zebrafish, where down-regulating a specific histonic demythylase that represses ribosomal RNA can rescue the CHARGE phenotype caused by CHD7 haploinsufficiency.[8] However, I think a therapy to prevent phenotypes caused by a pathogenic *CHD7* variant in humans will not be available soon. Mostly because the risk of a pathogenic *CHD7* variant is generally not known prior to a pregnancy, since pathogenic *CHD7* variants usually occur *de novo.*

6.2.2 *CHD7* variants

Most of the pathogenic *CHD7* variants are truncating single nucleotide alterations and small deletions or insertion in the coding region, which can be detected by Sanger sequencing (see chapter 2.1).[9] The sensitivity of Sanger sequencing depends on the primers used. For example, ten pathogenic variants were identified in the first Sanger sequencing of *CHD7* in 17 patients (59% detection), however, upon re-analysis using a new primer set, five additional pathogenic variants were found, increasing the mutation detection rate to 88%.[10,11] The sensitivity of heteroduplex analysis, a method previously used to screen for mutations in larger genes, also proved to be low. A reanalysis of two samples from clinically typical CHARGE patients who were *CDH7* negative when heteroduplex was used identified a pathogenic *CHD7* variant in both cases (chapter 2.3). As a result, reanalysis of *CHD7* is warranted in typical CHARGE patients if heteroduplex analysis was used. Furthermore, *CHD7* analysis is not complete without MLPA screening to detect larger copy number alterations such as exon deletions, exon duplications or whole gene deletions.[12] These alterations have been identified in around 1% of patients with pathogenic *CHD7* variants.[9]

Even with these methods, pathogenic *CHD7* variants may still be missed in a typical CHARGE patient in a diagnostic setting. This could partly be due to a classification problem. For instance, we identified two intronic variants to be pathogenic that had been previously classified as benign. This indicates that re-analysis of *CHD7* and the variants identified might be useful in patients with clinically typical CHARGE syndrome but in whom no pathogenic *CHD7* variant had been detected. Nevertheless, in a minority of patients (less than 5%) with typical CHARGE syndrome, no *CHD7* alteration can be found after re-analysis, most likely because these alterations may be located in deep intronic regions or the promoter region. There is no evidence thus far that variants in other genes can cause typical CHARGE syndrome.

Interpretation of missense variants can be difficult. In 2011 we developed a classification system to predict the effect of missense variants based on the application of a computational algorithm and a structural model to a combination of phenotypic and segregation data (chapter 2.2).[13] Since the introduction of our system, exome sequencing techniques have become widely available. DNA of individuals without CHARGE syndrome have been exome sequenced revealing variants in many genes including *CHD7*. This further necessitates the need for interpretation guidelines. The information from large open access databases can help in the classification of variants, especially in the identification of benign variants, although it should be noted that a mild effect of a variant is very difficult to exclude. Functional analysis

is also becoming more and more important for interpreting whether variants are pathogenic or benign. For CHD7, for instance, in vitro analysis of the effect of variants on remodeling activity have been done, but, unfortunately, a generally available functional analysis still needs to be developed.[5]

As CHD7 regulates gene expression, a possible general functional assay (read-out) might come from gene expression profiling, for example by RNA-sequencing. It can then be seen if a patient with a pathogenic *CHD7* variant has a different and recognizable gene expression pattern. Such a recognizable profile might be used for the interpretation of the >90 variants of unknown significance currently present in our online *CHD7* database (www.CHD7.org). Gene expression profiling may also be of use in the diagnostic work-up when no pathogenic *CHD7* variant is identified in patients with a typical CHARGE phenotype. Expression profiling in CHD7 expression cells of these patients might reveal a not-yet-identified (deep intronic) *CHD7* variant. This approach has, however, not been done so far.

Information for the interpretation of genetic variants should be easily accessible. Therefore, we have created an open access *CHD7* mutation database (www.CHD7.org) that contains published and unpublished *CHD7* variants identified in laboratories around the world. The database is patient-based so it includes not only frequency data on variants but also phenotypic and segregation data. It is important that everyone can easily share and insert data, and that the information from different sources about these variants is presented clearly. Mutation databases are valuable for clinicians as well as researchers. The development and maintenance of a mutation database is complex, not only for our *CHD7* mutation database, but for all databases on rare genetic disorders and variants, but the hope is that by setting up European reference networks, better central facilities will be created.

6.2.3 Clinical aspects of pathogenic *CHD7* variants

6.2.3.1 Guidelines for CHD7 analysis

Although next generation sequencing is increasingly being used to screen multiple genes at once in a patient, it is still useful to know in which patients a careful analysis of the *CHD7* gene is warranted. This is because, firstly, it is currently still more cost effective to perform single gene analysis if the *a priori* chance of finding a mutation is high. Secondly, although this might change in the future, Sanger sequencing of one gene still has better coverage than most next generation multiple genes screening methods currently used in clinical practice. Thirdly, screening of multiple genes increases the chance of finding variants of unknown significance

and may result in unsolicited findings, especially if the whole exome or genome is analyzed. We published guidelines to help clinicians decide if *CHD7* analysis is warranted (see chapter 3).[14] These guidelines are based on clinical experience and the phenotypes of a cohort of 280 patients with a *CHD7* mutation. To check the sensitivity of our guidelines, validation in an independent cohort of patients with pathogenic *CHD7* mutations was needed.

Recently, an independent study used our guidelines on a cohort of 28 patients suspected of CHARGE syndrome in whom *CHD7* analyses had been performed in a clinical setting.[15] Based on our guidelines, *CHD7* screening was recommended for 27 of their patients, and a mutation was found in 16. In one patient for whom our guidelines recommended temporal bone imaging as a first step, *CHD7* analysis was done and no mutation was identified. So our guidelines advised *CHD7* analysis in all patients in which a *CHD7* mutations was identified (high sensitivity), and a *CHD7* mutation was identified in almost 60% of the patients for whom our guidelines recommended mutational analysis. The same study reviewed 32 atypical CHARGE patients from 19 families with pathogenic *CHD7* mutations previously published in literature.[15] Because these patients were stated to have an atypical phenotype, it is interesting to know if our guidelines for *CHD7* analysis would have recommended an additional investigation. Based on the clinical information presented, which is not complete, additional investigations (*CHD7* analysis or temporal bone imaging) would have been advised in at least 30 of the 32 patients based on our guidelines. For six patients, supporting clinical information of a family member was needed before an additional investigation was advised. This strengthens the addition of this feature to our guideline. The results of this new study thus support our conclusion that our guidelines are helpful in identifying patients for *CHD7* analysis, however a larger cohort is still needed to more reliably assess sensitivity and positive predictive value.

6.2.3.2 Clinical surveillance

Literature agrees that follow up of patients with a pathogenic *CHD7* mutation or typical CHARGE syndrome should be done by a multidisciplinary expert team to ensure optimal treatment of this complex patient group.[16] In the Netherlands, the National multidisciplinary CHARGE outpatients' clinic is located at the University Medical Center Groningen. As of the end of 2016, more than 100 patients had been seen in this officially accredited center of expertise. We use the knowledge gained at our clinic to advise the individual patient and to perform patient-centered research. For example, a prospective study on immunological function was performed after we identified overlap with 22q11.2 deletion syndrome in which

T-cell dysfunction often occurs (chapter 5.1).[17,18] The main goal of our research is to optimize the individual patient's clinical care. We have established recommendation for clinical follow-up of CHARGE patients (chapter 3),[14] but there are other recommendations for management, treatment and surveillance of patients with CHARGE syndrome.[16,19] Since many organ systems can be affected in CHARGE syndrome, no set of recommendations is complete. For example, some recommendations, including ours, lack advice on immunological assessment, while others lack recommendations for anesthesia or a cerebral MRI.[14,16,19] In addition, while all recommendations are based on available research and practice experience, no evidence-based guidelines are available that include a systematic review of the evidence and an assessment of the benefits and harms. I think all experts in the field of CHARGE syndrome should work together to produce an international evidenced-based guideline for the clinical surveillance of CHARGE syndrome. This will help us provide the best clinical practice to all CHARGE patients. Careful review of evidence will also identify topics that need further investigation in this complex syndrome.

For clinical practice it is important that guidelines for surveillance are easily accessible and up to date, not only for CHARGE syndrome, but for all rare diseases. I think this can be done by creating an online environment in which all guidelines for rare diseases are collected, and these guidelines can be updated by experts in the field when new information becomes available. In this way doctors will easily find the latest recommendations and treat their patients based on the latest insights.

6.2.3.3 Clinical or molecular diagnosis?

CHARGE syndrome has been a clinical diagnosis since it was first recognized as an association of congenital anomalies in 1979.[20,21] The updated Blake criteria (2006) and Verloes criteria (2005) are the clinical diagnostic criteria that are most commonly used (see table 1 in chapter 3).[22,23] Since molecular diagnosis became available, the phenotype has broadened and there is debate on how to update these clinical diagnostic criteria. For example, Hughes et al. proposed family history and facial clefting as major criteria based on the history of one extended family with a missense *CHD7* variant,[24] while Hale et al. included a pathogenic *CHD7* variant as a major criterion, changed cranial nerve dysfunction from a major to a minor criterion, and added several minor criteria including renal, skeletal or limb anomalies, autism, dysphagia and feeding difficulties.[15]

The major question underlying this debate is if CHARGE syndrome is a clinical or molecular diagnosis. On one hand, CHARGE syndrome should remain a clinical diagnosis, since not all patients can have their DNA analyzed and a pathogenic *CHD7* variant may be undetectable with current techniques or unrecognized/difficult-to-interpret with current knowledge. However, the clinical diagnostic criteria that are used should be as specific as possible to minimize the overlap with other syndromes. When diagnosing a patient with CHARGE syndrome on a clinical basis, other causes of a CHARGE phenotype such as chromosomal aberrations and teratogens should first be carefully ruled out. On the other hand, a molecular diagnosis is especially helpful in atypical patients. We have shown that at least 14% of the patients with a pathogenic *CHD7* mutation do not fulfill the clinical diagnostic criteria for CHARGE syndrome.[14] This percentage is likely to rise further since pathogenic *CHD7* variants will be identified by next generation sequencing in patients without an *a priori* clinical suspicion of CHARGE syndrome. These patients might not be labeled as CHARGE syndrome from a scientific point of view, but they should be treated as patients with CHARGE syndrome from a clinical point of view. Therefore I suggest that, the spectrum of phenotypes caused by *CHD7* mutations should be renamed to '*CHD7*-related disorders'. These disorders will have CHARGE syndrome on one side of the spectrum and patients with fewer symptoms or a different presentation on the other.

6.2.4 *CHD7* and the heart

Congenital heart defects are one of the most frequent congenital anomalies, with multiple causes including *CHD7* mutations. We identified variable heart defects in 220 of 299 patients (74%) with a pathogenic *CHD7* mutation, with an overrepresentation of atrioventricular septal and conotruncal defects (chapter 4.1).[25]Because of the clinical consequences, every patient with a pathogenic *CHD7* mutation should have a cardiac ultrasound. Specific attention to arch vessel anomalies is needed, especially in patients with feeding or breathing problems (chapter 4.2).[26]

We did not identify pathogenic *CHD7* mutations in a small cohort of patients with an AVSD or conotruncal heart defect and one other feature of CHARGE syndrome (chapter 4.3).[27] This suggests *CHD7* mutations are not a major cause of non-selected syndromic heart defects. Identifying a pathogenic *CHD7* mutation provides important information for clinical surveillance, counseling and reproductive options. We also know that the phenotype caused by *CHD7* mutations can be mild. For instance, we know of a case of a girl with a heart defect and a characteristic external ear anomaly as the only features of her pathogenic *CHD7* mutation.[14] Heart defects are also usually identified shortly after birth, while some

other possible features of a *CHD7* mutation will only become visible later, hypogonatropic hypogonadism in girls for example. The question also remains how often *CHD7* mutations are the cause of a congenital heart defects. In a retrospective studies in a cohort of 310 patients with the arch vessel anomaly bicarotid trunk, CHARGE syndrome was diagnosed in three (1%).[28] In another retrospective study, two of the 257 patients (0.8%) with tetralogy of Fallot with pulmonary stenosis had CHARGE syndrome.[29]Exome sequencing of 362 parent-offspring trios with a child with a severe heart defects revealed a *de novo* truncating *CHD7* mutation in a child with syndromic tetralogy of Fallot.[30] We therefore advise clinicians and diagnosticians to include *CHD7* in gene panels for screening patients with congenital heart defects. In a diagnostic setting, the genomic laboratory at the Maastricht University Medical Center (MUMC) in the Netherlands has already begun including *CHD7* in their congenital heart disease gene panel of 41 genes.

The regulation of cardiovascular development, including the role of CHD7, has been well studied.[31] Studies on the function of CHD7 have focused on neural crest cells because CHARGE syndrome was thought to be a neurocrestopathy before identification of its molecular cause.[32] Mouse Chd7 has been shown to play a major role in the formation of multipotent migratory neural crest cells and the maintenance of the undifferentiated state of neural crest cells by regulating other genes.[33,34] However, CHD7 is also involved in other cell types. Chd7 is required for normal cardiovascular development in the ectoderm of the pharyngeal arch and in the anterior mesoderm in mice.[35,36] Based on these studies, the congenital heart defects caused by *CHD7* mutations are probably due to multiple cell lineage defects by changes in the expression of cardiac developmental genes.

6.2.5 Syndromes overlapping with CHARGE syndrome

CHARGE syndrome is a very variable multiple congenital malformation syndrome and the phenotype of *CHD7* mutations overlaps with many other conditions.

We have already summarized the multiple chromosomal aberrations that clinically overlap with CHARGE syndrome in table 1 in chapter 2.1.[9] Since publication of this table, new chromosomal aberrations have been identified that have clinical features overlapping with CHARGE syndrome.[37-40] We have now added 5q11.2 microdeletion syndrome to the list, with choanal atresia or stenosis, external ear defect, growth retardation, developmental delay, hearing loss, genital defect and heart defects as overlapping features (chapter 5.2).[41] A whole genome array is warranted in patients suspected of CHARGE syndrome when no *CHD7* mutation is identified.

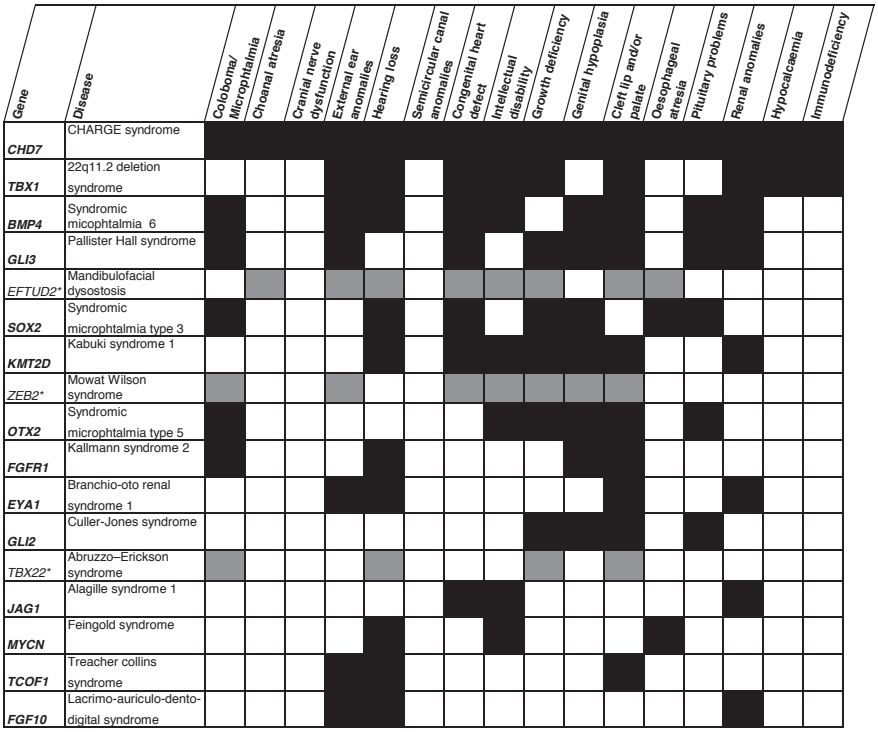
The most striking overlap occurs with 22q11.2 deletion syndrome. We showed in chapter 5.1 that a CHARGE phenotype may be caused by 22q11.2 deletion and, vice versa, a phenotype of 22q11.2 deletion may be caused by a *CHD7* mutation.[27] The overlap between phenotypes of 22q11.2 deletion syndrome and CHARGE syndrome can be explained by the link identified between the responsible genes: *TBX1* and *CHD7*.[18]

Causal genes are also linked in other syndromes that clinically overlap with CHARGE syndrome (see figure 1). For example, patients with molecularly proven Kabuki syndrome (MIM 147920), caused by *KMT2D* mutations (MIM 602113) among other causes, have wrongfully been labeled as having CHARGE syndrome in the past.[42,43] CHD7 and KMT2D have been shown to function in the same chromatin modification machinery, both interacting with members of a specific protein complex.[42]

For other syndromes that clinically overlap with CHARGE syndrome, a molecular link could not yet be identified. For example, multiple patients with mandibulofacial dysostosis caused by EFTUD2 mutations (MIM 610536 and 603892), Abruzzo-Erickson syndrome (MIM 302905 and 300307) and Mowat Wilson syndrome (MIM 235730 and 605802) have been diagnosed as having CHARGE syndrome based on their clinical features.[44-46] Since shared molecular pathways give overlapping phenotypes (figure 1), I expect there to be a molecular link between *CHD7* and these genes as well.

Figure 1 demonstrates that an overlapping phenotype can help in identifying genes in the same molecular pathway, and the inverse also true, genes with a molecular link may cause similar phenotypes and thus help in the identification of a causal variant in patients. This idea can be used for the identification of genes that cause a phenotype overlapping with CHARGE syndrome. Candidate genes are genes disrupted by chromosomal imbalances known to cause an overlapping phenotype or genes that interact with the CHD7 protein or *CHD7* gene. I expect that patients with a phenotype overlapping with CHARGE syndrome have mutations in genes that interact with CHD7 or the network in which CHD7 is involved. Identifying the cause in these patients may teach us more about the function of CHD7.

Figure 1. Syndromes overlapping with CHARGE syndrome



Multiple congenital anomaly syndromes have clinical overlap with CHARGE syndrome.
Here we show the overlapping clinical features of CHARGE syndrome with other known genetic syndromes. The expression of FGF10, FGFR1, OTX2, BMP4, TBX1 and PAX2 depend on *CHD7* in some tissues.[33,35,47-49] EYA1 and *CHD7* regulate the same neurogenetic cascade in the inner ear.[47] SOX2 and *CHD7* physically interact and have GLI2, GLI3, JAG1 and MYNC as common target genes.[1] The binding of the treacle protein encoded by TCOF1 to rRNA is dependent on the presence of *CHD7*.[3] KMT2D and *CHD7* function in the same chromatin remodeling machine, both interacting with a specific protein complex. Overlapping clinical features of causal genes that are linked to *CHD7* are indicated in black.
For the genes indicated with an asterix (*), a link with *CHD7* has not yet been published, but the clinical overlap shown by the grey boxes can be striking.[44-46] (This figure was adapted from [18]).

6.3 GENERAL CONCLUSION, SUMMARY OF RECOMMENDATIONS AND FUTURE PERSPECTIVES

This thesis has provided new insights into mutations occurring in the *CHD7* gene and made these data available via the open access online *CHD7* mutation database (www.CHD7.org). We have introduced guidelines to identify in which patients *CHD7* analysis is warranted. Replication in a small cohort showed a high sensitivity, but a larger cohort is needed to determine its value more reliably.

We focused on the phenotype of *CHD7* mutations, especially the heart and overlapping syndromes. We learned that heart defects occur in most patients with a *CHD7* mutation, with an overrepresentation of AVSD and conotruncal defect. *CHD7* mutations are not a major cause for these isolated heart defects but, based on the literature and clinical experience, we advise adding *CHD7* to multiple gene panels screening patients with structural congenital heart defects.

Thus far there is no evidence that other genes can cause typical CHARGE syndrome, but many syndromes have a phenotype that clinically overlaps with CHARGE syndrome, especially 22q11.2 deletion syndrome. Many syndromes that clinically overlap with CHARGE have a molecular link with *CHD7* as well. In patients with a phenotype that overlaps with CHARGE syndrome who do not have a *CHD7* mutation, good causal gene candidates for screening are genes that cause overlapping syndromes, genes that are molecularly linked to CHD7 or genes that are disrupted by chromosomal aberrations that produce a comparable phenotype. If the phenotype is very specific for CHARGE syndrome, we recommend re-analysis or re-evaluation of *CHD7*.

CHD7 mutations cause a variable phenotype that has now been shown to be broader than the features first outline in the acronym CHARGE and the clinical diagnostic criteria for CHARGE syndrome. I therefore suggest this spectrum of phenotypes should be renamed to '*CHD7*-related disorders'.

Future research should focus on developing a functional analysis to better interpret *CHD7* variants and on identifying *CHD7* defects missed in the currently used diagnostics, for example by gene-expression analysis. Last, but not least, although papers with recommendations on clinical surveillance have been published, it is important to establish international evidence-based guidelines for surveillance in *CHD7*-related disorders.

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CHAPTER 7

Addendum

7.1 NEDERLANDSE SAMENVATTING

Hoofdstuk 1 gaf een algemene introductie over CHARGE syndroom en *CHD7* varianten waarin het doel van dit proefschrift wordt gepresenteerd. Het hoofddoel van dit proefschrift was bijdragen aan de kennis over CHARGE syndroom veroorzaakt door varianten in het *CHD7*-gen met een speciale aandacht voor hartafwijkingen en om meer te leren over syndromen die klinisch met CHARGE syndroom overlappen.

Hoofdstuk 2 richtte zich op het *CHD7* gen. In **hoofdstuk 2.1** hebben we een overzicht gegeven van 528 pathogene *CHD7* varianten in 802 patiënten. Pathogene varianten waren gelijkmatig verspreid over het coderende gebied van het *CHD7* gen. De meest voorkomende pathogene varianten waren nonsense en frameshift varianten. De meeste varianten waren uniek, maar 94 kwamen herhaaldelijk voor, waaronder 27 arginine naar stop mutaties. Een pathogene variant ontstond meestal *de novo*, maar er zijn familiaire gevallen met een variabel fenotype beschreven door kiemcel- of somatische mosaïcisme. We hebben een locus-specifieke database gemaakt met daarin alle varianten die online beschikbaar is via www.CHD7.org.

Daarnaast hebben we op basis van het aantal pathogene *CHD7* varianten die zijn gevonden bij Nederlandse levend geboren kinderen geboren van 2006 tot 2010 en de algehele geboorte prevalentie in deze jaren, de geboorte incidentie van het CHARGE syndroom geschat tussen de 5.9 en 6.7 per 100.000 levend geboren kinderen per jaar.

In **hoofdstuk 2.2** introduceerden we een classificatiesysteem om de pathogeniciteit van de missense varianten te voorspellen door gebruik te maken van twee rekenkundige algoritmes en een structureel model in combinatie met gegevens over segregatie en fenotype. Door dit classificatiesysteem te gebruiken, hebben we 145 *CHD7* missense varianten geclassificeerd. De pathogene *CHD7* varianten bevonden zich met name in het midden van het coderende gebied van het *CHD7* gen. Bovendien gaven de pathogene missense varianten over het algemeen een milder fenotype in vergelijking met de pathogene truncerende *CHD7* varianten.

In **hoofdstuk 2.3** vonden we door middel van exome sequencing bij vier van de vijf CHARGE patiënten potentiële pathogene CHD7 varianten die eerder werden gemist bij de diagnostische analyse.

In **hoofdstuk 3** bestudeerden we het fenotypische spectrum van 280 patiënten met een pathogene *CHD7* variant. Vier kenmerken waren bijna altijd aanwezig bij patiënten met een pathogene *CHD7* variant: afwijkingen van het uitwendige oor, disfunctioneren van hersenzenuwen, hypoplasie van de semi-circulaire kanalen en vertraagde motorische mijlpalen. We onderzochten het milde einde van het fenotypische spectrum van pathogene *CHD7* varianten door het bestuderen van familiaire gevallen, mild aangedane patiënten met een bewezen pathogene *CHD7* variant en de resultaten van de *CHD7* analyse in cohorten met slecht één kenmerk van CHARGE of met gedeeltelijk overlappende syndromen. Op basis van deze resultaten maakte we richtlijnen voor de analyse van het *CHD7* gen, waaronder ook een advies wanneer de semi-circulaire kanalen zouden moeten worden bekeken. We gaven ook onze aanbevelingen over het klinisch vervolgen van CHARGE patiënten.

In **hoofdstuk 4** richtten we ons op de hartafwijkingen die worden veroorzaakt door *CHD7* varianten. In **hoofdstuk 4.1** bekeken we het cardiale fenotype van 299 patiënten met een pathogene *CHD7* variant. Hartafwijkingen kwamen voor 74% van deze patiënten. De typen hartafwijkingen varieerden, maar een atrioventriculair septum defect (AVSD) en een conotruncale hartafwijking waren oververtegenwoordigd bij *CHD7*-gerelateerde hartafwijkingen in vergelijking met een op EUROCAT gebaseerde groep patiënten met niet-syndromale hartafwijkingen.

We richtten ons in **hoofdstuk 4.2** specifiek op afwijkingen van de aortaboog, omdat deze afwijkingen de oorzaak kunnen zijn van behandelbare voedings- en ademhalingsproblemen bij CHARGE syndroom. Bij tenminste 14% van de patiënten vonden we een aortaboog afwijking, maar onze data waren ontoereikend om de voedings- en ademhalingsproblemen hieraan te koppelen.

In **hoofdstuk 4.3** verrichtten we een analyse van *CHD7* in 46 patiënten met een conotruncale hartafwijking en/of een AVSD en één ander kenmerk van het CHARGE syndroom, maar we vonden geen pathogene *CHD7* varianten. Dit wijst erop de analyse van *CHD7* niet routinematig moeten worden verricht bij patiënten met een conotruncale hartafwijking of een AVSD.

In **hoofdstuk 4.4** hebben we de klinische aspecten van de hartafwijkingen bij het CHARGE syndroom, zoals omschreven in de voorgaande hoofdstukken samengevat, en geven we een overzicht van de functie van *CHD7* in de cardiovasculaire ontwikkeling. Het blijkt dat *CHD7* belangrijk is in het reguleren van andere genen die betrokken zijn bij de cardiovasculaire ontwikkeling.

In **hoofdstuk 5** richtten we ons op twee klinisch overlappende microdeletie syndromen. In **hoofdstuk 5.1** bestudeerden we de klinische overlap tussen CHARGE syndroom en het 22q11.2 deletie syndroom op basis van de moleculaire diagnose. De fenotypes van beide syndromen overlappen vooral op aangeboren hartafwijkingen, gespleten gehemelte, ontwikkelingsachterstand, nierafwijkingen en gehoorverlies. Zelfs kenmerken die specifiek lijken voor CHARGE syndroom, zoals colobomen, choanen atresie en afwijkingen aan de semi-circulaire kanalen, komen voor bij een klein percentage van de patiënten met het 22q11.2 deletie syndroom. Andersom komen kenmerken die specifiek leken voor het 22q11.2 deletie syndroom, zoals hypoplasie van de thymus, voor in patiënten met pathogene *CHD7* varianten. Op basis van deze waarnemingen, laten we zien dat 22q11.2 deleties vastgesteld kunnen worden bij klinische CHARGE patiënten en pathogene *CHD7* varianten bij patiënten met fenotypisch het 22q11.2 deletie syndroom. Dit heeft gevolgen voor de genetische aanpak van deze beide groepen patiënten.

In **hoofdstuk 5.2** beschreven we de klinische fenotypes van zes patiënten met een 5q11.2 deletie. Het 5q11.2 microdeletie syndroom overlapt klinisch met CHARGE en 22q11.2 deletie syndroom door de aanwezigheid van choanen atresie en stenose, afwijkingen van het uitwendige oor, groeiachterstand, autisme, velofaryngeale insufficiëntie en hartafwijkingen. Het meest interessante kandidaat gen in het kleinste gebied van overlap is *DHX29*, dat codeert voor een eiwit dat belangrijk is in de vertaling van het mRNA. Desalniettemin hebben we niet voldoende bewijs om aan te tonen dat haplo-insufficiëntie van dit gen de oorzaak is van het fenotype 5q11.2 microdeletie syndroom.

Hoofdstuk 6 beschrijft wat we hebben geleerd door dit proefschrift in samenhang met wat we nu al weten, maar ook wat we nog moeten leren over de functie van CHD7, de identificatie en interpretatie van *CHD7* varianten en hun klinische effecten. De belangrijkste adviezen zijn het opnieuw analyseren van onze richtlijn voor *CHD7* analyse in een groot cohort, het opnemen van *CHD7* in gen panels voor aangeboren hartafwijkingen, heranalyse van *CHD7* bij klinisch typische patiënten, maar juist analyse van fenotypisch of genotypisch overlappende syndromen bij atypische patiënten. Ik stel daarnaast voor om het spectrum van fenotypes veroorzaakt door pathogene *CHD7* varianten '*CHD7*-gerelateerde aandoeningen' te noemen. Toekomstig onderzoek moet zich mijn inziens vooral richten op het ontwikkelen van een functionele analyse voor CHD7 en internationale evidence-based richtlijnen voor *CHD7*-gerelateerde aandoeningen.

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7.3 LIST OF PUBLICATIONS

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- **Corsten-Janssen N**, du Marchie Sarvaas GJ, Kerstjens-Frederikse WS, Hoefsloot LH, van Beynum IM, Kapusta L, van Ravenswaaij-Arts CM. CHD7 mutations are not a major cause of atrioventricular septal and conotruncal heart defects. Am J Med Genet A. 2014 Dec;164A(12):3003-9
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7.4 CURRICULUM VITAE

Nicole Corsten-Janssen werd op 27 april 1984 geboren te Zwolle. Na het behalen van haar atheneum diploma aan de Thorbecke scholengemeenschap te Zwolle in 2002 begon zij met de studie geneeskunde aan de Rijksuniversiteit Groningen. Tijdens haar studie werd zij geraakt door de klinische genetica en besloot ze haar wetenschappelijke stage en keuze co-schap te doen bij de klinische genetica in het UMCG.

In oktober 2008 behaalde Nicole haar arts-examen en is ze gaan werken als ANIOS bij de afdeling klinische genetica in het UMCG. Vanaf juni 2011 tot september 2016 werd zij daar opgeleid tot klinisch geneticus door Dr. J.C. Oosterwijk en Prof. dr. I.M. van Langen. Vanaf september 2016 werkt ze als klinisch geneticus bij de afdeling genetica in het UMCG.

Sinds juni 2009 combineert Nicole haar klinische taken en opleidingstaken met onderzoek naar CHARGE syndroom en *CHD7*-mutaties, onder leiding van Prof. dr. C.M.A. van Ravenswaaij-Arts, Prof. dr. L. Kapusta, en Prof. dr. R.M.W. Hofstra, wat heeft geleid tot dit proefschrift. Zij heeft diverse voordrachten gehouden op nationale en internationale wetenschappelijke congressen, maar ook op nationale en internationale patiëntbijeenkomsten.

Nicole is in 2012 getrouwd met Kristian Corsten, samen hebben zij 2 kinderen, Twan (2014) en Romee (2015).

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